Influence of tree canopy on N₂ fixation by pasture legumes and soil rhizobial abundance in Mediterranean oak woodlands

Carranca C^{2,*,**}, Castro IV², Figueiredo N², Redondo R³, Rodrigues ARF¹, Saraiva I², Maricato R² and Madeira MAV¹

¹Centro de Estudos Florestais, ISA/UL, Tapada Ajuda, 1349-017, Lisboa, Portugal
²INIAV, Qta Marquês, 2784-505 Oeiras, Portugal; *CEER-Biosystems Engineering, ISA/UL, Tapada Ajuda, 1349-017, Lisboa, Portugal; **ICAAM-UE, Aptdo 94, 7002-554 Évora, Portugal; corresponding author: Tel. +351 214403517, Fax +351 21446011, e.mail: corina.carranca@iniav.pt
³Laboratorio de Isotopos Estables, Universidade Autonoma, Madrid, Spain

Abstract

Symbiotic N₂ fixation is of primordial significance in sustainable agro - forestry management as it allows reducing the use of mineral N in the production of mixed stands and by protecting the soils from degradation. Thereby, on a 2 - year basis, N₂ fixation was evaluated in four oak woodlands under Mediterranean conditions using a split - plot design and three replicates. ¹⁵N technique was used for determination of N₂ fixation rate. Variations in environmental conditions (temperature, rainfall, radiation) by the cork tree canopy as well as the age of stands and pasture management can cause great differences in vegetation growth, legume N₂ fixation, and soil rhizobial abundance. In the present study, non - legumes dominated the swards, in particular beneath the tree canopy, and legumes represented only 42% of total herbage. A 2 - fold biomass reduction was observed in the oldest sown pasture in relation to the medium - age sward (6 t DW ha⁻¹ yr⁻¹). Overall, competition of pasture growth for light was negligible, but soil rhizobial abundance and symbiotic N₂ fixation capacity were highly favored by this environmental factor in the spring and outside the influence of tree canopy. Nitrogen derived from the atmosphere was moderate to high (54 – 72%) in unsown and sown swards. Inputs of fixed N₂ increased from winter to spring due to more favorable climatic conditions (temperature and light intensity) for both rhizobia and vegetation growth. Assuming a constant fixation rate at each seasonal period, N₂ fixation capacity increased from about 0.10 kg N ha⁻¹ per day in the autumn - winter period to 0.15 kg N ha⁻¹ per day in spring. Belowground plant material contributed to 11% of accumulated N in pasture legumes and was not affected by canopy. Size of soil fixing bacteria contributed little to explain pasture legumes N.

Keywords: above- and belowground organ, agro - forestry system, cutting frequency, light, *Quercus suber* L.

1. Introduction

Intercropping systems including pasture legumes have been used to recover degraded soils via their ability to improve physical, chemical and biological soil properties. This is the case of evergreen oak woodlands in the Mediterranean basin known by “montado” or “dehesa”, respectively in Portugal and Spain. These ecosystems consist of a mixed cropping which features *sensu lato* a widely spaced tree stand (20 - 80 trees ha⁻¹) and a ground cover of arable crops or permanent (natural or introduced) pastures. Typical trees are cork oak (*Quercus suber* L.), holm oak (*Q. rotundifolia* L.), mountain oak (*Q. pyrenaica*) and olive (*Olea europae* L.). Permanent biodiverse pastures consisting of more than twenty plant species, among legumes and non - legumes have been introduced in these systems in Portugal and are important for soil quality improvement and providing extensive grazing (Crespo, 2006). Persistence of newly sown pasture species must be considered as part of the decision managing process of the whole system. In Mediterranean regions such problem is evident since grasses are highly aggressive in competition with introduced legumes (Giller and Cadisch, 1995). Annual clovers with a re - seeding property such as the subterranean clover (*Trifolium subterraneum* L.) have been successfully used in these dry land pastures to provide grazing

1 during late winter and early spring. The proportion of legumes required to balance the nitrogen
2 (N) cycle of grazed swards depends on the appropriate choice of plant species, the rate of
3 pasture utilization, but also on the efficiency of N₂ fixation and the recycling of excreta, litter
4 and belowground material.

5 Light intensity affects the symbiosis by legumes since light enhances their photosynthetic
6 capacity, improving the plant nutritional status. Rhizobial in the symbiotic nodules (bacteroids)
7 needs a continuous supply of carbohydrates to produce the required energy and capture the
8 atmospheric N₂ (Carranca, 2013). Decreasing radiation reduces plant growth and yield (Smith
9 and Whiteman, 1983). This is demonstrated by diurnal variations in nitrogenase activity.
10 Mulongoy (1992) reported that a very few plants can grow and fix N₂ under shade. There is a
11 considerable literature on the subject of the distribution of radiation in plant communities, and
12 the influence of trees on light interception (Wilson and Ludlow, 1991), and on herbage biomass
13 (Jensen, 1987; Cubera et al., 2009). However, no data were found for the tree canopy influence
14 on the symbiotic N₂ fixation by pasture legumes and indigenous rhizobial population in agro -
15 forestry systems, in particular under the Mediterranean condition. The capacity of legumes to
16 intercept the solar radiation in these ecosystems is an important factor affecting their
17 competitiveness to survive (Cubera et al., 2009; Dubbert et al., 2014). Tree canopy influences
18 both the quality and quantity of light reaching the understory vegetation in a limited area, the
19 soil water content by intercepting the rainfall, and the plant evapotranspiration (Nunes et al.,
20 2002; Dubbert et al., 2014). The area below the canopy also concentrates a higher rooting
21 volume and contents of senescent leaves, fruits and decomposing fungi (ectomycorrhiza) which
22 may affect soil quality and pastures performance (Cubera et al., 2009).

23 In this context, a study was developed to test the effects of a cork oak tree in four
24 “montado” sites on the understory vegetation and rhizobial community. The study was
25 developed in one natural (unsown) and three improved (sown) mixed stands with different ages
26 at south Portugal, and the effects of a representative and well developed tree canopy were
27 evaluated for a two - year period on: *i*) the size of indigenous rhizobial population, *ii*) pasture
28 vegetation, and *iii*) N₂ symbiotic efficiency by associated legumes.

29 **2. Material and Methods**

30 **2.1. Sites description**

31 The study was developed in four “montado” sites located at south Portugal under rainfed
32 conditions, with annual rotational grazing pastures: a natural (unsown, control) pasture greater
33 than 25 – yrs - old and two improved (sown) pastures older than 5- and 12 – yrs - old were
34 chosen at Herdade do Olival (Estremoz - Évora, 38°51'42.66N, 7°32'32.83''W), and a sown
35 pasture greater than 30 – yrs - old was chosen at Herdade dos Esquerdos (Vaia Monte -
36 Portalegre, 39°07'-39°08'N, 7°29'-7°30'W). In both regions, the climate is of Mediterranean -
37 type. The mean monthly temperature does not differ significantly from each other and ranges
38 from 10 °C to 25 °C (Fig. 1A, B). Mean annual rainfall in Portalegre region, including
39 Vaia Monte is about 700 mm (Fig. 1A), whereas in Évora region including Estremoz, the annual
40 rainfall is about 670 mm (Fig. 1B).

41 Soils showed a loamy - sand texture and were classified as Dystric Cambisols and Eutric
42 Luvisols overlying granitic bedrock (IUSS, 2006), respectively at Vaia Monte and Estremoz.

43 **2.2. Layout of the experiments**

44 In 2010, six microplots (1.2 m² each) were arranged in each the aforementioned four “montado”
45 systems with annual rotational grazing pastures. All improved pastures were sown in the
46 autumn at time zero with a biodiverse mixture for grazing cattle, as proposed by Crespo (2006)
47 for Mediterranean rainfed environments. This mixture consisted of several cultivars and native
48 populations of legumes and grasses comprising perennials or self - reseeding annuals (Lelièvre
49 et al., 2008). The components of the mixture used were: *T. subterraneum* L., *T. vesiculosum*
50 Savi, *T. incarnatum* L., *T. resupinatum* L., *T. michelianum*, *Ornithopus sativus* Brot., *Lolium*
51 *multiflorum* Lam. and *Dactylis glomerata* L. at a rate of 25 - 30 kg seeds ha⁻¹. Legume seeds
52 were inoculated with *Rhizobium leguminosarum* bv. *trifolii*. at a rate of 10⁵ cells per seed. The
53 rhizobial population abundance in soils was studied in all pastures. To restrict the access of
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animals (cows, sheep, and pigs) for grazing the herbage during the study period, experimental sites were protected with fences and cages.

Each study site was chosen with a central, well established and representative cork oak tree (*Quercus suber* L.). The layout of the experiment was a split - plot design with completely randomized microplots. The main treatment was the cork oak canopy influence (beneath and outside the canopy) on pasture vegetation, N₂ fixation and soil rhizobial population size. The tree canopy influence was compared in four pastures with different age and composition. Two sets of microplots were installed in each site (pasture), one set consisting of three microplots randomly distributed beneath the tree canopy, and another set of three microplots randomized out of tree canopy influence. Experiments were repeated in 2012 using different microplots in each pasture.

Phosphorus (P) and potassium (K) were applied in 2010 as basal dressing at rates of 40 kg P ha⁻¹ and 50 kg K ha⁻¹, respectively. About one week after plant emergence (beginning of December 2010 and 2012), a rate of 3 kg N ha⁻¹ in the form of ¹⁵NH₄¹⁵NO₃ 5% atom ¹⁵N enriched was applied to each microplot for evaluation of symbiotic fixation according to the ¹⁵N dilution technique (Carranca et al., 1999). By the end of winter (February 2011 and 2013), complete plant samples (shoot + root + visible nodule) were harvested from a 1.0 m² area in each microplot. Much care was put when excavating roots from soil to avoid damaging the belowground plant material. After collecting the plant material, new microplots (1.2 m²) were immediately arranged in each site and the aerial plant material was cut and discarded to simulate the grazing. ¹⁵N fertilizer was added to these new microplots, at the same rate and form as previously described. In spring (April 2011 and May 2013), at bloom, plants were harvested as before. No more plant growth was observed after this last harvest.

2.3. Soil and plant sampling and analysis

Soil samples were collected in December 2010 and 2012 in each microplot for chemical and biological the soil characterization. For chemical analysis, four soil samples were taken from each microplot (0 - 20 cm depth) and bulked samples were air - dried, sieved (< 2 mm) and analyzed for pH_(H2O) in a soil : water suspension of 1 : 2.5 ratio and for total N and organic C by dry combustion (LECO). For biological studies, four soil samples were collected aseptically from each microplot at 0 - 10 cm depth and bulked samples were stored in sealed plastic bags under cooled conditions (6 °C) till microbial analysis processing. The size of rhizobial population was estimated by indirect count, using the Most Probable Number (MPN) method and a ten - fold dilution series (Somasegaran and Hoben, 1994). *Trifolium subterraneum* cv. Clare growing in Jensen's agar medium was used as host test plant (Vincent, 1970). Plants were grown for eight weeks in a controlled growth room (18 - 20 °C, 12 hours light / day). After this period, plants were harvested and roots were examined for nodulation. Results were transformed through log₁₀ to follow a normal distribution and were expressed as rhizobia bacteria number per gram of dried soil (Somasegaran and Hoben, 1994).

Plant material collected in the field was immediately transported to the laboratory and was washed with care using a sieve to recover all belowground material and was separated into legumes and non - N₂ fixing plants. Belowground material from legumes (visible roots + nodules) was easily separated from roots in the associated non - legumes. Botanical composition was described in 2011. Thereafter, plant samples were separated into above- and belowground (root and visible nodule) material and oven - dried at 65 °C for 48 h to determine the dry weight (g DW m⁻²), ground and sieved (< 0.5 mm) to determine total N (g kg⁻¹) and % ¹⁵N enrichment by dry combustion and mass spectrometry, respectively. The percentage of N derived from N₂ fixation (%Nda) was calculated using the equation: %Nda = (1 - (legume atom% excess / non-legume atom% excess)) × 100 (Carranca et al., 1999), where legume and the companion non - legume atom% excess was calculated by subtracting the ¹⁵N atom% enrichment of legume or companion non - legume from the air ¹⁵N atom% abundance (0.3663%). The amount of N₂ fixed (g N m⁻²) in the above- and belowground legume biomass was estimated by the product of %Nda and plant biomass (g DW m⁻²) divided by 100 (Carranca et al., 1999).

2.4. Statistical analysis

Results were analyzed by ANOVA (Statistics 6.0) using the General Linear Model to evaluate factors effects (years of study, tree canopy, sites, date of cut, plant species, plant organ) on vegetation biomass, %N derived from the atmosphere and N₂ fixation capacity. Soil chemical characteristics were also evaluated by ANOVA for soil pH_(H₂O), total organic C and N and rhizobial population (using the log₁₀ transformed data) in response to years of study, pasture type and the canopy effect. Means were separated using the Bonferroni's test at $p < 0.05$. Factor analysis (Principal Component Analysis - PCA) was applied to identify the main associations between the rhizobial abundance in soils, soil chemical characteristics and pasture legume features and also to discriminate the pattern of rhizobial population distribution by studied pastures.

3. Results

3.1. Soil chemical characteristics

Soil pH_(H₂O) in all pastures was slightly acid and did not differ significantly within years of study and among pastures (Table 1). Nevertheless, a higher value ($p < 0.001$) was determined outside the influence of cork tree canopy (5.8) compared with beneath the canopy (5.5). Similarly, total N and organic C did not vary significantly within the study period and among pastures (Table 1), but showed higher ($p < 0.001$) values under the canopy (1.8 and 23.4 g kg⁻¹, respectively for total N and organic C) compared with outside the tree influence (0.9 and 9.6 g kg⁻¹, respectively for total N and organic C).

3.2. Soil rhizobial population size

The abundance of soil rhizobial population declined in plots located beneath the tree canopy (Table 1), varying from an average value of about 3×10^3 bacteria g⁻¹ soil at Vaiamonte (> 30 - yrs) and Estremoz (> 12 - yrs) to a value of about 2×10^4 bacteria g⁻¹ soil outside the influence of tree canopy in both pastures (Fig. 2). The youngest pasture at Estremoz showed the highest rhizobial abundance, in particular outside the tree influence (5.2×10^4 bacteria g⁻¹ soil). Beneath the canopy, the unsown pasture (> 25 - yrs) had a similar population size to the existing outside the tree canopy (7×10^3 bacteria g⁻¹ soil), and similar to sown pastures greater than 12- and 30-yrs-old (Fig. 2).

No relevant relationships (PCA) were found between bacteria abundance and soil chemical characteristics (data not shown).

3.3. Pasture vegetation

After a long establishment (5- to > 30 - yrs period) and annual rotational grazing, plants community composition and diversity in sown swards was analyzed and compared with the mixture used at plantation, and compared with the natural vegetation in the unsown pasture. In 2011, *Lolium* sp. and *Phalaris* sp. were the dominant non - legume plant species in sown pastures at Vaiamonte and Estremoz, whereas composite plants and *Plantago* spp. were the dominant non - fixing plants in the control sward at Estremoz (> 25 - yrs - old). Among the species introduced, only *Lolium* sp. survived, in particular at Vaiamonte (> 30 - yrs - old) and in the youngest improved pasture (> 5 - yrs - old), at Estremoz. The medium - age pasture at Estremoz was mostly dominated by *Phalaris* sp., especially beneath the tree canopy (> 95% of non - legumes) (data not shown). After these periods, the dominant legume in all swards was the subclover (> 90% of legumes).

In general, non - legumes dominated the swards (Table 2) in particular beneath the tree canopy (Fig. 3). In the autumn - winter, legume production was significantly greater outside the tree canopy (90.0 g DW m⁻²) compared with the understory vegetation (22.5 g DW m⁻²) (data not shown); in spring, non - legume production was significantly higher (260.5 g DW m⁻²) under the tree canopy influence, but for legumes no significant differences were found in both positions (102.2 g DW m⁻²). Overall the two - years experiment, the total herbage production was 4335 g DW ha⁻¹. The average aerial biomass produced by legumes during the autumn -

winter in all pastures was equivalent to 409 kg DW ha⁻¹, i.e. 11% greater than the associated non-legumes (370 kg DW ha⁻¹) (data not shown), whereas in spring legume aboveground biomass (888 kg DW ha⁻¹) was 37% smaller than the non-N₂ fixing plant species (1413 kg DW ha⁻¹).

Nitrogen content in legumes (g N m⁻²) did not differ ($p \geq 0.05$) from the associated non-legumes (Table 2), although total N concentration was significantly higher in legumes (24 g N kg⁻¹ DW) compared with the non-legumes (16 g N kg⁻¹ DW) (Fig. 4). A similar result was observed for the tree canopy effect: herbage N did not vary under and outside the canopy influence (average of 0.98 g N m⁻², Table 2), but the vegetation N concentration differed significantly with a higher value measured beneath the tree canopy (19 g N kg⁻¹ DW) compared with the 18 g N kg⁻¹ DW outside its influence (data not shown). The same result was observed for legumes sole, and in particular the aboveground material: higher N concentrations were measured for the two cuts beneath the tree canopy (23.4 and 19.4 g N kg⁻¹ DW, respectively in the first and second cut), comparing with outside the canopy influence.

3.4. Symbiotic N₂ fixation in pasture legumes

As reported for plant biomass, estimates of N derived from atmosphere (%Nda) were affected by years of study, sites/pastures, cuts and several interactions, but not by the tree canopy (67% Nda) (Table 3). Contrasting to plant biomass, legumes fixed more atmospheric N₂ in 2012 / 2013 (72% Nda) compared with 2010 / 2011 (63% Nda) (Table 3). Estimates of %Nda were smaller ($p < 0.001$) in the oldest pasture (Vaiafonte) (54% Nda) compared with the others, which did not differ from each other (71% Nda). Unsown pasture showed a relevant symbiotic fixation with native soil rhizobia (68% Nda).

The interaction of pasture type and seasonal growth (cuts) was not significant for total N₂ fixed by legumes (Table 3), but as expected there was a trend for a significant increase on symbiotic fixation expressed as %Nda with the progress of the growing season, from autumn - winter (62% Nda) to spring (73% Nda) (Table 3), with the corresponding increase of fixed N₂ in spring, equivalent to an input of 9.2 kg N ha⁻¹ (Table 3), compared with the 6.2 kg N₂ fixed ha⁻¹ in the autumn - winter period.

Recovered belowground plant material of fixing plants (visible roots + nodules) did not vary by canopy effect (14.3 g DW m⁻²), for the mean effect of years of study, pastures and cuts, and represented 22% of aboveground legume biomass (65 g DW m⁻²) (data not shown). Belowground plant tissues fixed 52% Nda (Fig. 5), which represented 66% of N derived from atmosphere in aerial plant material (74%) and 11% of total N₂ fixed in the legume (Table 3).

Competition for light did not limit ($p \geq 0.05$) the legume growth (Table 2) and tissue N but limited N concentration (data not shown) and total N₂ fixed by understory vegetation (Table 3). This fact agrees with the previously described for rhizobial abundance in soil. Thereby the amount of fixed N₂ at the plant level beneath the tree canopy was equivalent to an average of 22.7 kg N ha⁻¹, lower than the significant input of 39.5 kg N ha⁻¹ fixed outside the tree influence (Table 3). However, the interaction of cuts with tree canopy showed that only in winter the N₂ fixed was significantly reduced beneath the canopy (Fig. 6).

Although the same pattern of response to canopy effect was observed for bacteria population in soils and total N₂ fixed by legumes, no relevant relationship (PCA) was found between rhizobial abundance in soils and pastures performance. Nevertheless, a weak relationship was observed between the N₂ fixed by legumes and the size of soil bacteria in sown pastures older than 5 - and 12 - yrs at Estremoz (Fig. 7A, B). In these pastures, rhizobial abundance also responded differently to supplemental variable "years of study": in the former there was a strong and positive relationship with this supplemental variable, whereas no association was observed in the latter. In the pasture older than 12-years, a strong and opposite relationship was found between this supplemental variable and total N₂ fixed by legumes, whereas no relevant association was noticed in the younger sown pasture. In the pasture older than 12-years, bacterial abundance in soil was strong and positively associated with the supplemental variable "canopy". This variable was strong and positively associated with the fixed N₂ in legumes of the pasture older than 5-years.

4. Discussion

4.1. Soil rhizobial population size

The importance of rhizobial population in soils where legumes are either present or have been recently cultivated has been largely reported (Hirsch, 1996; Zahran, 1999; Ferreira and Castro, 2011; Carranca, 2013). In the three sown pastures under study, rhizobial abundance measured in the soil in the autumn and outside the influence of the cork oak canopy was greater than 10^4 bacteria g^{-1} soil, but for the unsown pasture (7×10^3 bacteria g^{-1} soil) no significant variation in population size was observed for both positions. This result does not agree with Ferreira et al. (2010) who counted in the autumn about 5×10^4 CFU g^{-1} in soils over granites and schists (0 – 10 cm depth), outside the cork oak tree canopy influence, in the long term natural pastures of several “montado” systems located in the south Portugal, where the mean annual temperature ranged from 14 to 17 °C and the total rainfall varied from 500 to 700 mm per year. Bottomley (1992) and Pryor and Crush (2006) reported that bacterial population size fluctuates throughout the year, varying from < 10 to 10^7 bacteria g^{-1} soil, with higher values in spring and lower numbers in the autumn following a dry summer in the Mediterranean regions, but rarely exceeding 10^6 bacteria g^{-1} soil (Hirsch, 1996). Understory, where the soil pH averaged 5.5, only the youngest sown pasture soil (Estremoz) was richer than 10^4 bacteria g^{-1} soil. In soils with pH 5.5 - 7.5, Hirsch (1996) verified that the biovars of *R. leguminosarum* were present at 10^4 - 10^6 bacteria g^{-1} soil, but below or above this pH range the size of bacteria population was smaller.

Using the “whole - soil inoculation technique” proposed by Beck et al. (1983) and Brockwell et al. (1988), Soares et al. (2014) measured the indices of effectiveness (%E) of rhizobial selected in these pastures according to Ferreira and Marques (1992), with inoculation of subclover in a controlled environment and found that soil rhizobial populations were always effective ($E > 25\%$), and particularly high in the youngest sown pasture at Estremoz ($E = 88\%$). Soares et al. (2014) explained this result by the more recent seed inoculation of subclover with *R. leguminosarum* L. compared with other sown pastures. The lowest index of effectiveness was measured for the N_2 fixing bacteria in the unsown pasture ($E = 32\%$) which is in accordance with the lowest bacteria abundance in respective soils. Soares et al. (2004) using the ERIC-PCR technique (De Bruijn, 1992) also observed high rhizobial population diversity in all pasture soils ($D > 0.7$), especially in sown pastures outside the tree canopy ($D > 0.9$). These results indicate the existence of multiple molecular fingerprint patterns among rhizobia isolates, demonstrating that there was no selection for a single rhizobial genotype, even in the youngest sown pasture (Estremoz) previously inoculated with *R. leguminosarum* L., but demonstrating that some dominance was more evident beneath the tree canopy. Confirming present data for soil bacteria abundance in unsown pasture, rhizobial diversity was also not affected by tree canopy.

Suitable legume - *Rhizobium* associations are useful in providing a vegetation cover in degraded lands and valuable inputs of N that become available to intercrop non - fixing plants. The herbage dry matter production in the present study was site - specific due to differences in pasture age and composition. The medium age sown sward at Estremoz was the best situation. Unlike, edaphic conditions (chemical characteristics) and native soil rhizobial population size were not relevant to discriminate between pastures performance. Although soil rhizobial population is frequently associated with an efficient plant nodulation, present data could not fully support such a relation since no relevant association (PCA) was observed between the soil rhizobial population size and pastures biomass (either aerial or belowground) or N_2 fixing capacity. In fact, the highest value of rhizobial abundance was obtained in the youngest sown pasture (> 5 - yrs - old) at Estremoz (Table 1) where the herbage production was the lowest (equivalent to $3 \text{ t DW ha}^{-1} \text{ yr}^{-1}$) (data not shown). The smallest size of native soil N_2 fixing bacteria was able to form an efficient symbiosis with the wild legume in the unsown pasture as demonstrated by the moderate fixation rate (68% Nda, corresponding to $27 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) (Table 3). A single determination of the size of soil rhizobial population in the autumn was apparently not a good biological indicator to describe permanent pastures performance or symbiotic fixation capacity of pasture legumes, mainly the subclover under the Mediterranean

condition (Fig. 7A, B), since symbiotic fixation was determined by ^{15}N technique which gives an integrative measurement for the whole season. Macdonald et al. (2011) reported that the use of targeted gene probes for assessing environmental perturbations of indigenous soil rhizobial populations is probably more sensitive than the conventional plant bioassay and MPN methods.

4.2. Pasture vegetation

The herbage in all pastures was dominated by the non - legumes throughout the entire growing season, and in particular beneath the tree canopy. The proportion of subclover dry matter increased from the first to the second cut (data not shown) when it did not differ from non - legume species; it also increased outside the canopy especially in the autumn - winter, suggesting the effect of sunlight intensity. Jensen (1987) observed that the tree removal had no significant effect on early season of herbaceous vegetation including legumes, but had a highly significant effect on mid - season and end of season standing crops. Cleared plots yielded 28, 47, and 46% more than tree covered plots at early, mid, and end of season time of observation, while tree covered plots yielded 7, 8, and 12% less than open grassland plots at these times.

After a longer period than five years, overall legumes persistency represented 42% of total aboveground biomass, in the normal range of values reported in the literature (10 - 45% after 2 - 3 years) for Mediterranean environments (Giller and Cadisch, 1995; Rochon et al., 2004). The medium - age sown pasture at Estremoz showed a significantly higher proportion of legumes in the total herbage (54.6%) and produced the highest herbage biomass (6.3 t DW ha⁻¹ season⁻¹). Overall, total herbage DW production (legumes + non-legumes and above- + belowground material) in the whole season was greater than 4.3 t DW ha⁻¹ yr⁻¹, being much higher than that estimated (2.2 - 2.8 t DW ha⁻¹) by Cubera et al. (2009) for unsown pasture in evergreen holm oak woodlands in southern Portugal,

The study indicates that the influence of year - to - year variations in rainfall is an important factor contributing to the observed differences in herbage growth and symbiotic fixation rate. This suggests that scenarios of climate change (e.g. long dry periods) should be also taken into account for the choice of better adapted pasture to withstand extreme events.

The lack of variation with canopy treatment regarding harvested belowground biomass throughout the growing season is not in agreement with the finding reported by Huss - Danell et al. (2007), further studies being needed for deeper understanding on the subject.

Lolium sp. and *Phalaris* sp. were the dominant grasses in improved pastures, whereas *Plantago* and composite plants dominated the unsown sward. This trend agrees with Lelièvre et al. (2008) who reported that *Lolium* and *Phalaris* show the highest growth rates in the autumn and winter in Mediterranean conditions, especially when the average temperature is higher than 8 °C, and could produce two cuts / grazings in February and March / April.

4.3. Symbiotic N₂ fixation by pasture legumes

Present data have provided new insights in N₂ fixation in the oak woodland in Mediterranean systems, and particularly about the tree canopy influence.

The N content in legumes per unit area was highly dependent on legumes DW. Legumes and non - legumes accumulated equal amounts of N, equivalent to about 20 kg N ha⁻¹ yr⁻¹ by each group of plant (total amount of 40 kg N ha⁻¹ yr⁻¹ pasture⁻¹, data not shown), but N concentration was higher in legumes (22 g N m⁻²) than in non - legumes (15 g N m⁻²). These results agree with those reported by Huss-Danell et al. (2007) who observed that the N concentration was about twice as high in the clover as in the grasses. In case of legumes, harvested roots + nodules showed a N concentration (19.7 g N kg⁻¹ DW) similar to the reported in the literature for red clover cultivated in Sweden (19.3 g N kg⁻¹ DW) (Huss-Danell et al., 2007).

Estimated rates of N₂ fixation in the present study were moderate to high (54 - 72% Nda), in agreement with some literature (Tsialtas et al., 2004; Carranca, 2013). Nevertheless, they are greater than those observed by Carranca et al. (1999) for subclover intercropped with *Lolium perenne* L. and *Dactylis glomerata* L. (37 - 46% Nda, corresponding to an input of 12 - 27 kg N ha⁻¹ yr⁻¹) in ungrazed sward under similar environment, where the amount of rainfall was only 450 mm. Fixation rate in the unsown pasture (68%) was moderate and did not confirm the

lowest index of effectiveness ($E = 32\%$) determined by Soares et al. (2014) under controlled conditions and for a single selected subclover cultivar.

Amounts of N_2 fixed allocated to subclover herbage during the whole season were in the range of 2 to 7 g N m⁻² (Table 3). The high amount determined in the medium - age sown pasture at Estremoz was above the range (4 - 6 g N m⁻²) reported by Huss-Danell et al. (2007) for red clover (*Trifolium pratense* L.) in Sweden, under an unusually high rainy season. In this 12 - yrs- old sown pasture, *Phalaris* dominated the sward and was apparently a stronger competitor for soil N. Pasture legumes (mostly subclover) were forced to rely more on symbiotic fixation as N source to produce the highest plant N (66 kg N ha⁻¹ yr⁻¹, Table 3), compared with other pastures. This was particularly relevant outside the tree canopy influence (41 and 91 kg N ha⁻¹ yr⁻¹, respectively under and outside the tree canopy), where *Phalaris* represented more than 95% of total non - legumes (data not shown), the soil N was more depleted compared with soil beneath (Table 1), and light was not a limiting factor either for legumes and rhizobial growths. The dominant grass in the youngest pasture at Estremoz and the oldest one at Vaiamonte was the *Lolium* sp. (data not shown). According to Carlsson and Huss - Danell (2003), the *Lolium* sp. is a weaker competitor for inorganic soil N compared with the tall *Phalaris*.

Aboveground measurements of N_2 fixed give net cumulative seasonal values while root measurements rather give point - in - time values by the turnover of fine roots and leakage of root cells content. In the present study, no consistent data were observed for the variation of total N concentration in the belowground material with pasture age, but a tendency for a reduced amount of N_2 fixed was noticed: sown pasture older than 30 years showed the lowest %Nda (44% in belowground organ, compared with 55% Nda in other pastures, Fig. 5) and together with the unsown pasture older than 25 years had the lowest amount of fixed N_2 (0.13 g N m⁻², compared with 0.28 g N m⁻² in the medium - age sown pasture) (data not shown).

In the literature, most estimates of symbiotic N_2 fixation rely on the shoot N (Carranca et al., 1999; Peoples et al., 2001). Data on the contribution of belowground tissue for total N_2 fixed by legumes (either pasture or grain legumes) are rare (Høgh - Jensen and Schjoerring, 2000; Carranca et al., 2009, 2013; Sierra and Daudin, 2010). The limited knowledge on the contribution of roots + nodules to this process is partly due to difficulties in undertaking quantitative studies on belowground inputs. Recent studies have shown that the N contribution by root harvest (visible roots + nodules) can be greater than previously thought (< 10% of plant N). As demonstrated in the present experiment, 11% of total N_2 fixed was accounted by the harvested belowground tissue. This proves that studies relying on the shoot N have greatly underestimated the role of legumes in maintaining the N fertility in soils.

Few studies have also examined the effect of cuttings / grazing frequency on the development of the aboveground as well as the belowground organs, with emphasis on below - harvest contribution to the total N_2 fixation in mixed stands. The variations in the amounts of N_2 fixed during the present seasonal courses corresponded well to the seasonal changes in subclover biomass. Symbiotic fixation increased significantly from winter to spring due to more favorable climatic conditions for both rhizobia and vegetation growths, in particular the temperature and light intensity. The presence of active indeterminate nodules in the infected roots of indeterminate crops also contributed significantly to the continuous fixation rate by pasture legumes (mostly subclover). The N_2 fixing capacity varied from 0.10 kg N ha⁻¹ per day in the autumn - winter period to 0.15 kg N ha⁻¹ per day in spring, assuming a constant daily fixation rate in each period. This value was similar to that estimated by Carranca et al. (1999) in the young sward (1 - 2 - yrs - old) based on subclover mixed with *Lolium* and *Dactylis* for the May - June period (0.2 kg N ha⁻¹ day⁻¹). It was also similar to the daily N allocation to red clover herbage determined in Sweden by Huss-Danell et al. (2007) for the periods 21 May to 24 June and 31 August to 8 October. But a much greater daily fixation rate (0.6 to 1.0 kg N ha⁻¹ per day) was also estimated by these authors (2007) in the same study for the mid - season (29 June to 20 August). For the interaction between the seasonal growth and tree canopy assuming the mean effects of other factors, the amount of N_2 fixed in the understory vegetation was lower in the autumn - winter period (0.6 g N m⁻²) than in the spring (1.6 g N m⁻²), but the cork oak tree

canopy did not affect the total N₂ fixed in the spring growth (2.0 g N m⁻²) (Fig. 6) since light intensity was high enough to promote fixation in both positions. No reference data were found to sustain our findings. The oldest sown pasture showed a constant fixation rate along the season (54% Nda), whereas other pastures increased ($p < 0.001$) the fixation rate in spring (79% Nda) compared with 53% Nda in the autumn – winter period (data not shown). Therefore studies should be developed to assess the pasture age effect on N fixation rate along the growing season.

Overall, present results suggest that competition of pasture growth for light was negligible, but soil rhizobial abundance and symbiotic N₂ fixation capacity by legumes were highly favored in the spring and outside the tree canopy influence. Under controlled conditions, Armas and Pugnaire (2011) observed that legumes growth was not affected by light, whereas in temperate latitudes, Sprent (1999) and Vitousek et al. (2002) observed that legumes were often shade - intolerant. Further studies are recommended to consolidate the present findings on the effects of shading and rainfall interception by widely - spaced tree canopies on the vegetation cover for extensive grazing.

Conclusions

Nowadays, legumes are considered important components of the strategy for increasing production and sustainability of “montado” ecosystems by protecting soils from degradation and by providing N to the soils. Thus, factors affecting negatively the symbiotic N₂ fixation should be taken into account and avoided. The appropriate choice of plant species to be used in the Mediterranean area is relevant and must include the subclover, *Lolium* and *Phalaris* plants. These species persisted for the long term producing a vegetation biomass higher than the unsown pasture which is appropriate for an extensive grazing. The light and rainfall interception by the widely – spaced cork oak trees in particular during the autumn – winter season did not affect the vegetation beneath, but reduced the rhizobial population size and the capacity of N₂ symbiotic fixation by legumes. In the spring, when the light intensity and air temperature are high and more favorable for plants and bacteria growth, no negative effect of tree shade was observed on the symbiotic fixation. The widely – spaced trees are then fully recommended for an appropriate management of “montado” agro - forestry systems.

Although legume capacity for N₂ fixation and soil rhizobial abundance responded in the same way to the tree canopy effect, the size of soil fixing bacteria contributed little to explain the accumulated N in pasture legumes denoting that a single sampling in the autumn for determination of rhizobial population size in the soil was not a good biological indicator to describe the permanent pasture performance, in particular the symbiotic fixation determined by ¹⁵N technique which gives an integrated measurement for the whole season. Another reason could be the MPN method used for rhizobial abundance in soil. The use of targeted gene probes for assessing environmental perturbations of indigenous soil rhizobial populations might be more sensitive than the conventional plant bioassay and MPN methods.

Further studies are recommended to consolidate the present findings on the effects of shading and rainfall interception by widely - spaced tree canopies on the vegetation cover for extensive grazing, and by using the molecular techniques for rhizobial population to better understand the role of this parameter as a biological indicator for pasture legumes performance.

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Table 1. Some chemical and biological characteristics of pasture soils by the influence of study period (years of study), pasture type and cork oak canopy

Source of variation	pH _(H2O)	Total N (g kg ⁻¹)	Total organic C (g kg ⁻¹)	Rhizobial population (bact. number g ⁻¹ dry soil)
<i>Years of study</i>				
A	5.7a	1.35a	16.7a	14x10 ³ a
B	5.7a	1.34a	16.9a	18x10 ³ a
<i>Tree canopy</i>				
Under	5.5b	1.81a	23.37a	8x10 ³ b
Out	5.8a	0.87b	9.56b	24x10 ³ a
<i>Pasture type</i>				
4	5.5a	1.39a	16.72a	11x10 ³ b
3	5.7a	1.16a	14.89a	11x10 ³ b
2	5.7a	1.34a	16.83a	37x10 ³ a
1	5.7a	1.46a	17.41a	7 x10 ³ b

ANOVA	F - value	F - value	F - value	F - value
Years of study	ns	ns	ns	ns
Tree canopy	20.58***	97.17***	84.72***	9.13**
Pasture type	ns	ns	ns	6.76**
Interaction	ns	ns	ns	ns

Years of study (yrs): A = 2010/2011, B = 2012/2013; ANOVA = Analysis of Variance; ns, **, *** = F - values not significant ($p \geq 0.05$), and significant for $p < 0.01$ and $p < 0.001$, respectively; for each factor, means with different letters in the same column differed significantly according to Bonferroni test for $p < 0.05$. (1 = Estremoz, natural > 25 - yrs, 2 = Estremoz, improved > 5 - yrs, 3 = Estremoz, improved > 12 - yrs, 4 = Vaiamonte, improved > 30 - yrs).

Table 2. Means comparison and ANOVA results for seasonal and annual plant biomass (g DW m⁻²) and seasonal plant N (g N m⁻²) in four pastures at south Portugal, during 2010 / 2011 and 2012 / 2013

Source of variation		Seasonal plant biomass (g DW m ⁻²)	Annual plant biomass (g DW m ⁻²)	Seasonal plant N (g N m ⁻²)
<i>Years of study (Y)</i>				
	A	68.87a	510.97a	1.34a
	B	44.51b	356.08b	0.81b
<i>Tree canopy (T)</i>				
	Under	54.90a	439.22a	0.94a
	Out	53.68a	427.83a	1.01a
<i>Pasture (P)</i>				
	4	42.32b	338.58b	0.75bc
	3	78.69a	629.48a	1.60a
	2	42.74b	341.92b	0.61c
	1	53.01b	424.12b	0.95b
<i>Cut (C)</i>				
	Winter	30.75b	-	0.71b
	Spring	77.63a	-	1.23a
<i>Plant species (S)</i>				
	Legume	39.60b	-	0.97a
	Non-legume	68.78a	-	0.98a
<i>Plant organ (O)</i>				
	Aboveground	77.03a	-	1.50a
	Belowground	31.35b	-	0.45b
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ANOVA		F - value	F - value	F - value
Years of study (Y)		28.7***	23.01***	20.7***
Tree canopy (T)		ns	ns	ns
Pasture (P)		22.6***	17.87***	37.4***
Cut (C)		168.1***	-	51.3***
Plant species (S)		65.1***	-	ns
Plant organ (O)		159.5***	-	214.7***
Relevant interactions:				
	YxC	ns	-	19.92***
	YxP	20.3***	ns	ns
	PxC	4.3**	-	ns
	PxS	ns	-	10.52***
	TxC	18.6***	-	8.17**
	TxP	8.1***	6.77**	5.67**
	CxS	49.4***	-	7.15**
	TxS	57.6***	-	52.65***
	YxCxP	11.1***	-	10.29***
	YxPxT	4.6**	3.68*	3.18*
	YxPxS	7.4***	-	ns
	PxTxS	12.9***	-	10.75**
	TxPxCxS	9.6***	-	5.17**
	YxTxPxS	ns	-	3.03*

Years of study (yrs): A = 2010 / 2011, B = 2012 / 2013; ANOVA = Analysis of Variance; DW = dry weight; ns, *, **, *** = F - values not significant ($p \geq 0.05$), and significant for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; for each factor, means with different letters were significantly different ($p < 0.05$) according to the Bonferroni test (1=Estremoz, unsown > 25 - yrs, 2 = Estremoz, sown > 5 - yrs, 3 = Estremoz, sown > 12 - yrs, 4 = Vaiamonte, sown > 30 - yrs).

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Source of variation		Seasonal plant biomass (g DW m ⁻²)	Annual plant biomass (g DW m ⁻²)	Seasonal plant N (g N m ⁻²)
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	A	68.87a	510.97a	1.34a
	B	44.51b	356.08b	0.81b
<i>Tree canopy (T)</i>				
	Under	54.90a	439.22a	0.94a
	Out	53.68a	427.83a	1.01a
<i>Pasture (P)</i>				
	4	42.32b	338.58b	0.75bc
	3	78.69a	629.48a	1.60a
	2	42.74b	341.92b	0.61c
	1	53.01b	424.12b	0.95b
<i>Cut (C)</i>				
	Winter	30.75b	-	0.71b
	Spring	77.63a	-	1.23a
<i>Plant species (S)</i>				
	Legume	39.60b	-	0.97a
	Non-legume	68.78a	-	0.98a
<i>Plant organ (O)</i>				
	Aboveground	77.03a	-	1.50a
	Belowground	31.35b	-	0.45b
<hr/>				
ANOVA		F - value	F - value	F - value
Years of study (Y)		28.7***	23.01***	20.7***
Tree canopy (T)		ns	ns	ns
Pasture (P)		22.6***	17.87***	37.4***
Cut (C)		168.1***	-	51.3***
Plant species (S)		65.1***	-	ns
Plant organ (O)		159.5***	-	214.7***
Relevant interactions:				
	YxC	ns	-	19.92***
	YxP	20.3***	ns	ns
	PxC	4.3**	-	ns
	PxS	ns	-	10.52***
	TxC	18.6***	-	8.17**
	TxP	8.1***	6.77**	5.67**
	CxS	49.4***	-	7.15**
	TxS	57.6***	-	52.65***
	YxCxP	11.1***	-	10.29***
	YxPxT	4.6**	3.68*	3.18*
	YxPxS	7.4***	-	ns
	PxTxS	12.9***	-	10.75**
	TxPxCxS	9.6***	-	5.17**
	YxTxPxS	ns	-	3.03*

Years of study (yrs): A = 2010 / 2011, B = 2012 / 2013; ANOVA = Analysis of Variance; DW = dry weight; ns, *, **, *** = F - values not significant ($p \geq 0.05$), and significant for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; for each factor, means with different letters were significantly different ($p < 0.05$) according to the Bonferroni test (1=Estremoz, unsown > 25 - yrs, 2 = Estremoz, sown > 5 - yrs, 3 = Estremoz, sown > 12 - yrs, 4 = Vaiamonte, sown > 30 - yrs).

Table 3. Means and ANOVA results for N derived from the atmosphere (%Nda) and seasonal and annual N₂ fixed (g N m⁻²) by legumes produced in 2010 / 2011 and 2012 / 2013 in four pastures at south Portugal

Source of variation		%Nda	Seasonal legume N ₂ fixed (g N m ⁻²)	Annual legume N (g N ₂ fixed m ⁻²)
<i>Years of study (Y)</i>				
	A	63.0b	0.88a	3.53a
	B	71.5a	0.66b	2.69a
<i>Tree canopy (T)</i>				
	Under	64.8a	0.56b	2.27b
	Out	68.6a	0.99a	3.95a
<i>Pasture (P)</i>				
	4	54.1b	0.45b	1.90b
	3	70.8a	1.65a	6.58a
	2	76.0a	0.32b	2.26b
	1	68.2a	0.68b	2.70b
<i>Cut (C)</i>				
	Winter	61.8b	0.62b	-
	Spring	72.7a	0.92a	-
<i>Plant organ (O)</i>				
	Aboveground	74.3a	1.38a	-
	Belowground	51.9b	0.17b	-
ANOVA		F - value	F - value	F - value
Years of study (Y)		4.70*	4.8*	ns
Tree canopy (T)		ns	18.3***	14.23***
Pasture (P)		6.54***	35.8***	6.47**
Cut (C)		15.33***	8.9**	-
Plant organ (O)		59.5***	143.8***	-
Relevant interactions:				
	YxC	ns	16.2***	-
	YxP	8.05***	3.3*	ns
	PxT	ns	7.8***	6.47**
	PxC	7.60****	ns	ns
	TxC	7.23**	4.1*	-
	YxO	12.59***	ns	ns
	CxO	3.98*	11.8**	-
	PxO	ns	28.0***	-
	TxO	ns	12.5**	-
	YxPxT	4.95**	ns	ns
	YxPxC	ns	8.4***	ns
	YxCxO	4.06*	4.2*	-
	PxTxO	ns	7.1***	-

Years of study (yrs): A = 2010 / 2011, B = 2012 / 2013; ANOVA = Analysis of Variance; ns, *, **, *** = F - values not significant ($p \geq 0.05$) and significant for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; for each factor, means with different letters are significantly different according to the Bonferroni test for $p < 0.05$. (1 = Estremoz, unsown > 25 - yrs, 2 = Estremoz, sown > 5 - yrs, 3 = Estremoz, sown > 12 - yrs, 4 = Vaiamonte, sown > 30 - yrs).

Table 3. Means and ANOVA results for N derived from the atmosphere (%Nda) and seasonal and annual N₂ fixed (g N m⁻²) by legumes produced in 2010 / 2011 and 2012 / 2013 in four pastures at south Portugal

Source of variation		%Nda	Seasonal legume N ₂ fixed (g N m ⁻²)	Annual legume N (g N ₂ fixed m ⁻²)
<i>Years of study (Y)</i>				
	A	63.0b	0.88a	3.53a
	B	71.5a	0.66b	2.69a
<i>Tree canopy (T)</i>				
	Under	64.8a	0.56b	2.27b
	Out	68.6a	0.99a	3.95a
<i>Pasture (P)</i>				
	4	54.1b	0.45b	1.90b
	3	70.8a	1.65a	6.58a
	2	76.0a	0.32b	2.26b
	1	68.2a	0.68b	2.70b
<i>Cut (C)</i>				
	Winter	61.8b	0.62b	-
	Spring	72.7a	0.92a	-
<i>Plant organ (O)</i>				
	Aboveground	74.3a	1.38a	-
	Belowground	51.9b	0.17b	-
ANOVA		F - value	F - value	F - value
Years of study (Y)		4.70*	4.8*	ns
Tree canopy (T)		ns	18.3***	14.23***
Pasture (P)		6.54***	35.8***	6.47**
Cut (C)		15.33***	8.9**	-
Plant organ (O)		59.5***	143.8***	-
Relevant interactions:				
	YxC	ns	16.2***	-
	YxP	8.05***	3.3*	ns
	PxT	ns	7.8***	6.47**
	PxC	7.60****	ns	ns
	TxC	7.23**	4.1*	-
	YxO	12.59***	ns	ns
	CxO	3.98*	11.8**	-
	PxO	ns	28.0***	-
	TxO	ns	12.5**	-
	YxPxT	4.95**	ns	ns
	YxPxC	ns	8.4***	ns
	YxCxO	4.06*	4.2*	-
	PxTxO	ns	7.1***	-

Years of study (yrs): A = 2010 / 2011, B = 2012 / 2013; ANOVA = Analysis of Variance; ns, *, **, *** = F - values not significant ($p \geq 0.05$) and significant for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively; for each factor, means with different letters are significantly different according to the Bonferroni test for $p < 0.05$. (1 = Estremoz, unsown > 25 - yrs, 2 = Estremoz, sown > 5 - yrs, 3 = Estremoz, sown > 12 - yrs, 4 = Vaiamonte, sown > 30 - yrs).

Figures caption

Fig. 1. Climatic characteristics for (A) Portalegre (including Vaiamonte) and (B) Évora (including Estremoz) regions. (R = mean monthly rainfall; Tav = mean monthly temperature; Tmax = maximum monthly temperature; Tmin = minimum monthly temperature).

Fig. 2. Rhizobial population abundance (expressed as bacteria number per gram of dried soil) in four pastures in the “montado” agro - forestry system, under and outside the influence of a cork oak canopy.

Fig. 3. Total (above plus belowground) plant biomass for legumes and non - legumes (g DW m^{-2}) by the interaction of pasture and the cork oak canopy effect, and for the mean effects of years of study (yrs) and cuts (1 = Estremoz, unsown > 25 - yrs, 2 = Estremoz, sown > 5 - yrs, 3 = Estremoz, sown > 12 - yrs, 4 = Vaiamonte, sown > 30 - yrs).

Fig. 4. Interaction between legumes and non - legumes and cuts for the aerial biomass (g DW m^{-2}) and total N concentration ($\text{g N kg}^{-1} \text{DW}$), assuming the mean effect of years of study and tree canopy.

Fig. 5. Interaction effect of pasture and plant organ on fixation rate (%Nda), assuming the mean effects of years of study, cuts and tree canopy.

Fig. 6. Interaction of cuts with the tree canopy on N_2 fixation capacity by legumes above- and belowground biomass (g N m^{-2}) overall the study period and pastures at south Portugal.

Fig. 7. Principal Component Analysis (PCA) for the relationships between soil rhizobial population size and total N_2 fixed by legumes in (A) pasture 2 (sown sward older than 5 - yrs) and (B) pasture 3 (sown sward older than 12 - yrs), at Estremoz (south Portugal).

Figures caption

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Fig. 1

Fig. 2

Fig. 3.

Fig. 4

Fig. 5

Fig. 6

Figure
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A

B

Fig. 7.