

Legumes Cropping and Nitrogen Fixation under Mediterranean Climate

The Case of Montado/Dehesa System

Fernando Teixeira

Abstract

Climate change contributes to the environmental pressures that the Montado/Dehesa systems are experiencing, leading to an impoverishment of the floristic composition of the understorey. The strongly acidic soils of these systems are associated with nutrient deficiencies, nutritional disorders and the toxicity of metals, especially Mn and Al; these problems are discussed with emphasis on the antagonism between Fe and Mn and the relationship between K concentration and Mg uptake and concentration. The potential for the use of the legume-rhizobia symbiosis to increase biological nitrogen fixation and avenues for research are discussed. The co-colonization of the roots of legumes with arbuscular mycorrhizal (AM) fungi and the effects on P and Mn uptake are discussed. A better understanding of the relationships between soil pH, organic matter content (SOM), microbial community, soil P content and the plant strategies to mobilize it, as well as plant effects on the soil solution concentrations of Mn, is important for the management of these systems. The increase of biological nitrogen fixation in these systems, through the breeding of tolerant cultivars to acidic soils and a stepwise legumes enrichment, alongside soil fertility management, may contribute to increasing biomass production, SOM content and overall ecological plasticity.

Keywords: sustainable agriculture, Montado/Dehesa, legume, biological nitrogen fixation, acid soil, Mediterranean climate

1. Introduction

Plant biomass production is strongly correlated with nitrogen (N) availability which, in most farming systems, is dependent on the use of N-fertilizers. These N-fertilizers are obtained, with few exceptions, from the Haber-Bosch industrial process of atmospheric N₂ fixation which is energy demanding and responsible for 1.44% of the global emissions of carbon dioxide (CO₂) [1]. Contrastingly, most plants of the family Fabaceae (legumes), which comprises 751 genera and 19,500 species [2], can establish symbiotic relationships with rhizobia bacteria capable of fixing atmospheric N₂ into ammonia (NH₃), through the development of root nodules that host the bacteria (bacteroids). This symbiosis has been explored by humankind since the early beginning of agriculture and it still is an essential part of

many traditional agriculture farming systems (e.g., see [3]). In the Mediterranean basin and Europe at large, the rise of modern agriculture, which cannot be decoupled from relatively cheap N-fertilizers, has driven the abandonment of legumes in the farming systems. Still, legume usage in the frame of mixed pastures, and forages, did not decline over time as steeply as grain legumes did [4].

The Montado (in Portugal) or Dehesa (in Spain), is an agro-silvopastoral system, typical of the Southwestern part of the Iberian Peninsula, characterized by a savannah-like landscape, where the main tree species are cork and holm oak (*Quercus suber* and *Quercus ilex*, respectively), where it occupies an area of ca. 3.5 Mha [5]. The Montado/Dehesa is the result of the interaction of humans with the land, and it would not exist without it; cork and firewood harvesting, livestock, farming, pastures and cereal crops, among others, are activities that help to maintain the landscape features [5] and contribute to the rich biodiversity [6]. These ecosystems are presently under significant environmental pressures. Projections of the climate change in the Mediterranean basin show that in the decades to come the Iberian Peninsula will experience a reduction in precipitation and higher temperatures throughout the year (e.g., see [7]). Models suggest that these climatic changes will affect the distribution of the cork and holm oak, with an important reduction in the presence of these trees in the regions where they are presently found (e.g., see [8]). Other important environmental pressures on these ecosystems arise from the soil properties, affecting their resilience, namely, the strongly acidic reaction ($\text{pH} < 5.5$). In these soils, manganese (Mn) toxicity is often pointed out as the main cause of the low biomass productivity of the pastures (e.g., see [9]). Legumes may help to improve N content and P availability (organic P) through their rich underground biomass and surface plant residues and, thus, increase SOM content and counteract soil acidification. This chapter focuses on the legume-rhizobia symbiosis under rainfed farming in the acidic soils of the Montado/Dehesa systems, conditioned by the Mediterranean climate. The legume-rhizobia and tripartite symbiosis with AM fungi and a set of factors that have been identified as particularly challenging for legumes production are briefly reviewed. Possible avenues of research are identified that may allow, in the future, to enhance biological N-fixation and biomass production in these systems through a stepwise, low-input, legumes enrichment strategy.

2. Root-nodule symbiosis as mitigation of environmental pressures

The biological N-fixation produced by the legume-rhizobia symbiosis may have a profound effect on the Montado/Dehesa ecosystem by increasing the N content of the system and its availability to grasses and other forbs, increasing the overall biomass production and the soil organic matter (SOM) content. The term rhizobia designate diazotrophic bacteria of two different classes of Proteobacteria, encompassing species and strains well beyond those of the genus *Rhizobium*. Rhizobia N_2 -fixation only occurs in the frame of the symbiotic relationship with legumes [10]. Legume-rhizobia symbiosis is energy demanding for the plants, and thus, it only happens if there's not enough nitrogen available (nitrate and ammonium) in the soil to meet the plants' needs (e.g., see [11]). The bacteria in the symbiosis receive in exchange photosynthates as a carbon source. The plants control the symbioses, and nodule formation, through regulatory mechanisms, such as the "autoregulation of nodulation" (AON), carbon and nitrogen regulation of nodulation, among others (e.g., [11]). For the symbiosis to occur, both the legume host and the microsymbiont must be compatible [12]. The soil and climate conditions found in the Montado/Dehesa will dictate if legumes sowed, even when inoculated with compatible

rhizobia, will produce functional nodules, as the survival and thriving of both symbionts in the following years will only occur if both can cope with those conditions. In the next paragraphs, these environmental pressures are discussed along with the contribution of successful legume-rhizobia symbioses to mitigate them.

2.1 Soil reaction and toxicity of metals

Increasing SOM content may help to counteract soil acidification, to the extent that SOM constitutes an important proton buffer, and SOM depletion and low calcium (Ca^{2+}) saturation of the cation-exchange capacity (CEC) of the soil [13] may constitute one of the main reasons for soil acidification in the Montado/Dehesa system. The high concentration of protons in the soil solution leads to the solubilization of heavy metals that may become toxic to the plants, namely, aluminum (Al^{3+}) and Mn^{2+} (e.g. [14, 15]). The concentration of these toxic elements that plants may endure will vary with species and cultivars but often they have much lower thresholds than their wild counterparts (e.g., [16]). In low pH soils, nodule formation and nodule weight can be reduced by percentages above 90% and 50%, respectively [17]. Rhizobia bacteria can be found in a wide range of proton concentrations, with species (strains) surviving at pH values as low as 4 [25]. Nonetheless, soil acidification might have a profound effect on the survival of the bacterial strains present and thus on the occurrence of matching symbionts [26]. *Bradyrhizobium* spp. are, generally, more pH-resistant (tolerant) than *Rhizobium* spp. [17].

Proton [H^+] concentration in soil solution and the interaction with other elements, namely Al^{3+} and Mn^{2+} , affect plant growth. Aluminum [Al^{3+}] has no known biological function (e.g., [18]) but it can impair plant growth when in relatively high concentrations in the soil solution. The major factor affecting Al^{3+} concentration in soil solution is proton concentration and the presence of other ions that react with the dissolving/precipitating surfaces [15], namely, SOM (e.g., [19]). pH values above 4.5–5.5 are considered as leading to the precipitation of Al^{3+} which in relatively high concentrations affects root elongation and root hair formation likely due to the binding to the pectic matrix of the cell walls, substituting Ca, and hence cell wall thickening and rigidity (e.g., [20, 21]). The aerial part of the plant is also affected by Al^{3+} via induced nutrient deficiencies of magnesium (Mg), Ca and P, phytohormones imbalances and drought stress [20], but transport to the shoots, with some exceptions, is usually limited [22]. Plant Al-tolerance is characterized by the production of root exudates, organic acids and mucilage capable to chelate Al^{3+} , and by a lower CEC of the surface cell walls [20]. Pasture/forage legumes have different tolerance to different Al^{3+} concentrations. For example, the genus *Trifolium* has a higher tolerance than species of the genus *Medicago* (e.g., [23]), and very tolerant species, like *Lupinus luteus* (e.g., [24]), are capable of coping with Al^{3+} concentrations more than 20-fold than the most sensitive legumes. Wood et al. [25], working with *Trifolium repens* (white clover), observed an inhibitory effect of Al^{3+} on root hairs formation and root elongation, at concentrations of 50 μM and at pH 4.3 and 4.7, and no multiplication of *Rhizobium trifolii* and reduced nodulation for Al^{3+} concentrations of 50 μM at pH 5.5. Different rhizobia strains have been shown to grow at much higher Al^{3+} concentrations than the host [27]. Manganese [Mn^{2+}] plays an important role in plant growth, as a cofactor in many processes, from photosynthesis to the control of oxidative stresses (e.g., [28]); plant requirements of Mn are very low and a concentration of 50 $\mu\text{g Mn. g}^{-1}$ shoot DM is considered sufficient for normal plant growth [29]. Mn^{2+} concentration in soil solution is pH related, with concentrations reducing sharply above pH values of ca. 5–5.5 (e.g., [30]), but it is also dependent on the oxidation-reduction conditions of the soil (e.g. [14]), plant characteristics, namely, carboxylate exudation

behavior [30], and the microbiological activity (e.g. [31]). In studies with nutrient solutions, with similar ranges of pH and Mn^{2+} concentrations, it has been reported the inhibitory effect of Mn on the formation of root hairs of important commercial crops, such as soybean (e.g., [32]). Other studies, with similar Mn^{2+} concentrations, did not find any effect of Mn on root hairs formation or root elongation, e.g. in *T. repens* (white clover) [25]. Chen et al. [32] suggest that the soybean responses to Mn toxic concentrations, leading to the inhibition of root elongation, may be due to root cell wall modification and lignification. Many transporters can transport excessive amounts of Mn into the root cells, such as the iron-regulated transporters (IRT1), the “natural resistance-associated macrophage protein” (NRAMP), and many others [28]. The mechanisms of plant Mn-tolerance involve both, the ability to excrete and to store Mn in the cells. Nazeri et al. [33] observed a sharp decrease of Mn concentration in the roots of non-mycorrhizal *Trifolium subterraneum* after the supply of P, consistent with the excretion of Mn as no change in concentration of Mn in the shoots was observed. Although the mechanisms for Mn storage in the shoots are not known for most species, the ability to increase the concentration of carboxylate anions in the cells to chelate Mn is a possible explanation at least for some species [29]. Wood et al. [25] did not detect any effect of Mn at 200 μM on nodule formation in *T. repens*, for a pH range from 4.3 to 5.5. On the other hand, Izaguirre-Mayoral and Sinclair [34] observed that Mn at concentrations of 70 and 90 μM inhibited growth and nodulation of a soybean Mn-sensitive genotype but not on a tolerant genotype. Critical toxicity concentrations for Mn in the above-ground biomass range from 200 to 3500 $\mu g \cdot g^{-1}$ dry weight [35]. Some legume species are exceptionally tolerant to high leaf concentrations of Mn, above 7000 $\mu g \cdot g^{-1}$ dry weight (e.g., *Lupinus albus*) [29]. Keyser et al. [36] found no effect of Mn^{2+} (200 μM solution) in the growth of 23 strains of cowpea rhizobia and 10 *Rhizobium japonicum* (*Bradyrhizobium japonicum*), although a slowed growth was observed when Ca^{2+} concentrations were also low. Wood et al. [25] did not observe any effect of Mn^{2+} (200 μM solution) on the numbers of *R. trifolii*, and no interaction with Ca.

2.2 Soil reaction, nutrient deficiencies and nodulation

Phosphorus [P] is an important element in molecules participating in the intracellular buffering system (the conjugate acid-base pair $H_2PO_4^- - HPO_4^{2-}$), in the energy metabolism of the cells (e.g., ATP, adenosine triphosphate), in the formation of nucleic acids, among others. In acidic soils, low available P in soil solution is mainly due to its retention as adsorbed P on the surface of soil particles of Al- and Fe oxides [37]. Some plant species can exudate to the rhizosphere important amounts of carboxylates that are capable to mobilize Al- and Fe-oxide-sorbed P and also organic P. The organic P is then hydrolyzed by phosphatases, which are exudate to the rhizosphere. The inorganic P uptake by the plant occurs through a high-affinity inorganic P transporter in the plasma membrane of the root cells, belonging to the PHT1 gene family [38]. This strategy of P-mobilization is accompanied by the mobilization of other nutrients such as Mn [29]. Another strategy most plants follow is the promotion of symbiosis with arbuscular mycorrhizal (AM) fungi capable of scavenging phosphorus (available P) [39]; this strategy will be discussed further ahead. The relative importance of each of these strategies of P uptake, for each plant species/cultivar, and the interactions with the environment, may have an impact on the availability of other nutrients, namely, Mn and their uptake. Plants must possess adequate levels of phosphorus (P) otherwise the N-fixation rate by the microsymbiont will be conditioned by P-availability. For example, the molybdenum-dependent nitrogenase requires for each mol of N_2 reduction, 16 mol of ATP [40]. Nodulating plants allocate a substantial part of the P uptake to the nodules in soils with low

available P [41] and P fertilization may have an important effect on biologically-fixed N (e.g. [42]).

Iron [Fe^{2+}] is essential for biological N-fixation, for example, due to its role in the FeMo cofactor of nitrogenase [43] and the prosthetic group of the leghemoglobin. Fe content and availability to plants in acidic soils are usually high, but plant Fe-deficiency can occur in sandy soils with high concentrations of Mn^{2+} in soil solution [44]. Legumes, like all dicots, mobilize Fe through the acidification of the rhizosphere; the mobilized Fe^{3+} is then reduced to Fe^{2+} by plasma membrane reductases and the uptake happens through plasma membrane iron-regulated transporters (IRT1), in what is known as the strategy I of iron uptake [45]. Mn and Fe antagonistic relationship has been observed in many studies with legumes and non-legumes (e.g. [46]). Izaguirre-Mayoral and Sinclair [34] observed that: (i) a higher Mn concentration in the leaves of two soybean cultivars when in the presence of low Fe and high Mn concentrations in the culture solution and; (ii) a lower concentration of Fe in the leaves with increasing Mn concentrations in the culture solutions with high Fe concentration. In acidic soils, the Mn-induced accumulation of Fe in the roots may affect nodulation and nitrogenase activity.

Calcium [Ca^{2+}] is an essential nutrient in plant cells, namely, by its structural role in the cell walls and membranes, and the signaling role in the cytosol [47]. Calcium also plays many roles in the nodulation process of legumes, viz., in the root hair deformation and entrapment of rhizobia soon after nod factor release by the rhizobia [48]. The uptake of Ca^{2+} is mediated by plasma membrane transporters, the Ca channels [47]. These Ca channels may be permeable to Mn [28]. Nitrogenase activity can be reduced in acidic soils, particularly, if Ca concentration is low and at the early stages of plant development in common bean (*Phaseolus vulgaris* L. Dobruganca) [49]. Liming, to increase soil pH from 5.2 to 7.3, was shown to increase nodulation, root and shoot weight in 14 lucerne cultivars (*Medicago sativa*) [50]. Muofhe and Dakora [42], working with rooibos (*Aspalathus linearis*), observed a 27.2% increase in biologically-fixed N in response to Ca supply.

Magnesium [Mg^{2+}], besides its role in the chlorophyll molecule, and in a multitude of enzymes, also plays an essential role in ATP; ATP, to become biologically active requires binding with Mg (e.g., [51]). Several studies show a negative effect of K on Mg concentration in the shoot tissues (for reviews see, e.g., [52, 53]). This interaction of K x Mg may be of significance because, in the acidic soils of the Montado/Dehesa, K availability might be high, and low Mg concentration in the plant shoots may have a significant effect on plant growth and nutritional value as feed. The Mg^{2+} transporter(s) responsible for uptake into the root cells is(are) poorly known (e.g., [52]), although there is evidence of Mg^{2+} transport through Ca-channels [47]. Reduced translocation of Mg from the roots to the shoots, in presence of high K^+ concentration, might be the cause [53]. According to an analysis performed by Rietra et al. [52] on 94 peer-reviewed papers and 117 interactions (synergistic, antagonistic or zero-interactions) on crop yields, no interactions were found between Mg and Mn.

Molybdenum [Mo] is essential for some enzymes found in plants, involved in nitrogen metabolism and phytohormones synthesis [54]. Mo, as seen for Fe, is essential for biological N-fixation due to its role in the FeMo cofactor of nitrogenase [43]. A molybdate transporter type 1 (MTR1), that is a molybdate-specific transporter, has been identified in *Medicago truncatula*, and their expression in the nodules was determined [55]. Mo availability to plants in the soil solution correlates positively with decreasing proton concentration, being highest for soils with $\text{pH} > 6.6$, and with the percentage of soil particles with diameters smaller than 20 μm [56]. Adhikari and Missaoui [50], working with 14 Lucerne cultivars (*M. sativa*), a species particularly sensitive to low pH, observed that plants grown

in soils with a pH of 5.2 and Mo supplementation, had a statistically significantly higher number of nodules (53% more nodules) than the control.

2.3 Temperature

In the Montado/Dehesa, biomass accretion happens from fall through winter and spring. The length of the growing period will vary as there is no consistent rain-fall pattern from year to year. The daily minimum soil temperatures in the Winter months are often well below 5°C at 2 cm depth (e.g., [57]). In mid-Winter, as the growth rate of legumes increases in responding to favorable temperature and water availability so increases the potential for biological N-fixation. Biomass accretion of the annual species of the understorey ends in late May or early June after soil-available water has been used and the air temperatures are still relatively mild.

The tolerance of rhizobia to low temperatures varies, with different minimum temperatures for growth as low as 5°C, and survival –10°C [58]. Gibson [59] studied the effect of time and temperature in nodule formation of four subterranean clovers (*T. subterraneum*) cultivars and three *R. trifolii* strains, and observed inhibition of nodule formation below root temperature of 7°C, and an increased time to nodule formation as temperatures decreased below 22°C (from 4.1 to 5.7 days at 22°C to 20.2 to 24.2 days at 7°C); the author also observed that for plants with roots at 12°C, time to detect leghaemoglobin in nodules varied between 5 and 8 days (2–4 days for plants with root temperature of 22°C). Peltzer et al. [60], in a study with *Lupinus angustifolius* cv. Yandee, observed that nodule initiation at temperatures between 7 and 12°C failed due to insufficient exudation of flavonoids from the legume to activate nod factors of *Bradyrhizobium*. However, nitrogenase activity in nodules formed at adequate temperatures may occur at a much wider range of temperatures. Dart and Day [61] observed that nitrogenase activity, of nine different species, had a maximum for root temperatures of around 20 to 30°C, and that some species sustained nitrogenase activity for temperatures from 2 to 40°C; these authors also observed that at the temperature range of 2 to 10°C, this activity was only slightly reduced for *Vicia sativa* and *T. subterraneum*. In the winter months, low temperatures and relatively low light exposure of the understorey, as encountered in the Montado/Dehesa, is likely to affect the photosynthetic activity of legumes, and the carbohydrate content in the nodules (e.g., see [11]), affecting plant growth and nitrogenase activity.

2.4 Water stress

Extended periods of low or no precipitation during the growing season are very common in the Montado/Dehesa region and can affect symbiosis. Unsaturated soil conditions, and soil texture (especially in clayey soils), conditioning the diameter and continuity of saturated soil pores, affect rhizobia motility [62]. Thus, in the presence of a low concentration of rhizobia per gram of soil, the initiation of symbiosis may be dependent on transient saturated conditions after rainfall. N-fixation of nodulated legumes may be severely impaired by drought, well before photosynthesis is reduced, and the mechanisms for this response are species-specific and not fully understood; O₂ limitation, C availability and N feedback mechanisms have been proposed as playing an important role in the regulation of nitrogenase activity during drought periods [63]. A better understanding of these mechanisms would allow faster and smarter breeding for drought-tolerant legume species. On the other hand, the Montado/Dehesa systems are located in peneplains, and waterlogging is a common problem in some areas. Waterlogging has a profound effect on aeration and the redox conditions of the soil that can impose high Mn²⁺ availability over time [14]. The nodules, in saturated soils, will be deprived of free O₂, essential for the

oxidation of the carbohydrates to produce the energy needed for the nitrogenase activity; also the diffusion of CO₂ and H₂, gases that can inhibit nitrogenase activity, will be hindered [61]. Roberts et al. [64] discuss the model/role of a gas diffusion barrier in the nodules, capable to maintain a microaerobic state, ca. 20 nM O₂, under normal atmospheric conditions, that assure nitrogenase activity at suboptimal rates; changes of the O₂ partial pressure of the atmosphere lead to short term changes of the gas diffusion barrier permeability and the rapid inhibition of the nitrogenase activity (transient and fully recoverable), or long term changes, leading to changes in the cellular and subcellular morphology, including the formation of lenticels and secondary aerenchyma on the surface of the nodules. Depending on the severity of the hypoxic conditions and the exposure time, the adaptation of the legume, regarding the number of nodules and nitrogenase activity, may not be sufficient and, depending on the species/cultivars, the recovery and survival might be compromised. Pampana et al. [65] observed that 5 days of waterlogging during the flowering period were sufficient to reduce the number of pods and seeds of white lupin plants almost three-fold, as well as seed weight and shoot and root dry matter. On the other range of the spectrum, Pugh et al. [66] observed that white clover (*T. repens*) grown under saturated conditions from germination had, after 9 weeks, higher shoot dry matter than normally watered plants; the authors also observed that the plants normally watered had a substantial reduction of the acetylene reduction activity (an indicator of nitrogenase activity) when waterlogged (a reduction to 4%, when compared to previous activity) and that the acetylene reduction activity increased when permanently waterlogged plants were suddenly drained (a 250% increase). Both drought and waterlogging in the Montado/Dehesa are likely to affect the biological N-fixation although the effect of N-fixation on biomass yield requires further experiments allowing the separation of other effects on biomass yield (photosynthetic activity, nutrient uptake and translocation, root anoxia/hypoxia, and so on).

2.5 The importance of tripartite symbiosis

Legumes, besides symbioses with rhizobia bacteria, can establish symbioses with AM fungi in mutualistic relationships where the fungi increase the plant uptake of water and nutrients, in particular phosphorus, and receive photosynthates in exchange [39]. Most plants are co-colonized by multiple AM fungi species and endemic AM fungi, well adapted to the soil conditions, will compete with inoculated AM fungi for mycorrhization of the roots [39]. These symbioses may be important for N-fixation if in the presence of low concentrations of plant-available P. The mycorrhizal component may account for much of the P uptake of legumes and the direct uptake can be residual. Nazeri et al. [33] showed that mycorrhizal plants of *T. subterraneum*, grown under low P-available conditions, had higher P concentration in the roots and shoots, and lower Mn concentrations, when compared with non-inoculated plants, indicating alternative strategies to acquire P. Alho et al. [67], studying the effect of intact extraradical AM propagules, in undisturbed soils, on the infection of *T. subterraneum* by the fungi, observed that plants infected with intact propagules had statistically significant higher P and N concentrations in the shoots (214 to 515% and 203 to 479%, respectively), higher shoots and nodules dry weight (274 to 618% and 398 to 640%, respectively), and much lower concentration of Mn in the roots (34 to 56%) when compared to control (disturbed soil) 42 days after growth started; these authors observed also that the preceding plants, i.e. the plants grown to establish the mycorrhiza, being more or less mycotrophic, affected the infection of *T. subterraneum*, with non-mycorrhizal species producing statistically significantly lower values for all those variables when

compared with plants infected with intact propagules produced by mycotrophic species.

3. Gaps in current research

To increase the soil productivity in the Montado/Dehesa ecosystem, the correction of the soil reaction by liming is expensive but, where economically viable, it is effective, either with calcitic or dolomitic limes (e.g., [13]). However, the economic and social benefits of liming must be balanced with the ecological impact of this practice. From an ecological point of view, liming contributes to the emissions of greenhouse gases (GHG) from mining, transporting and incorporating the lime into the soil. Additionally, liming causes a marked stratification of the soil profile pH and the effects on the forest stand and acidophilic endemic species, in the long term, are unknown. On the other hand, liming potentially yield higher carbon sequestration (SOM), the improvement of several topsoil properties, higher feed production and quality (protein content), just to name a few. Unfortunately, although there are many metric approaches to quantify these variables there is no reliable model to assist in the decision to correct the soil reaction through liming in the Montado/Dehesa.

Alternatively, and although in a wider time frame, the benefits of liming can be achieved through higher SOM content (increasing CEC and the soil buffering capacity) and the management of soil fertility and plant nutritional deficiencies. Endemic legumes species, with cultivars selected for the traits of interest, can increase the N content of the system and N availability to other forbs and grasses, and, along with the correction of plant nutrient deficiencies, enhance biomass production and SOM content. Seeding with no-till systems would allow the preservation of the SOM content, without the exacerbation of microbial activity. It would also allow a sequential introduction of the cultivars of interest, beginning with those species/cultivars that can tolerate the soil conditions and boost soil organic matter (cultivars selected aiming acid soils reclamation and tolerant to the low light conditions of the understory), creating favorable conditions for the survival of the rhizobia of interest (already present or inoculated) and the preservation of AM fungi, in what can be defined as the first step in a **stepwise legume-enrichment**. These first introduced species/cultivars would be kept through self-seeding by allowing narrow bands to grow to maturity (seed formation) when cutting the pasture for fodder (hay or silage), or by grazing the legume-improved pastures only in the Summer. After achieving a design threshold of SOM content, correlated with higher nutrient availability and soil buffering capacity, pH-sensitive cultivars, capable of higher biomass accretion and adapted for the multi-diverse environments of the Montado/Dehesa, namely, the light/shade exposure, could be sowed. This low-input strategy for legume-rich forage in the Montado/Dehesa would require multidisciplinary research. The next paragraphs will discuss avenues of research readily identifiable: (i) legume species and phenotypic traits; (ii) micro-symbionts and symbioses; (iii) soil fertility and nutritional problems.

Legume species and phenotypic traits. In the Montado/Dehesa system, and conditioned by the spatial variability (environmental variability) caused by the forest stand, the best approach to improve biological N-fixation is through the use of mixtures of legumes with different phenotypical traits, capable of occupying these different environments. The plants' genera and species that should be the subject of plant breeding, are not dissimilar from those in the mixtures of the *Sown Biodiverse Permanent Pasture Rich in Legumes* system (see [68]), namely, the genus *Trifolium*, which has many species that are, at least, naturalized in the Iberian Peninsula, and

several other endemic genera, including *Ornithopus*, *Lotus* or *Lupinus*; however, breeding for the acidic conditions of the Montado/Dehesa should include traits such as low pH tolerance, Al tolerance, Mn tolerance (the storage capacity or exclusion of Mn), shade tolerance (photosynthetic efficiency), drought tolerance, waterlogging tolerance, high nutrient use efficiency, diseases and pests tolerance, matching rhizobia (the persistence in the soil) and the potential to mycorrhizal symbiosis.

Pastures sowed with mixtures of legumes in the Montado/Dehesa, in soils with pH in water between 4.9 and 5.94, increased the biomass production by more than three-fold, as well as the SOM content, and the protein content of grasses and non-legume forbs [69]. However, the positive effects observed in this study decreased continually from the first year onwards, suggesting the inadequacy of the cultivars sowed. From a stepwise legume-enrichment perspective, lupins may play an important role in the first steps of legume enrichment. The Mediterranean basin is the place of origin of important annual lupin species, with an important genetic pool for plant breeders. For example, in 2009, the number of accessions (landrace and wild types) distributed among different institutions totalled 1804 in Portugal and 5057 in Spain [70]. Lupins are tolerant to acidic soils, with low available P, and can cope with very high concentrations of Mn in the shoot tissues (e.g. [71]). Thus, at least conceptually, well-adapted cultivars of lupins, with good biomass accretion, mixed with other highly tolerant hardy cultivars of other genera could be sowed, increasing SOM and nutrient availability, and establishing/increasing the microsymbionts population, and their ability to survive. In this respect, lupins do not possess very high specificity to their rhizobia microsymbiont, being able to establish symbiosis with several species of *Bradyrhizobium* [72].

Microsymbionts and symbioses. Through screening of acid-tolerant rhizobia strains present in these soils, their matching legume hosts and N-fixation efficiency may lead to the expansion of the area of legume-ameliorated pastures in the Montado/Dehesa systems. In this respect, *Bradyrhizobium* species (and their hosts) may be of particular interest due to their higher tolerance to low pH soils [17]. Concerning AM fungi, when breeding legumes for improved biomass yield, the best cultivars are likely to be deprived of the genetic apparatus that favors symbiosis or alter the regulatory mechanism (the thresholds), increasing the specificity or decreasing susceptibility with the microsymbionts (e.g., [73]). Thus, at least conceptually, breeding new cultivars of legumes from endemic wild types may preserve the ability of these cultivars to establish symbiotic relationships with the different AM fungi present in these soils. In mycorrhizal legumes, the symbiosis may have a profound effect on P and Mn uptake and concentration (e.g. [33, 67]). The work of Alho et al. [67], studying plants and their mycotrophic character, and the highly positive effect of intact mycorrhizal on the infection of *T. subterraneum*, supports the concept of a **stepwise enrichment of legumes in the Montado/Dehesa**, based on the plant species present at the beginning of the process, and by the effect of no-till direct seeding of new cultivars to maximize mycorrhizal symbiosis. For annual legume crops, and especially under the Mediterranean climate and acidic soils of the Montado/Dehesa, the benefits from a tripartite symbiosis may be synergic, with an effect on biomass accretion caused by improved P uptake and N-fixation, much higher than the simple addition of the isolated effect of the microsymbionts, but this is yet to be demonstrated.

Soil fertility and plants' nutritional problems. The management of soil fertility is paramount for increasing the productivity and sustainability of these systems. Where total P is extremely low, P fertilization is needed and may induce higher N-fixation. Nevertheless, as observed by Hernández-Esteban et al. [69], P-fertilization has only a limited effect on pasture productivity, and produced a higher effect when applied to sown legume pastures; the reasons for the low effect

of P on natural pastures may have to do with the phenotypical traits of the wild flora which have evolved adaptation mechanisms to thrive in these poor and very dynamic environments. Even in strongly acidic soils, where the P-fertilizers are quickly adsorbed/precipitated in relatively insoluble forms, they will enter the soil's P-pool and will be made available by the plants and microbes in the future. The P-mobilizing strategies of legumes, non-legumes and the microbial community (e.g. [74]), and their effects on Mn availability and uptake of the different groups (legumes, other forbs and grasses), justify a comprehensive study of the relationships between these and other variables. In this respect, the P distribution within the plant (P allocation to the shoots, roots and nodules) can become, as suggested by [37], a tool for the determination of the symbiotic efficiency and/or the adaptation of the legumes (host-bacteria symbiosis) to the environmental conditions. Other plant nutritional disorders that can be detrimental to plant growth and biological N-fixation, such as the Fe and Mn antagonism, or the inhibitory effect of high K^+ uptake on Mg^{2+} uptake and concentration in the shoot tissues, should be further researched, as they can define new approaches to nutrient management, floristic composition of pastures, plant breeding, and others. The complexity of the relationships between different nutrient uptake and the concentrations of these elements in the plant tissues poses many challenges, namely, for screening candidate cultivars. A high-throughput ionomic approach, and the correlations between these elements in the plant tissues, which are highly species- and environmental-specific, can be a very useful tool (e.g. [75]).

4. Concluding remarks

The potential for biological N-fixation with legumes in the Montado/Dehesa systems is lower than in more northern regions in Europe due to the erratic rainfall patterns and the relatively low temperature during part of the growing season, and the poor and strongly acid soils. Increasing the potential N-fixation through liming is expensive and, in these sensitive biodiverse systems, with unknown consequences in the long term.

Legumes bred for tolerance to acid soils and associated metal toxicity, for drought and waterlogging, and for the low light conditions in Winter, could provide biodiversity and the potential to increase N-fixation in the multi-diverse environment, both spatial and temporal, of the Montado/Dehesa. A **stepwise approach**, through the use of no-till direct seeding, starting with the introduction of mixtures of hardy tolerant legume species/cultivars, and adequate soil and plant nutrient management can potentially create the soil conditions necessary for a second phase introduction of more sensitive legumes, but with higher biomass and N-fixation potential. Such a low-input strategy for legume-rich forage has the potential to increase the sustainability and productivity of these systems, by increasing the contents of N, C and organic P.

The avenues of research that are needed may prove beneficial beyond the natural borders of the Montado/Dehesa, by identifying legume cultivars and rhizobia strains tolerant to strongly acidic soil conditions useful in other regions of the world.

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Conflict of interest

The author declares no conflict of interest.

Author details

Fernando Teixeira
MED – Mediterranean Institute for Agriculture, Environment and Development,
Institute for Advanced Studies and Research, Universidade de Évora, Polo da Mitra,
Évora, Portugal

*Address all correspondence to: fteixeir@uevora.pt

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