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N_2 fixation by two lupine species under different soil management systems. Transfer of fixed N_2 from legume to intercropped eucalyptus

A fixação do N_2 por duas espécies de tremoceiro em diferentes sistemas de gestão do solo. Transferência do N_2 fixado pela tremocilha para o eucalipto em consociação

Corina Carranca[1], Manuel Madeira[2] and Maria Odete Torres[3]

[1] *Instituto Nacional de Investigação Agrária e Veterinária [INIAV], Centro de Engenharia dos Biossistemas [CEER], Quinta do Marquês, Av. República, Nova Oeiras, 2784-505 Oeiras, Portugal. E-mail: corina.carranca@inav.pt, author for correspondence.*
[2] *Universidade Técnica de Lisboa, Instituto Superior de Agronomia [UTL/ISA], Centro de Estudos Florestais [CEF], Tapada da Ajuda, 1349-017 Lisboa, Portugal*
[3] *Universidade Técnica de Lisboa, Instituto Superior de Agronomia [UTL/ISA], Centro de Engenharia dos Biossistemas [CEER], Tapada da Ajuda, 1349-017 Lisboa, Portugal*

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ABSTRACT

The rate and pattern of symbiotic fixation and N credit by white lupine as affected by soil tillage, and yellow lupine affected by soil amendment with eucalyptus harvest residues were evaluated; also, fixed N transferred from yellow lupine to the intercropped eucalyptus was assessed. Lupine species growing in sandy soils with low nutrient status fixed ≥94% N_2 with the indigenous soil rhizobium, in two sites (Pegões, Óbidos). Soil disturbance and harvest residues incorporation did not affect the fixing capacity of both lupines. Positive N inputs (≥+69 kg N ha^{-1}) were estimated for both lupines, which is relevant in a rotation or association with non-legume crops. Nitrogen concentration in newly-planted eucalyptus was 23% lower in harvest residues amended soil and 36% greater by the addition of mineral-^{15}N compared with organic fertilization (yellow lupine). Mineral-N use efficiency by eucalyptus seedlings was low (4%) which may be associated with the small root development (shoot:root ratio=4.3). Organically fertilized trees showed ^{15}N enrichment thirteen times smaller than mineral-^{15}N fertilized trees.

Keywords: Haplic Podzols, harvest residues, *Lupinus albus* and *Lupinus luteus*, N benefit, tillage

RESUMO

Avaliou-se a fixação simbiótica e crédito em N pelo tremoceiro doce em resposta à mobilização do solo, e pela tremocilha com incorporação no solo de resíduos de abate de eucalipto; avaliou-se também a transferência do N_2 fixado pela tremocilha para o eucalipto consociado. As espécies de tremoceiro estudadas fixaram >94% N_2 com o rizóbio indígena em solos arenosos, pobres em nutrientes, das áreas de Pegões e Óbidos. A mobilização do solo e os resíduos de abate não afectaram negativamente a fixação simbiótica pelos dois tremoceiros. Observaram-se benefícios em N (≥+69 kg ha^{-1}) por ambas as proteaginosas, o que é relevante em sistemas de rotação ou consociação. A concentração do N nos eucaliptos recém-plantados foi 23% menor no solo com resíduos.

Palavras-chave: Benefício em N, *Lupinus albus* e *Lupinus luteus*, mobilização do solo, Podzóis Háplicos, resíduos de abate

Introduction

Grain legumes are grown worldwide and cultivated primarily for their grains which are harvested at maturity and marketed as fresh or dry products, rich in proteins for human consumption and animal feeding. However, their cultivation has been neglected for many years in the European Union (EU), which only produces about five million tonnes, especially peas (3 Mt), fababeans (1.2 Mt) and soybeans (0.8 Mt), and imports two-thirds of protein required for feed uses (Crépon *et al*., 2004). Grain legumes are expected to increase the eco-efficiency of agricultural production due to their ability to produce proteins of high quality without using nitrogen (N) fertilisers (Gaillard and Nemecek, 2004). In this way, it is supposed that energy use by grain legumes and N losses (nitrate, ammonia, etc.) are much lower than in non-legume crops. The current European Common Agricultural Policy favours the introduction of grain legumes in crop rotations where they are economically beneficial for farmers in several regions by their value to the next crop, but no specific financial support has been decided yet. Legumes have crucial functions in the crop rotation *via* their N and non-N characteristics (pathogen inocula size, weed seed bank, soil microbial activity, soil aeration, etc.) for succeeding crops. In a grain legume-non-legume (e.g. cereal) sequence, legume N benefits are related to N carry-over of unused inorganic soil N by legume and legume residues N after the maturity phase, which may reduce the need for mineral N supply in the subsequent crop (cereal), potentially contributing to build up soil fertility. The significance of N benefit in the short- and long-term cropping systems is determined by the crop N balance, the soil N dynamics after the legume has stopped to take up N from soil and the N demand of the succeeding crop (Criozat, 2004; Jensen, 2004; Carranca, 2013b). The N balance for legume-based systems can be both positive and negative, depending on the relationship between the fraction of N derived from the atmosphere (%Ndfa) and N removal (Carranca, 2013a). A positive N balance is most likely to occur when the proportion of N derived from N_2 fixation is close to 100% (Carranca *et al*., 2009).

Lupine is an interesting seed pulse from which protein for human diet (sweet/white lupine) and fodder (yellow lupine) can be obtained. It may nodulate with slow growing soil *Bradyrhizobium* sp. or by inoculation with fast growing *Rhizobium* sp. bacteria strains, but limited research has been conducted on management options to enhance lupine ability to fix atmospheric N_2. The amount of N_2 fixed is controlled by the effectiveness of the rhizobium-plant symbiosis, plant sink strength, pre-existing soil N pools and fluxes, and environmental and nutritional constraints (Carranca, 2013a). Nitrogen fixation can be increased by management practices aimed to increase plant N demand, but some legumes may delay nodulation until they sense a lack of available soil N. Changes of soil management practices like the intensity of tillage will alter the edaphically chemical and biophysical factors, and therefore may indirectly influence symbiotic fixation (Pálmason *et al*., 1992; Carranca, 2013a). When tillage is minimized, lower rates of N mineralization and nitrification, coupled with increased N immobilization, will lead to a decrease in soil available N which stimulates N_2 fixation capacity. Tillage may also destroy nodulated roots, reducing the fixation rate (Goss and De Varennes 2002). A promising development to avoid build-up of extra N in the soil may be perennially based cropping systems, intercropping, or eventually including perennials in annual cropping systems (Fuhrer, 2004; Carranca, 2013b).

Legume growth and symbiotic N_2 fixing microorganisms may be inhibited by the amount and nature of allelophatic chemicals contained in residues from woody species, particularly leaves and roots (De Varennes *et al*., 2007; Rocha and Santos, 2007; Carranca, 2013a). For instance, essential oils in eucalyptus species, especially present in leaves show a complex composition, variable during the day and with time, and contain organic phenols, acetones, alcohols and antibiotics, among others (Ferguson and Rathinasabapathi, 2009). Plant growth and symbiotic N_2 fixing microorganisms may be inhibited by the allelophatic chemicals contained in the residues of tree species (Carranca, 2013a), though Heckman and Kluchinski (1995) did not observe any allelophatic inhibition on nodulation or N_2 fixation by soybean associated with heavy application to soil of oak, maple, sycamore or walnut leaves. Eucalyptus residues have substances that cause injures in the development of some leguminous plants, as well as the infection by the rhizobium. In fact, research regarding identification of allelopathic compounds in leachates of bark, fresh leaves and leaf litter of *Eucalyptus tereticornis* L., *E. camadulensis* L., *E. polycarpa* L. and *E. microtheca* L. showed a negative influence of phenolics and leachates on the germination, crop yield and nitrogenase activity of redgram [*Cajanus cajan* (L.) Millsp.], a pulse widely used in India (Sasikumar *et al*., 2001).

In this context, two field experiments were developed aiming *i)* to evaluate the rate and pattern of N_2 fixation and N credit of white lupine (*Lupinus albus* L.) as affected by soil tillage, and of yellow lupine (*Lupinus luteus* L.) as affected by eucalyptus harvest residues management, and *ii)* to determine the transferred N_2

fixed by *L. luteus* L. to the intercropped *Eucalyptus globulus* L. in a new plantation**.**

Material and Methods

Sites and experimental layout

Experiment A

A field experiment (A) was carried out in 2001/2002 and 2003/2004 at Pegões, central Portugal (38º 24'N, 8º 35'W), to evaluate the N_2 fixing capacity and potential N credit of white lupine (*Lupinus albus* L.) in two blocks (190 m^2 each), with two tillage treatments (conventional tillage (CT) and no-till (NT)), each treatment being replicated four times. The experimental field was established in a long-term use for fallow before the experiment started. Four plots (5 m x 1 m = 5 m^2) were delimitated in each block for both legume and non-legume treatment, and till treatments were randomly distributed. No-till plots were not tilled at any time during the experiment, whereas CT plots were rotavated by chisel up to 20 cm depth just before seeding. The soil (Haplic Podzol) was sandy and up to 20 cm depth showed a $pH_{(H2O)}$ of about 6, a cation exchange capacity of 0.88 $cmol_{(+)}$ kg^{-1}, and 7 g organic C kg^{-1} and 0.6 g Kjeldahl N kg^{-1}. Basal dressing corresponding to 60 kg ha^{-1} for phosphorus (P) and for potassium (K) was applied just before tillage.

The climate of the region is of Mediterranean type, with hot and dry summers and mild autumns and winters. Average minimum and maximum air temperatures during both growth periods did not differ significantly and ranged from a minimum of 6ºC in December and January to a maximum of 23-26ºC in April, May and October. In January 2002, there were six days with temperature about 0ºC, with the formation of frost. Monthly rainfall in 2001/2002 varied from a maximum value of 126 mm in January/02 to the lowest amount of 20 mm in February/02, and in 2003/2004 it ranged from 179 mm in October/03 to 29 mm in April/04.

A non-legume crop, oat (*Avena sativa* L.), was used as a reference crop to estimate the N_2 fixed by the A-value method (Carranca *et al*., 1999b). A seeding rate of 60 plants m^{-2} was used for lupine and 12 g seeds m^{-2} for oat, and the sowing date was late September 2001 and 2003 for both crops. Legume nodulation was assessed by the native soil bacteria. In year 2003/2004, new blocks and plots were established as above. Ammonium nitrate doubly enriched with 5 atoms% ^{15}N was annually applied in a liquid form to the plots, one week after plant emergence, at rates of 10 and 20 kg N ha^{-1} for lupine and oat, respectively. Molybdenum (Mo) and boron (B) were applied as topdressing in November of 2001 and 2003, at rates of 200 g Mo ha^{-1} and 3 kg B ha^{-1}.

Weeds were chemically controlled by applying terbutrin before seeding the lupine. At post-emergence, weeds in each plot were controlled by hand. Glyphosate was applied in oat plots before seeding and "Oxytril M" was used at three-leaf stage. Standard methods were used for pests control during crop growth.

Experiment B

A field experiment (B) with yellow lupine (*Lupinus luteus* L.) was laid out at Óbidos - west Portugal (39º 20'N, 9º 13'W) in 2004/2005 and 2005/2006, in an area where the soil (Haplic Podzols associated with Haplic Arenosols) was amended with eucalyptus (*Eucalyptus globulus* L.) harvest residues (leaves, branches, twigs, bark), to evaluate the N_2 fixing capacity of legume and its potential credit N. The trial was designed in two blocks with two rates of eucalyptus residues (0 and 43 t ha^{-1}). Each residue treatment was replicated four times. This site was also in a long-term use for fallow before the experiment. The soil (0-20 cm) was sandy and showed a $pH_{(H2O)}$ of 6.9, very low cation exchange capacity (0.58 $cmol_{(+)}$ kg^{-1}), 3 g organic C kg^{-1} and 0.2 g Kjeldahl N kg^{-1}. Harvest residues from a neighbour eucalyptus clear-felled site consisted of 20% leaves, 74% branches+twigs and 6% bark, and were chopped into pieces of 10 cm length and incorporated by harrowing in the soil at 0-20 cm depth, before sowing the yellow lupine. Amounts of mass and nutrients in harvest residue components are shown in Table 1. Basal dressing corresponding to 60 kg P ha^{-1} and 60 kg K ha^{-1} was applied after incorporation of harvest residues into the soil. After harvest residues incorporation in the soil, the C/N ratio in the top soil layer (0-10 cm) increased from 15 to about 26.

The climate at Óbidos site is of Mediterranean type, but tempered by the ocean proximity. Mean annual air temperature at the site, taking into account data from a meteorological station which existed in the neighborhood (Caldas da Rainha) is 15.2ºC (Reis and Gonçalves, 1981), ranging from a monthly average of 10.4ºC (January) to 19.8ºC (August). The 30-yr mean rainfall is about 610 mm, but less than 10% occurs between May and September. Relative air humidity is about 80% along the year, both at 9 A.M and 6 P.M.

Lupine was late sown in January 2005 at a rate of 60 plants m^{-2}. Re-seeding of legume occurred naturally from shattering pods and plant germination took place by November 2005 for the second-year experiment. Oat (*Avena sativa* L.) was used as a reference crop to estimate N_2 fixed by the legume using the A-value method and was sown annually (January and November in 2004/2005 and 2005/2006, respectively) at a seeding

Table 1 – Amounts of mass (t DM ha^{-1}) and nutrients (kg nutrient ha^{-1}) of eucalyptus harvest residues incorporated in the soil at the Óbidos site.

Residues	Mass	N	P	Ca	Mg	K	Mn
	(t ha^{-1})	(kg ha^{-1})					
Leaves	8.6	108.9	5.8	93.1	11.9	24.9	5.0
Bark	2.6	5.2	0.8	39.6	3.6	5.9	1.0
Branches	4.3	11.3	0.7	40.2	3.6	2.9	1.6
Twigs	27.3	43.1	5.1	144.4	16.3	30.5	15.8
Total	42.8	168.4	12.3	317.3	35.3	64.2	23.4

N=nitrogen; P=phosphorus; Ca=calcium; Mg=magnesium; K=potassium; Mn=manganese.

rate of 12 g seeds m^{-2}, in each amendment treatment and four replications. Root nodulation in legume was assessed by natural soil bacteria. Plots were marked for legume (3 m x 3 m) and oat (5 m x 1 m) in the two soil amendment treatments. In 2005/2006, new plots were marked for each soil amendment treatment. Ammonium nitrate doubly enriched with 5 atoms% ^{15}N was annually applied in the liquid form to each plot, one week after plant emergence, at rates of 10 and 20 kg N ha^{-1} for lupine and oat, respectively.

Experiment C

A field trial (C) was laid out for one year (2005/2006) in the Óbidos site to evaluate the symbiotically fixed N_2 transferred from yellow lupine (*L. luteus* L.) to the associated eucalyptus (*Eucalyptus globulus* L.) in a new plantation (3 m x3 m spacing), by comparison with sole eucalyptus in a forest system. The layout of the experiment was designed in two blocks with two soil amendments (0 and 43 t eucalyptus residue ha^{-1}) and plots with three N fertilizer treatments (organic, mineral and control), which were replicated four times. Harvest residues collected in a neighbour eucalyptus clear-felled site consisted of 20% leaves, 74% branches+twigs and 6% bark, and were chopped into pieces of 10 cm length and incorporated in the soil by harrowing at 0-20 cm depth one year before (November 2004). The mass and nutrient amounts in residues are shown in Table 1. Basal dressing corresponding to 60 kg P ha^{-1} and 60 kg K ha^{-1} were applied after residue amendment.

In October 2005, one eucalyptus seedling was planted in the centre of each sub-plot (3 m x 3 m) containing the associated fodder lupine (organic fertilizer) or as a sole crop fertilized with mineral ^{15}N. Control trees were also planted in each soil amendment as unfertilized sole crops. Lupine undergone natural re-vegetation and plant emergence occurred in November 2005. One week after legume emergence, ammonium nitrate doubly enriched with 5 atom% ^{15}N was distributed in each legume sub-plot, in the liquid form at a rate of 10 kg N ha^{-1}, avoiding the area of direct tree influence (about 30 cm from the seedling). At the same time, the same fertilizer type was applied at a rate of 20 kg ^{15}N ha^{-1} in 1 m x 1 m areas surrounding the sole trees planted. Trees in organic sub-plots (in both soil amendment plots) were not fertilized with mineral ^{15}N.

Sampling, measurements and laboratory procedures

Experiments A and B

Complete plant samples (legume and oat) were taken from ^{15}N labelled areas (1 m^2) in each sub-plot, at different legume growth stages: 1-vegetative, 2-bloom, 3-pod formation, and 4-pod-filling or physiological maturity to establish the phenological phase of maximum fixation capacity. Plant material was separated into different plant organs (shoots, pods (when present) and visible roots (plus nodules in case of legume), which were washed with deionised water, dried at about 75ºC for 24 h to determine the DM yield (kg DM ha^{-1}), and ground (<0.5 mm), avoiding any cross contamination, to evaluate the N concentration (g kg^{-1}) and %^{15}N enrichment by dry combustion in an automatic N analyzer-mass spectrometer (Universidad Autonoma de Madrid, Spain). Nitrogen yield (kg N ha^{-1}) was calculated as the product of DM yield and N concentration. Percent N derived from the atmosphere (%Ndfa) was estimated by the A-value method (Carranca *et al.*, 1999b) using the reference value of 0.3663% for air natural ^{15}N abundance. Fixed N_2 (kg N ha^{-1}) was calculated as the product of %Ndfa and N yield. Potential contribution of legumes to soil N was calculated as the subtraction of the amount of total N_2 fixed in the legume (kg N ha^{-1})

from the amount of N yield (kg N ha^{-1}) in the pods (seeds included) (Carranca *et al.*, 1999a). A positive difference indicated a potential gain to soil N. Nitrogen harvest index (%NHI) was also estimated as the ratio between N in the pods+grain and aboveground plant N (Lecoeur and Sinclair, 2001).

Experiment C

One complete tree was harvested from each sub-plot at legume physiological maturity (June 2006) to establish the symbiotically fixed N_2 transferred from lupine to the intercropped eucalyptus. The control trees were also harvested at the same time. Trees were separated into above- and belowground plant material which was washed with deionised water and dried at about 75ºC for 48 h to evaluate dry matter yield (g DM tree^{-1}). Plant samples were then ground (<0.5 mm), avoiding any cross contamination. In each plant sample, N concentration (g kg^{-1}) and %^{15}N abundance were determined as mentioned above. Plant N (mg tree^{-1}) was calculated as the product of DM yield and N concentration. ^{15}N transferred from lupine to the planted trees was evaluated by the atom% ^{15}N excess determined in the tested trees minus the ^{15}N in the control trees (unfertilized). Percent N derived from mineral fertilizer (%Ndff) in the sole eucalyptus tested was determined as the ratio between atom% ^{15}N excess (a.e.) in these trees and the %^{15}N a.e. in the mineral N fertilizer (4.6337% a.e.) times hundred (Carranca *et al.*, 1999b). ^{15}N recovered in these trees (mg ^{15}N tree^{-1}) was estimated by the product of %Ndff and plant N (mg N tree^{-1}). Fertilizer N use efficiency (%FUE) was estimated by the ratio between ^{15}N recovery and mineral ^{15}N fertilizer input (20 kg N ha^{-1}, i.e., 2 g N m^{-2} tree^{-1}) times hundred.

Statistical analysis

Analysis of Variance by General Linear Model using the Statistica 6.0 program was performed to evaluate the effects of factors (year, tillage, amendment, date of sampling, plant organ, fertilizer treatment) on measured variables. Means were compared using the Bonferroni's test, at $p<0.05$.

Results

Experiment A

Crop yield

Average yield (aboveground, pods, roots) of white lupine during the growth cycle was not significantly affected by soil disturbance (average 2.3 t DM ha^{-1}) but was affected by climate, with a significantly higher biomass in 2003/2004 (2.6 t DM ha^{-1}) compared to year 2001/2002 (2.2 t DM ha^{-1}) (Table 2).

Table 2 - Dry matter yield (DM), N concentration (NC), N yield (NY), Ndfa and fixed N_2 (FN) as affected by year, tillage system, date of sampling and plant organ in the two-year field experiment with white lupine (*Lupinus albus* L.) cropped at Pegões site. Plant organs: SH (shoots), VRN (visible roots+nodules), PG (pods+grain); sampling date (D): 1=vegetative, 2=bloom, 3=pod formation, 4=pod-filling growth phases.

Source of variation		DM (kg DM	NC (g kg^{-1}DM)	NY (kg ha^{-1} yr^{-1})	Ndfa (%)	FN (kg ha^{-1} yr^{-1})
Year (Y)	2001/2002	2164b	26.2b	65b	89b	61b
	2003/2004	2571a	29.4a	71a	99a	70a
Tillage (T)	Tillage	2240a	27.5a	65a	94a	62a
	No-till	2436a	27.8a	70a	94a	68a
Sampling date (D)	1	656b	36.5a	27b	95b	25b
	2	1078b	29.2b	37b	86c	34b
	3	4262a	23.5c	126a	92bc	117a
	4	4319a	20.9c	110a	100a	110a
Plant organ (P)	SH	3556a	33.1a	89a	94b	89a
	VRN	571b	21.8c	7b	92b	7b
	PG	288b	28.6b	9b	100a	9b
Y x D		ns	ns	7.0***	ns	ns
T x D		ns	ns	ns	ns	ns
Y x T		ns	ns	ns	ns	ns
Y x P		9.1	ns	ns	ns	ns
D x P		39.6***	ns	39.7***	8.9***	49.5***
Y x D x P		ns	ns	ns	4.3**	ns

%Ndfa= %N derived from atmosphere; for the same source of variation, means in a column followed by the same letter are not significantly different by the Bonferroni's test at $p<0.05$; ns, $^{*, **, ***}$= *F*-values not significant and significant for $p>0.05$ and $p<0.05$, $p<0.01$, $p<0.001$, respectively.

Crop N

Soil disturbance did not affect the average legume N concentration (28 g kg^{-1} DM) and %Ndfa (94%) which was mainly dependent on the year weather conditions, with a higher rate in 2003/2004 (99% Ndfa) compared to 2001/2002 (89% Ndfa). A significant reduction of %Ndfa was found in date 2 of 2001/2002 (86% Ndfa), increasing thereafter to the pod-filling in both years (100% Ndfa) (Table 2). Nitrogen concentration was significantly higher in shoots (33 g N kg^{-1} DM) and pods (29 g N kg^{-1} DM) compared to visible roots+nodules (22 g N kg^{-1} DM), and declined throughout the growth cycle from an average value of 37 g N kg^{-1} of total plant DM at vegetative phase to 21 g N kg^{-1} total DM at pod-filling stage. Nitrogen concentration was also significantly greater in year 2003/2004 (29 g kg^{-1}DM) compared to year 2001/2002 (26 g kg^{-1}DM).

Unlike total N, percent N derived from the atmosphere did not differ in shoots and visible roots+nodules (93% Ndfa), but reached 100% in pods (Table 2). Average fixation capacity in year 2003/2004 was significantly higher (70 kg N ha^{-1}) than in 2001/2002 (61 kg N ha^{-1}) with evidence of preferential allocation to the aboveground (shoots+pods+grain) biomass (98 kg N ha^{-1} year^{-1}) compared to 7 kg N ha^{-1} year^{-1} in the visible roots+nodules (Table 2). Fixed N_2 was substantially smaller in the first and second growth stages (29.5 kg N ha^{-1}), compared to the pod-filling phase (115.5 kg N ha^{-1}) sustained by the increase of crop yield and fixation rate.

N credit and harvest index

Because of the focus on aboveground biomass N, the contribution of grain legumes to N budget in rotation is generally underestimated. In this study, we included root N for the N balance. Lupine roots contained about 7% of aboveground N and accounted for 7% of total N_2 fixed in the plant, and thus the potential N input was estimated by including shoots and visible roots+nodules biomass N. Nitrogen yield in pods at filling stage (9 kg N ha^{-1}) was subtracted to estimate the credit N of white lupine. An apparent positive soil N input of +96 kg N ha^{-1} (93% of crop N) was estimated. At physiological maturity, a smaller N benefit by the sweet legume is expected since a higher N mobilization to the grain must occur. The apparent N harvest index determined at pod-filling stage in this pulse was only 9.4%, though the %NHI at maturity should result in a higher value.

Experiment B

Crop yield

Average DM yield of yellow lupine was significantly higher in 2005/2006 (4.4 t ha^{-1}) compared to 2004/2005 (2.8 t ha^{-1}) (Table 3), and in average about 1 t ha^{-1} greater than the white lupine biomass (Table 2). The incorporation of eucalyptus harvest residues in the soil did not affect the biomass produced by the fodder crop in both study periods (3.8 t DM ha^{-1}) (Table 3), with a similar response of sweet lupine to tillage, but with a lower rate (Table 2).

Crop N

Incorporation of harvest residues did not affect the yellow lupine N concentration (27 g N kg^{-1} DM) and N_2 fixation capacity (103 kg N ha^{-1}) (Table 3). Fixation rate was also unaffected by the incorporation of harvest residues in the soil (99%). Greater N_2 fixed was measured in shoots and pods (66 kg N ha^{-1} year^{-1}), but lower than in white lupine (96 kg N ha^{-1} year^{-1}). Fixation increased since the vegetative phase (57 kg N ha^{-1}) to the pod formation (144 kg N ha^{-1}), than decreasing to the maturity phase (83 kg N ha^{-1}). This seasonal variation showed a similar trend as in white lupine, though the N_2 fixed at bloom in the yellow lupine was more related to the value measured at pod-forming in sweet lupine. Fixation rate by *L. luteus* at maturity (99% Ndfa) was slightly higher than by *L. albus* at pod-filling (95% Ndfa). Maximum fixation (100% Ndfa) by fodder legume was obtained at pod formation, earlier than in the sweet pulse (pod-filling).

Credit N and N harvest index

Potential N benefit from *L. luteus* was estimated including the above- and visible belowground biomass N at maturity and amounted to +69 kg N ha^{-1} (50% of crop N) showing a smaller fertilizer N value than sweet lupine at pod-filling stage, but still a high N input for the associated or subsequent crop in rotation. Nitrogen harvest index was similar to the N credit (50%).

Experiment C

After eight months of growth, soil amendment did not affect the control eucalyptus seedlings which only accumulated 8 g total DM tree^{-1}, and had a N concentration of 10.4 and 4.8 g N kg^{-1} DM, respectively in the top and root organ, and a natural ^{15}N abundance of 0.006% and 0.015% a.e., respectively in the above- and belowground plant material (data not shown). Dry matter yield (top+root) of fertilized 8-month old eucalyptus did not vary significantly with fertilizer type (organic or mineral) or with the 2-year soil amendment treatment, and presented a mean value of 32 g total DM tree^{-1} (Table 4), four times greater than control trees. Tree N also did not vary significantly with fertilizer

Table 3 - Changes in dry matter yield (DM), N concentration (NC), N yield (NY), Ndfa and N_2 fixed (NF) as affected by year, soil amendment, date of sampling and plant organ in a two-year field experiment with yellow lupine (*Lupinus luteus* L.) cropped at Óbidos site. Amendment (A): WR (with residues), NR (no-residues); Sampling date (D): 1=vegetative, 2=bloom, 3= pod formation, 4= physiological maturity growth phases; Plant organs: SH (shoots), VRN (visible roots+nodules), PD (pods).

Source of variation		DM (kg DM ha^{-1})	NC (g kg^{-1} DM)	NY (kg ha^{-1} yr^{-1})	Ndfa (%)	NF (kg ha^{-1} yr^{-1})
Year (Y)	1	2823b	28.9a	70b	98b	69b
	2	4353a	26.9a	109a	99a	108a
Amendment (A)	WR	3837a	27.1a	104a	98a	102a
	NR	3850a	27.4a	105a	99a	104a
Sampling date (D)	1	1533c	38.0a	58c	98c	57c
	2	4005b	29.1b	117b	99ab	115b
	3	6508a	22.1c	144a	100a	144a
	4	3505b	24.1c	85bc	98c	83bc
Plant organ (P)	SH	2883a	22.2c	64a	99a	63a
	VRN	422c	25.8b	6b	98b	6b
	PD	1467b	46.6a	68a	99a	68a
Y x D		16.9***	6.7***	34.8***	117.9***	34.5***
A x D		5.5*	ns	10.1**	ns	10.1**
D x P		35.5**	39.5***	ns	8.5***	48.9***
Y x D x P		4.9*	4.9**	9.1**	4.2*	9.3**

%Ndfa=%N derived from atmosphere; in each column and for each source of variation, means with the same letter are not significantly different by the Bonferroni's test at $p<0.05$; ns, *, **, ***= *F*-values not significant and significant for $p>0.05$ and $p<0.05$, $p<0.01$, $p<0.001$, respectively.

Table 4 - Dry matter (DM), C concentration (CC), N concentration (NC), tree exported N (TN), atom ^{15}N excess as affected by fertilizer type and soil amendment in a eight-month field experiment with *Eucalyptus globulus* L. intercropped with *Lupinus luteus* L., at Óbidos site. (HR-harvest residues).

Source of variation variation	DM (g DM $tree^{-1}$)	NC (g N kg^{-1} DM)	TC (mg C $tree^{-1}$)	TN (mg N $tree^{-1}$)	Atom ^{15}N (%)
Fertilizer N (F)					
Lupine	31a	7.5b	-	272a	0.0996b
Mineral	32a	10.4a	-	280a	1.3195a
Amendment (A)					
With HR	29a	8.1a	7.74a	188b	0.6203a
No HR	35a	9.8a	9.37a	366a	0.7988a
Plant organ (P)					
Top	26a	9.9a	13.69a	253a	0.7157a
Root	6b	8.0b	1.06b	46b	0.7034a
F x A	ns	ns	-	ns	ns
F x P	ns	6.1*	-	ns	ns
A x P	ns	ns	-	ns	ns
F x A x P	ns	ns	-	ns	ns

- =Not determined; in each column and for each source of variation, means with the same letter are not significantly different by the Bonferroni's test at $p<0.05$; ns, *=not significant and significant F-values for $p>0.05$ and $p<0.05$, respectively.

treatment (276 mg N tree^{-1}) whereas woody residue in soil reduced significantly the exported N (188 mg N tree^{-1}) compared to 366 mg N tree^{-1} in unamended sub-plots (Table 4). These results are mainly a consequence of tree biomass since the N concentration responded positively to mineral N fertilization (10.4 g kg^{-1} DM), in contrast with the organic treatment (7.5 g kg^{-1} DM), and only slightly to soil amendment (8.1 g kg^{-1} DM) in comparison with untreated sub-plots (9.8 g kg^{-1} DM). Residues in soil under organic fertilization did not significantly affect the total C assimilated (8.6 g C tree^{-1}) (Table 4).

As expected, the %^{15}N a.e. in the eucalyptus receiving mineral N (1.32% a.e.) was thirteen times greater than in organically fertilized trees (0.10% a.e) (Table 4), and corresponded to 29% Ndff. These mineral-^{15}N fertilized trees only contained 80 mg ^{15}N tree^{-1}, showing a low FUE (4%) for the first eight months after planting. Harvest residues only reduced tree ^{15}N enrichment by 23%.

Discussion

Fixation capacity and potential soil N input by lupine species

Results of the present study confirm that systems involving legumes represent a sustainable alternative to conventional agriculture by capture of atmospheric N_2, being an adequate strategy for organic fertilization and rotation efficiency improvement. In fact, fixation rate by sweet lupine was high (≥89%). The fodder crop with shattering pods was supplied with N_2 from the air considerably better than the sweet pulse (Table 3), as already observed by Merbach and Schilling (1980). Both studied lupine species, for human diet and fodder production fixed N_2 from the atmosphere very efficiently (94-99% Ndfa) in symbiosis with the indigenous rhizobium in coarse textured soils, poor in organic matter (OM) and available N. The difference observed in %Ndfa for the two-year experiment was mainly due to the low value (86% Ndfa) determined in the second sampling date in year 1 (flowering stage, in January), which may be explained by the occurrence of six days of extremely low soil temperature with frost. The lower fixation rate by sweet pulse in the growth stage 2 and 3, respectively in January and February (86-92%), when the daily mean air temperatures were as low as 5-6ºC, agrees with the value of 92% Ndfa measured by Pálmason *et al*. (1992) for sweet-blue lupine cropped at the mean temperature of 10ºC. In fact, low temperature reduces plant growth and controls the geographical distribution of soil rhizobia (Breitbarth *et al*., 2006), which are mesophiles and mostly do not grow below 10ºC and nodule growth is restricted (Carranca *et al*., 2009; Carranca, 2013a).

In the present study, fixation rate increased from the vegetative phase (one month after sowing) to the pod formation/filling stage (100% Ndfa) in both lupine species, in much larger rates than those observed for other grain legumes (*Vicia sativa* L., *Vicia faba* L., *Ciccer arietinum* L.) cropped under similar rain fed ecological conditions, which fixed 30-82% Ndfa (Carranca *et al*., 1999a). Fixation rates observed in the present study are also higher than those reported by Howieson *et al.* (1998) for *L. albus* L. (68-85% Ndfa in Australia, 44-92% in Germany, and 80% in France), by Chalk *et al.* (1993) for *L. angustifolius* L. (79% Ndfa), and by Halvorson *et al*. (1992) for *L. lepidus* and *L. latifolius* (60% Ndfa). The high fixation rates observed in experiments A and B may were favoured by poor soil N and the presence of efficient indigenous soil bacteria with the tested local lupine varieties.

Merbach and Schilling (1980) reported that the yellow lupine fixed N_2 up to the ripeness, but the white lupine on the contrary finished fixation in the flowering phase by a possible competition of growing seeds and nodules for the photosynthates, and legume should be supplied with mineral N at this phenological stage. This trend was not observed in the present study (experiment A, see Table 2) as the rate of fixation by white lupine significantly increased from the flowering phase (92% Ndfa) to the pod-filling stage (100% Ndfa), and the NHI was very low (9.4%), showing that N mobilization in the plant to the grain may be later than this phase.

Disturbance associated with soil tillage did not reduce the symbiotic fixation by sweet pulse (94% Ndfa) because microbial communities were apparently not greatly affected, though a slightly higher N_2 fixation capacity (6% greater) was measured in conservative practice in line with the accumulation of plant biomass (Table 2); also, no great soil disruption occurred in the sandy soil by tillage since aggregation in this soil is negligible. Moreover, N fluxes resulting from mineralization were probably not relevant to inhibit fixation since the soil only contained 12 g OM kg^{-1}. Horn *et al.* (1996) and Shah *et al.* (2004) did not find any significant effect of tillage system on the rate of N_2 fixation by chickpea (*Ciccer arietinum* L.) and lentil (*Lens culinaris* Medikus), respectively. However, former authors observed a greater N accumulation in no-till (66 kg N ha^{-1}) comparing with conventional tillage (44 kg N ha^{-1}) system, while the latter found an apparently greater crop N yield under tillage than under no-till.

Incorporation of harvest residues from *Eucalyptus globulus* L into the soil, at Óbidos site (experiment B), showed no significant effect on the fixation rate by *L. luteus* (99% Ndfa) with the indigenous soil rhizobium (Table 3), and only a 3% reduction of fixed N_2 was noted in presence of those residues. This indicates that allelopathic compounds of those residues were not able to depress the leguminous growth and symbiosis, following the trend reported by Heckman and Kluchinski (1995) for soil amended with oak, maple, sycamore or walnut leaves, where no indication of an allelopathic inhibition on nodulation or N_2 fixation by soybean (*Glycine max.* (L.) Merr.) was observed. Our results are not in agreement with effects of allelopathic compounds in leachates of bark, fresh leaves and leaf litter of *E. tereticornis* L., *E. camadulensis* L., *E. polycarpa* L. and *E. microtheca* L., which showed a negative influence on the germination, DM yield and nitrogenase activity of redgram [*Cajanus cajan* (L.) Mills] (Sasikumar *et al.*, 2001). Findings of the present study suggest that *E. globulus* L. harvest residues may be innocuous for the indigenous rhizobium and legume germination and growth.

A great reduction in shoot N concentration during the growth cycle of sweet lupine (41 to 26 g N kg^{-1} shoot DM, from vegetative to pod-filling phases, respectively) (data not shown) was associated with a dilution effect caused by the crop development and the withdrawn of N to the reproductive organs (29 g kg^{-1} pod DM) after the onset of the flowering stage. A decrease in N concentration was also observed in roots during the growth cycle (32 to 12 g kg^{-1} DM) mainly by the dilution effect, roots decay, and nodules detachment and necrosis. Similarly to *L. albus* L., shoot N concentration in yellow lupine decreased with the plant age from the vegetative phase to maturity (37 to 10 g kg^{-1} DM) (data not shown), whereas the pod N increased from pod-filling to the harvest (32 to 62 g kg^{-1} DM, respectively). Also, the N concentration decreased from 39 to 16 g kg^{-1} DM in roots during the growth cycle. This trend is in agreement with Howieson *et al.* (1998) who reported a decrease of shoot N from 44 at vegetative to 15 g N kg^{-1} DM at ripening stages by *L. angustifolius* L.. The distribution of N in shoots, roots and pods has important implications for the %NHI and fertilizer N value of legume residue for the associated crop or the subsequent crop in a rotation. Nitrogen harvest indices were 9% and 50%, respectively for the white and yellow lupine species, though in the case of the former the estimation was done at pod-filling stage and it should result in a higher NHI value when calculated at maturity stage.

According to Jensen and Hauggaard-Nielsen (2003), the incorporation of residues from grain legumes contributes to maintain soil N level, but the overall benefit is much less than from legume-based pastures because of high NHI in pulses. But because of the focus on aboveground biomass N, the contribution of grain legumes to the N budget in rotation is generally underestimated. In addition, maximum contribution to the soil N pool occurs when the legume is 100% symbiotically dependent, since in this situation soil N is not taken up by the legume. Nitrogen output in the pods+grain of *L. luteus* at maturity (68 kg N ha^{-1}) was used to evaluate the N credit of this crop. Visible roots+nodules contained about 4% of aboveground N and accounted for 4% of total N_2 fixed in the crop. The potential soil N input by this pulse was estimated by including the above- and belowground visible roots-nodules at the end of ripeness and amounted to +69 kg N ha^{-1} (50% of crop N) showing a positive fertilizer N value for associated crop or subsequent crop in rotation. Considering the pods+grain at pod-filling stage of white lupine, a soil N input of +96 kg N ha^{-1} (93% of crop N) was estimated, but a smaller gain of N to the soil at crop maturity is expected due to a higher %NHI in the crop at this phase, caused by a greater N mobilization in the plant from shoots to pods. If crops are grown for the sole purpose of providing N in organic farming, the N contribution of white pulse at pod-filling (100% Ndfa) may reach +110 kg N ha^{-1} (see Table 2) and +144 kg N ha^{-1} (100% Ndfa) by the fodder lupine at pod formation (see Table 3). These values are of the same magnitude or even greater than the maximum values reported by Howieson *et al*. (1998), Jensen *et al.* (2004) and De Varennes *et al.* (2007) who found a soil N benefit by white lupine in Europe and Australia varying from +32 to +96 kg N ha^{-1}, depending on the growth phase, and do not agree with the N deficit found in North America (–41 kg N ha^{-1}), which can be explained by the exclusion of fallen leaves in the estimate of N_2 fixation at maturity (Howieson *et al*., 1998). These authors also reported a net N input by *L. angustifolius* L. (+95 kg N ha^{-1}) similar to that estimated in the present study for *L. albus* L. at pod-filling, whereas Chalk *et al.* (1993) reported an annual input to the soil N by *L. angustifolius* L. of only +34 kg N ha^{-1}.

Mayer *et al*. (2004) found a large variation (+7 to +64 kg N ha^{-1}) of potential N derived from sweet pulse residues (stems, leaves, visible and decayed roots, and rhizodeposits**)**. Estimates of N balances based on standard root harvesting data may be confounded by neglecting decayed roots, detached nodules and rhizodeposition (exsudates and products of root and nodule necrosis), which may add significant amounts of N to the soil (22-68% of the total plant N for pulses), as supported by Fuhrer (2004), Yasmin *et al.* (2006), De Varennes *et al*. (2007), Mathieu *et al.* (2007) and McNeill and Fillery (2008). We assume that N inputs by study pulse crops can exceed the aforementioned value**s,** as decayed roots and rhizodeposits were not accounted for in the estimation of N benefit.

Transfer of symbiotically fixed N_2 from yellow lupine to eucalyptus seedlings

Nitrogen concentration in the newly-planted *E. globulus* L. was 19% lower in harvest residues amended area (Table 4) in comparison with trees planted in unamended sub-plots, which may be associated with soil N immobilization caused by the relatively high soil C/N (26) after harvest residues incorporation. On the other hand, N concentration in mineral N treated eucalyptus seedlings was significantly higher than in organically (leguminous) fertilized seedlings (see Table 4), for the average effect of soil amendment, but exported N did not differ significantly between both treatments (276 mg N tree^{-1}). In organically fertilized seedlings, the ^{15}N a.e. coming from labelled fallen leaves, roots, nodules and root rhizodeposits of associated fodder legume was about thirteen times smaller than the ^{15}N a.e. in mineral treated seedlings (Table 4). Nevertheless, the N transferred from legume to associated seedlings showed a potential benefit of slow N transfer from *L. luteus* to the associated young eucalyptus for medium-term productivity. The low FUE (4%) estimated for eucalyptus is in line with the values observed in drip-fertigated orchards of 8-month-old orange and pear trees (6%), planted in similar environmental conditions, by Menino *et al*. (2007) and Neto *et al*. (2008). These low FUE values can by explained by the small root development in transplanted plants, where the ratio between the above- and belowground biomass was 4.3 in eucalyptus and 1.5 in fruiting trees. Despite the low FUE values observed for Eucalyptus seedlings, it is expected a positive *Lupinus* effect at a later stage of forest plantations. In fact, in a field experiment, close to the site of the present study, foliar N and P concentrations were greater in 5-yr old trees which were associated with *Lupinus* (Madeira *et al*., 2010) during the early plantation phase, following results observed in tropical regions for mixed forest plantations (J. Leonardo Gonçalves, personal communication).

Although there is a high N input by *Lupinus* species, their use to improve tree growth in forest plantations is questionable under Mediterranean conditions. In fact, in the aforementioned field experiment, the intercropped *Lupinus* negatively affected the early eucalyptus growth either when the harvest residues were incorporated or removed from the soil (Madeira *et al*., 2010), where the understory biomass in treatments with yellow lupine was 2.1-7.5 times greater than in other treatments. This early tree growth suppression agrees with results observed in other studies where leguminous cover reduced tree growth, especially when the drought season was more severe (Malik *et al*., 2001; Mendham *et al*., 2004). Such trend may be related to competition between trees and understory vegetation for water resources, as eucalyptus root mass, at early growth stage, is mostly located in the 30-cm top soil layers (Jones *et al*., 1999) and therefore may be more sensitive to summer drought in Mediterranean conditions. However, the negative effect of *Lupinus* on tree growth decreased with time, which may be associated with reduced competition between trees and the cover crop, as tree roots may have explored deeper layers (Fabião *et al*., 1987; Madeira *et al*., 2010). In fact, at middle rotation (five years after plantation) of the aforementioned field study, a positive effect of legumes was observed, in the absence of harvest residues, which may be ascribed to inputs of N through leguminous and P due to superphosphate application (Madeira *et al*., 2010). Although small or nil *Lupinus* effects are reported on tree growth under Mediterranean conditions, it is expected a positive *Lupinus* effect at a later stage of forest plantations by improving at least nutrient availability and soil quality (Madeira *et al*., 2010). Therefore, sharing of N sources between the intercropped N_2 fixing plants and the non-fixing trees may contribute to a better use of N natural resources and to counteract nutrient removal by harvesting. Also at the early forest plantation phase, legumes plants may reduce leaching of N and base cations which tend to occur after tree harvesting.

Conclusions

Under Mediterranean conditions, white and yellow lupine species behaved as beneficial legumes when cropped in sandy soils, low in OM and available N. Tillage showed little impact on fixation rate by these pulses, but no-till slightly increased total amount of fixed N_2 by increasing plant biomass. Eucalyptus harvest residues in soil did not depress the legume growth, and activity or efficiency of symbiotic bacteria, and only induced an apparent reduction on symbiotic dependence. Although rhizodeposits were not accounted for the determination of soil N benefit by the two lupine species in the present study, relevant N inputs were found ($\geq$+69 kg N ha^{-1}), which are of great importance in a legume/cereal rotation or an association with non-legume crops, particularly when legume was cropped for organic farming and fixation was 100% (fixation at pod formation/pod-filling stages). Symbiotically transferred N_2 from the fodder legume to newly planted *E. globulus* L. was apparently favourable for the sustainability of forest systems. Results emphasized the need of continuing the study to confirm the positive effect of using the fodder lupine as organic supplier for the eucalyptus planted in soils with a low OM status, especially looking into the influence of different rooting patterns in the early forest plantation development. Also, studies on possible benefits of soil amendment with harvest residues from eucalyptus plantations should proceed in order to evaluate the physical and chemical changes in soil, and its long-term influence on symbiotic N_2 fixation.

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