Plant Science 182 (2012) 121–133

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Ecological and agronomic importance of the plant genus *Lotus*. Its application in grassland sustainability and the amelioration of constrained and contaminated soils

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ARTICLE INFO

Article history:
Received 30 November 2010
Received in revised form 16 March 2011
Accepted 24 March 2011
Available online 21 April 2011

Keywords:
Lotus
Symbionts
Abiotic stress
Restoration

ABSTRACT

The genus *Lotus* comprises around 100 annual and perennial species with worldwide distribution. The relevance of *Lotus japonicus* as a model plant has been recently demonstrated in numerous studies. In addition, some of the *Lotus* species show a great potential for adaptation to a number of abiotic stresses. Therefore, they are relevant components of grassland ecosystems in environmentally constrained areas of several South American countries and Australia, where they are used for livestock production. Also, the fact that the roots of these species form rhizobial and mycorrhizal associations makes the annual *L. japonicus* a suitable model plant for legumes, particularly in studies directed to recognize the mechanisms intervening in the tolerance to abiotic factors in the field, where these interactions occur. These properties justify the increased utilization of some *Lotus* species as a strategy for dunes revegetation and reclamation of heavy metal-contaminated or burned soils in Europe.

1. Taxonomy and geographical distribution of the genus *Lotus* (*Fabaceae*)

The *Fabaceae* is the third largest family of Angiosperms. This family shows an incredibly biological diversity, with approximately 720 genera and more than 18,000 species worldwide [1,2]. Most of legume species take profit from root symbiosis with mycorrhizal fungi, a trait shared with almost 90% of plant taxa (see Section 4.3). However, legume species are characterized by their ability to establish symbiotic interactions with nitrogen-fixing bacteria [1–8]. These associations turn them more competitive in nutrient-poor soils, so they are usually regarded as "pioneer" plants. These advantages of legumes stimulated their adoption in the ancient agriculture and then became an important part of sustainable agricultural systems [9].

The tribe *Loteae* DC. is a monophyletic group [10–12] composed by four genera [13]. Plants in this tribe produce flowers characterized by standard claw with thickened infolded margins, diadelphous stamens and a style hardened from the base [12,14]. The name *Lotus* was introduced by Linnaeus in 1753 and since then, there were several changes in the species delimited by this name [10,12]. Actually, the *Lotus* circumscription is considered one of the most problematic issues within the *Loteae* taxonomy [11,13], in part due to the limited number of appropriate morphological traits [15]. Fortunately, recently developed molecular tools have significantly contributed to unravel this issue. In fact, the 100–130 species currently included within *Lotus*, have been widely explored using molecular markers such as Nuclear Ribosomal Internal Transcribed Spacer (nrITS) sequences [10,11,16–19], Random Amplification of Polymorphic DNA (RAPD) [20,21], Restriction Fragment Length Polymorphism (RFLP) [22], Amplified Fragment Length Polymorphism (AFLP) [23] and DNA-Chloroplast analysis [24].

Most *Lotus* species are native to Europe, Asia, Africa, Australia and some to the Atlantic and Pacific Ocean Islands [14,17]. Few species were described as native to the New World, but they were later segregated in four [25–27] or two [28] non-*Lotus* genera. However, *Lotus* species have a worldwide distribution, except in very cold regions and certain tropical areas of Southeast Asia and Central America [14,17]. This worldwide distribution is partially due to their introduction to non-native areas by human activities and its adaptability to different environmental stresses [29,30].

Abbreviations: DM, dry matter; Pas, proanthocyanidins; DMACA, p-dimetylaminocynamaldehide; FP, flooding pampa; AM, arbuscular mycorrhiza; ALP, alkaline phosphatase.

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0168-9452/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2011.03.016

The main centre of *Lotus* diversity has been located in the Mediterranean region [14,17,31]. In particular, the Macaronesia presents the greatest number of endemic taxa (approximately 17 species), mainly distributed along the Canary Islands. Six of these endemisms are currently considered under critical situation and have been included in the red list of the Spanish vascular flora [14,16,32–35].

2. Agronomic importance of *Lotus* species

The beneficial effects of legume forages on animal production have been recognized since long time ago [36]. Pastures comprising legumes have higher digestibility and crude protein amount, less neutral detergent fiber and show more homogeneous yield distribution over the season than pastures that are exclusively based on grasses [37–40]. Several members of *Lotus* are economically important species since they are used as highly productive crops in pasture systems in a diverse range of landscapes, including some often subjected to extreme environments and soil conditions [30,31].

From the agronomical point of view, *Lotus corniculatus* L. (birdsfoot trefoil), *Lotus uliginosus* Schkuhr. (greater lotus), *Lotus tenuis* Waldst et Kit. (=*L. glaber* Mill., narrowleaf trefoil) and *Lotus subbiflorus* Lagasca (hairy birdsfoot trefoil) are the most important *Lotus* species [30,31]. Moreover, *L. corniculatus* is considered one of the major forage legumes after lucerne (*Medicago sativa*) and white clover (*Trifolium repens*) [9].Yields reported for *L. corniculatus* cropped in agricultural lands range between 8000 and 10,000 kg DM ha^{-1} $year^{-1}$ [38,41–43], which represents around 50–80% that of alfalfa performance [30]. As an example, in agricultural soils of Iowa (EEUU), monocultures of *L. corniculatus* yielded 10,000 kg DM ha^{-1} $year^{-1}$, versus 13,000 and 8000 kg DM ha^{-1} $year^{-1}$ for alfalfa and kura clover (*Trifolium ambiguum* Bieb), respectively [38]. In farming soils of Southern Paraná (Brazil) [43], 6000 and over 10,000 kg DM ha^{-1} $year^{-1}$ were reported for *L. subbiflorus* and *L. corniculatus* respectively, in contrast with 8000 and 3500 kg DM ha^{-1} $year^{-1}$ for red and white clover, in that order. Similarly, in agricultural soils of Michigan (USA), it was informed a 6500, 7500 and 8500 kg DM ha^{-1} $year^{-1}$ yield for *L. corniculatus*, alfalfa and red clover, respectively [42]. In Chilean soils with aptitude for cropping rice, 9000–11,000 kg DM ha^{-1} $year^{-1}$ yields for different *L. corniculatus* cultivars were noted [41]. In the last study, the author also registered 6000–10,000 kg DM ha^{-1} $year^{-1}$ for two *L. uliginosus* cultivars and 7500–10,000 kg DM ha^{-1} $year^{-1}$ for two *L. tenuis* cultivars. In Argentinean Vertisols, the last species produced 8000 kg DM ha^{-1} $year^{-1}$ [44].

The nutritional value of *Lotus* species is considered to be similar or even superior to those of alfalfa and white clover. The DM digestibility for *Lotus* species fluctuated between 72% and 78%, whereas DM crude protein ranged between 20% and 24%, and that of acid detergent fiber varied between 24% and 30% [30,41,42,45]. An interesting advantage of *Lotus* species as a forage source is that plants can either be grazed by livestock or used as hay or silage, in contrast with several other legumes that do not offer these possibilities [30,46]. A disadvantage however, is that *Lotus* species generally have poor seedling vigor and therefore, do not compete well with broad-leaf weeds or aggressive grass weeds [31,46–48]. On the other hand, their persistence in grasslands depends on the species involved and their natural reseeding [30], although some species and cultivars have more persistence due to rhizome development [49]. *Lotus* seeds production is a complicated activity, since within the plant population, pods mature spontaneously and dehisce at different times causing decreases in seed harvest [50,51].

Main regions where *Lotus* species are exploited for agronomical purposes are South America, North America, and Europe, with 1.85, 1.39 and 1.38 million ha sown, respectively [31]. Ninety percent of the *Lotus* sown area is concentrated in only 10 countries, where *L. corniculatus* occupied 90% of this area [31]. However, *L. tenuis* is being increasingly used for forage production in temperate or subtemperate areas in Argentina, Chile, Uruguay and USA, mostly in Western and Northeastern states, the total sown area spans over 160,000 ha [30,45,46]. In turn, *L. uliginosus* is sown in around 100,000 hectares of New Zealand and coastal Southeast Australia [30,52], whereas *L. subbiflorus*, the only annual *Lotus* species with agricultural importance, is primarily sown in Uruguay, where this crop reaches about 50,000 ha [30,31,53].

2.1. The contribution of proanthocyanidins to forage quality

One of the most attractive characteristics of some *Lotus* species is their capacity to accumulate proanthocyanidins (Pas), also known as condensed tannins, in leaves. This trait differentiates *Lotus* species from traditional forage legumes such as *Medicago spp.* and *Trifolium spp.*, whose shoots completely lack these metabolites [31,42,54].

Ruminants fed on forages containing moderate amounts of Pas (around 5 mg Pas/g DM) reduce protein fermentation to ammonia in the rumen and methane gas emissions. This increases the quantity of protein digested in the small intestine and allows the control of rumen bloat and internal parasite infections, without using chemicals addition [55–66]. However, forages with excess Pa levels have been reported to negatively affect animal fitness by decreasing palatability and feed intake, and diminishing nutrient utilization [55,67–69].

The Pas level among *Lotus* species is highly variable. Using the BuOH-HCl assay, Pa contents were 7.2%, 3.0% and 0.8% of dry matter for *L. uliginosus*, *L. corniculatus* and *L. tenuis*, respectively [70], whereas by using the DMACA-HCl assay [71], contents were 0.54 and 10.1 mg Pas/g of DM for *L. tenuis* and *L. corniculatus*, in that order. In *L. uliginosus* plants, Pas levels are considered generally too high [72]. In contrast, *L. tenuis* presents low Pas levels, whereas the Pas content found in *L. corniculatus* is generally around the desirable one for feeding rumiants [54,55,70,73–75]. On the other hand, Pas level is a heritable character in the mentioned species [74]. This stimulated the use of traditional breeding techniques (such as mass selection) to manipulate Pa concentration in *L. corniculatus* and *L. uliginosus* shoots [72,74].

Several studies on the effect of Pas on ruminant feeding have been carried out in *L. corniculatus* and *L. uliginosus* [56,67,68,76–84]. In both species, Pas have been extensively studied at the biochemical and molecular levels [70,85–98]. It has been shown that Pas from *L. corniculatus* and *L. uliginosus* differ considerably in concentration and chemical structures [56,99,100], but their whole biosynthesis pathway still waits to receive full understanding [58,66,98,101–103].

Pas are flavonoid polymers composed by several catechin units, generally procyanidin and prodelphinidin, which are functionally defined through their capacity to bind proteins and metal ions [101,104–106]. These metabolites are considered as plant chemical defenses against pathogens and herbivores [66]. Furthermore, it has been hypothesized that protection against fungi and microorganisms may have played a major role in the evolution of plant tannins [55,66,107]. However the information available about pathogens and herbivore resistance in *Lotus* species is limited to very few works. No microbial pathogen is available for laboratory studies [108]. On other hand, virus-sensitivity among several *Lotus* accessions and species has been discussed in relation with their geographical origin [109].

Finally, Pas also contribute to control seed permeability and dormancy [110], and are also involved in root nodulation [111–113].

2.2. Relationship between species of the L. corniculatus group

The most economically and scientifically important *Lotus* species, *L. corniculatus*, *L. tenuis* and *Lotus japonicus* are included, along with several others, within the *L. corniculatus* group (Fig. 1). Species within this group have different ploidy levels with a basic chromosome number equal to six [17]. The morphological similitude among these species, particularly at the seed and seedling stages, often makes contaminations difficult to avoid, so commercial stocks of *L. tenuis* seeds result frequently contaminated by seeds of *L. corniculatus* [44,114]. Several experimental approaches have been reported to identify crop species of the *L. corniculatus* group in a fast and practical way. They include the analysis of flavonoid patterns by two-dimensional thin-layer chromatography on cellulose plates [115], the analysis of seed storage proteins by capillary gel electrophoresis and SDS polyacrilamide gel electrophoresis [116,117], staining of leaf proanthocyanidins [71] and karyological studies [118].

There is a great deal of controversy surrounding the topic of phylogenetic relationships among species of the *L. corniculatus* group [119,120]. Notwithstanding this, some breeders have used these relationships as a tool to generate interspecific hybrids for agronomical purposes, e.g.: optimization of pranthocyanidin levels [121]; obtaining rhizomatous cultivars to increase persistence [119,122,123]; reduction of seed shattering [51,119,124]; improving seedling vigor [119,125] and tolerance to insect and foliar diseases [119,125]. *L. uliginosus* is not included within the *L. corniculatus* group, but it is closely related to *L. corniculatus* in the phylogeny of the genus [24]. Moreover, crossing of these two species resulted in the generation of fertile hybrids [119,126].

3. *Lotus* species and the ecological restoration of damaged landscapes

It is estimated that about 15% of the total land area in the world is facing serious erosion problems caused by physical or chemical factors, including soil salinization and contamination by heavy metals [127]. The restoration of disturbed lands, by means of planting tolerant species has been proposed as an alternative to cope with these environmental threats. Introduced plants may protect soils from natural and anthropogenic erosion, and contribute to long-term site stability and productivity [29,128,129]. The adaptive characteristics shown by several *Lotus* species make them good candidates for restoration and phytoremediation of degraded environments [29]. Among them, the species with the higher potentials are *Lotus creticus* L., *L. tenuis*, *L. uliginosus* and *L. corniculatus*. In the following paragraphs, we discuss the current use of these species in affected areas.

3.1. L. creticus in saline and drought environments

L. creticus is a perennial species belonging to the *Pedrosia* subgenera [130], which displays a considerable tolerance to drought, salinity and abrasive wind conditions [131–140]. For these reasons, it is considered a good alternative for soil revegetation of dry and saline environments [29,141–144]. This species has been used to fix Spanish coastal dunes at the Natural Park of the Valencian Devesa de la Albufera (Fig. 2A). In these dunes, *L. creticus* plays an important role as a pioneer species by improving soil structure and providing symbionts inocula for further plant succession [141]. In addition, *L. creticus* decreases the effects of the severe Mediterranean winds, thus fulfilling a primordial function in ecosystem protection and preservation [141]. Another use of *L. creticus* is as forage in Tunisia, where salinity imposes a serious threat to cattle production [133,135].

3.2. L. tenuis, "keystone" species in flooded and saline environments

Very few species of agricultural relevance are able to grow under combined conditions of waterlogging and salinity [145–148]. The survival of *L. tenuis* to long-term flooding and saline conditions is higher than that of other forage legumes [29,149–152], including the salt-tolerant *L. corniculatus* [150,152]. This higher salt-stress tolerance of *L. tenuis* was ascribed to low chloride accumulation in shoots, high aerenchyma formation and low radial O_2 loss in roots [29,140,149,151–156]. In addition, *L. tenuis* grows well at 6–9 soil pH [157,158], has efficient phosphorus utilization [159–161], establishes symbiotic association with rhizobia and mycorrhiza fungi under waterlogged conditions [162] and has a high nutritional quality, even when grown under saline stress [163].

In the Flooding Pampa (Argentina), an area affected by waterlogging, saline and alkaline soil conditions [164,165], cattle production is supported mainly on natural grasslands [45], where the persistence and yield of common legume species as red and white clover and lucerne are significantly decreased [45,166,167]. *L. tenuis* was introduced in this area (Fig. 2B), in an attempt to add sustainability and profitable restoration to the affected soils, and then became quickly naturalized [152,168]. The over sowing with *L. tenuis* is a recommended strategy to cope with poor soil drainage and salinization [169]. This species is currently considered a "keystone species" because of its contribution to forage production in the region and its influence on growth of associated plant species, what leads to a progressive improvement of edaphic conditions [155,159,166,170–174]. Likewise, some authors suggested the use of *L. tenuis* as an option to improve and revegetate some regions in Southern Australia with saline and waterlogging environments [152,168,175]. However, different *L. tenuis* ecotypes greatly varied in their tolerance to salinity [176], suggesting than some ecotypes might be more appropriate than others for the above mentioned purposes.

3.3. L. uliginosus in strongly acidic soil environments

L. uliginosus is an aluminium (Al) tolerant species [30,106]. In particular, the tetraploid cultivar "Maku" has shown a higher degree of tolerance to acidic soils than other forage legumes of the same genus [177]. Stoutjesdijk [106] suggested that in *L. uliginosus*, Al detoxification is achieved through a mechanism involving the formation of Al-proanthocyanidins complexes. *L. uliginosus* has an interesting potential for ecological and agricultural uses in acidic soils [30] and is sown in southern Australia and New Zealand [52,178]. Under this situation, Al, which is very abundant in the Earth crust, becomes soluble and limits the growth of plants that are Al sensitive. In fact, Al toxicity is considered to be a major growth-limiting factor for plants in most acidic soils [179].

Finally, *L. uliginosus* also demonstrated high tolerance to excess of manganese [30], is able to grow and nodulate efficiently in flooded soils [180] or contaminated with mercury (Hg) and arsenic (As, Fig. 2C) [181] and grows well on mine refuses [30,182].

3.4. L. corniculatus in diverse marginal environments

L. corniculatus is a worldwide distributed species that grows under a wide range of environmental conditions. It is probably the Lotus species most commonly employed for ecological restorations of soils affected by nutrient deficiency, salinity, drought, or contaminants (Fig. 2D) [29,31,182–187].

The origin of *L. corniculatus* has been a subject of controversy [20,22,119,188,189]. Some theories support a scenario where *L. corniculatus* was originated from a chromosome doubling event in a diploid ancestor, or from a hybridization event between two highly

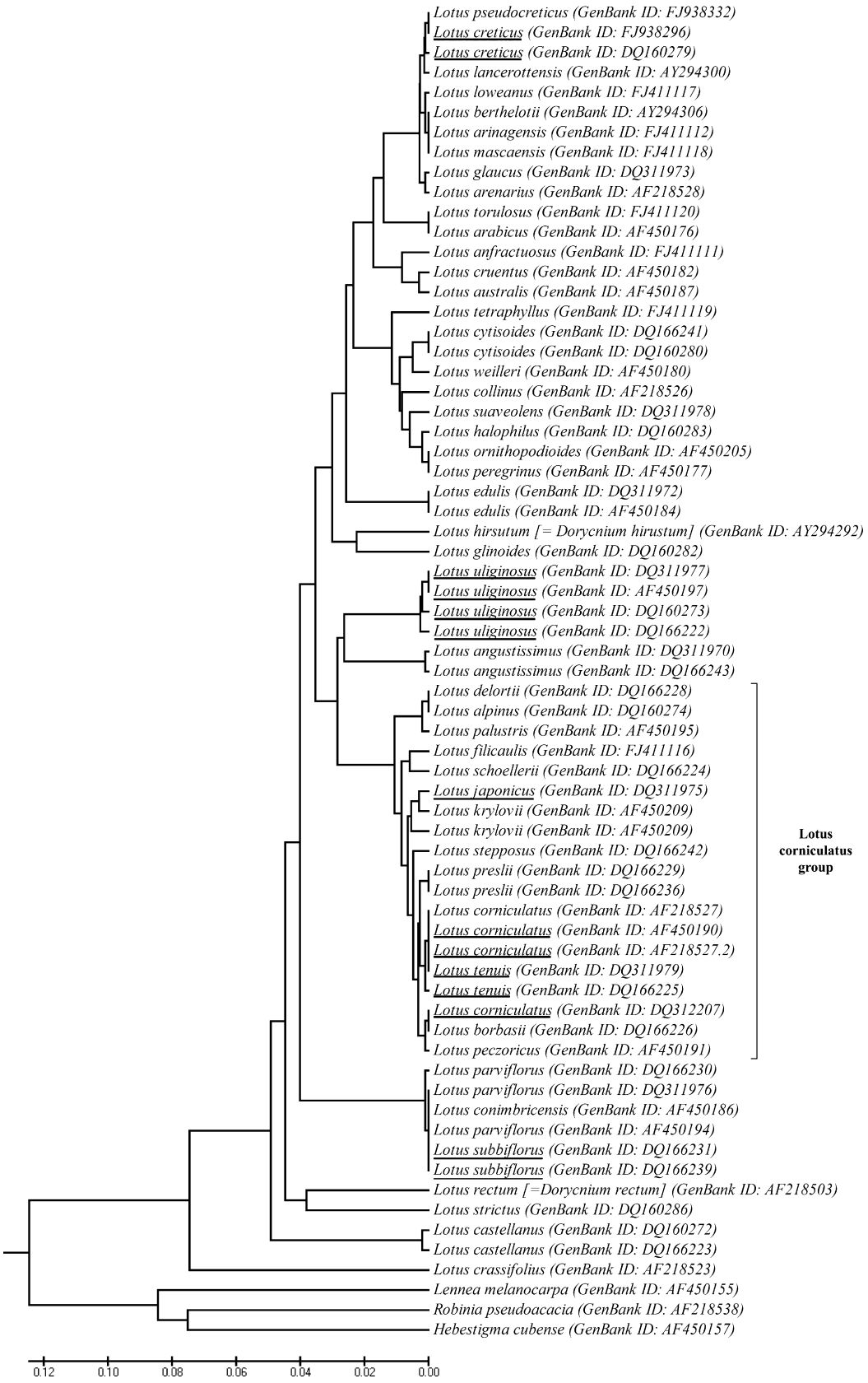

Fig. 1. Phylogeny of *Lotus* species based on Nuclear Ribosomal Internal Transcribed Spacer (nrITS) gene. The tree was constructed from the nucleotide sequence data by using the UPGMA algorithm and the phylogenetic distances were computed by using the *p*-distance method. The horizontal branch lines are proportional to the different nucleotides and indicate the *p*-distance. In parentheses the GenBank number of nrITS nucleotide sequence.

Fig. 2. *Lotus* species growing in marginal environments. (A) *L. creticus* in Mediterranean coastal dunes of Spain. (B) *L. tenuis* in flooding soils of Salado River Basin (Argentine). (C) *L. uliginosus* in heavy metals contaminated soils of Estarreja (Portugal). (D) *L. corniculatus* in saline-alkaline soils of Valencia (Spain).

related diploid progenitors. The latter is the most accepted theory, given that *L. corniculatus* is an allotetraploid species, being *L. tenuis* the female progenitor and other species, presumably *L. uliginosus* the male progenitor [24]. The high diversity observed in *L. corniculatus* phenotypes may be due to frequent intercrossing and the formation of hybrids with a high potential of adaptation to a wide range of environments [119,190]. Intraspecific and interspecific hybridizations are among the processes of genetic recombination that result in the production of new gene arrangements that give an extraordinarily rich source of genetic material on which natural selection may operate [119,191].

L. corniculatus naturally exhibits a high degree of genotypic diversity [192]. It has been proposed that differences in phenotype and fecundity between geographically separated populations of *L. corniculatus* may lead to differences in survival and fitness when seeds are sown in a restoration environment [193]. However, the adaptive variability of wild *L. corniculatus* genotypes has been hardly employed in breeding programs so far [186]. In fact, a great deal of tolerance to constrained environments has been lost in commercial cultivars of *L. corniculatus*, because breeding emphasis has been centered on dry matter production and persistence under non-restrictive conditions [31,187]. It has been shown that wild *L. corniculatus* genotypes can be crossed with commercial varieties using conventional crossing methods [186]. This fact, along with the occurrence of a wide range of wild genotypes [192] and the high number of commercial cultivars registered [194] suggests that these plant materials could be used as genetic source for the development of new cultivars, destined to restoration and forage production in diverse marginal environments [186,187,190].

4. Symbiosis

4.1. Nitrogen fixation by Lotus spp

Most legumes can establish nitrogen-fixing mutualistic symbioses with soil bacteria collectively known as rhizobia. Infection of legume roots by rhizobia leads to the development of a new organ, the nodule. Two types of nodules are developed by legume roots: determinate or indeterminate. The former are spherical in shape, while the latter are cylindrical. In addition, to these morphological features of both types of nodules, their anatomical structure is also different. The type of nodule developed in response to rhizobial infection, is host-specific, and does not depend on the bacterial partner. *Lotus* spp develop determinate nodules, as occurs with soybean (*Glycine max*) and *Phaseolus* spp. [195]. Root nodules are colonized by rhizobia, and a metabolic cooperation is established between both partners, so that bacteria reduce atmospheric nitrogen to ammonia, which is provided to the plant for further incorporation into organic compounds, and the plant provides the bacteria with carbohydrates derived from the photosynthetic process, which can be used as carbon and energy sources [196]. Thus, this symbiotic process allows legumes to use atmospheric nitrogen, which renders them able to colonize soils with low nitrogen contents and also contribute to a better adaptation of legumes to adverse conditions [197,198]. As described above, several *Lotus* spp either naturally grow or are cultivated in restrictive soils, hence symbiotic rhizobia are expected to contribute to the adaptation of their *Lotus* hosts to these environments. Moreover, rhizobial strains able to establish efficient nitrogen-fixing nodules under restrictive conditions could be used for the formulation of inoculants that pos-

itively impact on field production of this crop, as shown for other legume-rhizobia symbioses [198].

4.2. Diversity of Lotus rhizobia and its applications

Inoculant formulation requires previous selection of adequate rhizobial strains. Such strains not only need to be efficient in the nitrogen fixation process itself, but also to favorably compete with previously existing, native rhizobial populations in the field. Thus, knowing the diversity and abundance of *Lotus*-nodulating rhizobia in fields is a valuable approach for further selection and application of inoculant strains. In this regard, *Mesorhizobium loti* and *Bradyrhizobium* sp (*Lotus*) have been traditionally considered as the typical rhizobial symbionts of *Lotus* spp. [199,200]. Some species of this plant genus, such as *L. corniculatus*, *L. tenuis*, *Lotus filicaulis* and *L. japonicus* establish symbiosis with *M. loti*. On the contrary, *Bradyrhizobium* sp. (*Lotus*) strains either do not nodulate or form ineffective nodules in such *Lotus* spp, but are able to nodulate *L. uliginosus*, *Lotus angustissimus* or *L. subbiflorus*, species that in turn do not establish effective symbiosis with *M. loti* [201–204]. Whereas there is very little information available about the *Lotus* bradyrhizobia, much more is known about *M. loti*. This has been considered the type symbiont of *L. japonicus* and related cultivated *Lotus* spp. For instance, the complete genome sequence of strain MAFF303099 is known since several years ago, together with Nod factors (bacterial molecules required for nodulation) produced by several strains [205]. However, recent reports indicate that bacteria nodulating *Lotus* may be much more diverse than initially thought. Estrella et al. [206] investigated the genetic diversity and host range of rhizobia nodulating *L. tenuis* in soils of the Salado River Basin in Argentina, an area in which this legume is widely used as a forage resource. They found *Mesorhizobium*-like isolates closely related to *Mesorhizobium amorphae*, *Mesorhizobium mediterraneum*, *Mesorhizobium tianshanense* or the broad-host-range strain NZP2037 but unexpectedly, few of them were related to the *M. loti* type strain NZP2213. They also found a large amount of *Rhizobium*-like strains that were related to *Rhizobium gallicum*, *Rhizobium etli* or *Rhizobium tropici*, species that commonly have *Phaseolus vulgaris*, but not *Lotus*, as a host. Furthermore, a single isolate related to *Aminobacter aminovorans* was found. Although the genus *Aminobacter* is phylogenetically related to *Mesorhizobium*, it was not previously known to nodulate legumes [207]. The genus *Aminobacter* comprises several pesticide- and herbicide-degrading bacteria isolated from agricultural soils, which are able to grow on methylated amines, $ClCH_3$, CH_3Br and CH_3I as the sole carbon and energy sources [207]. In this way, the potential of *Aminobacter* spp for bioremediation purposes, along with the ability to establish symbiosis with legumes render this genus an interesting candidate for further biotechnological applications.

In another study, Lorite and coworkers found several *Mesorhizobium* species, such as *M. tarimense*/*M. tianshanense*, *M. charcoense*/*M. albiziae* and *M. alhagi*, as the usual *Lotus* rhizobia in saline soils of Southern Spain [208]. Interestingly, a high number of isolates from a particular location behaved as salt-dependent bacteria, requiring salt-enriched (NaCl or $CaCl_2$) media for optimal growth and symbiotic performance. Many of the isolates characterized in this work were able to nodulate *L. corniculatus* and *L. tenuis* and exhibited different levels of efficiency, demonstrating that rhizobia native from highly restrictive environments can efficiently nodulate cultivated *Lotus* spp.

Twenty-four *Lotus* spp, 17 of which are endemic, have been reported to be present in the Canary Islands, a hot spot of plant biodiversity [209]. An interesting feature of *Lotus* populations in this place is that some species are confined to a limited area within a single island [16]. In addition, some of these species are in serious danger of extinction. Thus the potential impact of rhizobial symbionts on survival in these environments, as well as the possible relationship between rhizobial specificity and the particular pattern of geographical distribution of *Lotus* spp, prompted studies on the diversity of rhizobial *Lotus* symbionts in these islands. A recent characterization of rhizobial symbionts of *Lotus* spp endemic to the Canary Islands detected at least ten different species of the genera *Mesorhizobium*, *Ensifer* and *Rhizobium*/*Agrobacterium* [210]. As part of this study *Ensifer meliloti* bv. lancerottense was found the preferred symbiont of endemic *Lotus* spp. growing on arid soils [211]. Nearly all the *Ensifer* isolates characterized in this work tolerated NaCl concentrations up to 400 mM, as well as alkaline conditions (pH 9).

Rhizobial symbionts can contribute to the adaptation of legumes not only to saline and alkaline soils, but also to a wider range of adverse conditions [198]. In their natural environments, some *Lotus* spp are exposed to water deficit, either permanently or periodically [30]. Moreover, in some cases they have to cope with simultaneous conditions of soil salinity, alkalinity and drought [30,45,46,155]. *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* symbionts of *Lotus* spp such as *L. creticus*, *L. argenteus* and *L. roudairei* have been identified in an arid region of South Tunisia [212], this being an example of a potential source of rhizobial diversity for the isolation of strains well adapted to dry soils. Moreover, the presence of *Mesorhizobium* symbionts of *Lotus* spp in alkaline and saline soils of Xinjiang province (China), a region with low annual rainfall [213] suggests that the potential sources of rhizobia well adapted to adverse conditions are widespread, far beyond the regions in which *Lotus* spp are highly abundant. In this way, further efforts to select rhizobial inoculants for *Lotus* species in arid soils should take advantage of the diversity of *Lotus* rhizobia present in marginal regions such as those previously mentioned.

In this regard, it is worth mentioning current efforts to select for novel inoculant strains of *Lotus tenuis*, an important forage source in natural grasslands of the Flooding Pampa (FP) in Buenos Aires Province, the most important area devoted to cattle breeding in Argentina [165]. The introduction of *L. tenuis* in the FP is relatively recent, the knowledge about the impact of rhizobial symbionts in the adaptation of this legume to the conditions of this ecosystem thus being scant. As a consequence, the development of high quality rhizobial inoculants for *L. tenuis* in the FP is still incipient. Indeed, *L. tenuis* seeds are in many cases inoculated prior to sowing, by using rhizobial formulations based on strains whose taxonomic identity has not even been established. Moreover, such strains have been selected for their ability to symbiotically fix nitrogen in environments very different to those of the FP. Therefore, the adaptation, survival and symbiotic efficiency of such strains can be affected by adverse factors such as soil salinity [214] a typical condition in an important proportion of the soils in this region [165]. Furthermore, introduced rhizobial inoculants can then be outcompeted by native rhizobia, well adapted to prevailing conditions [215]. Thus, inoculants based on native or naturalised rhizobial strains from this region could be an affordable and sustainable approach to improve *L. tenuis* productivity. In this regard, the symbiotic performance of some *L. tenuis* rhizobia native from the FP has recently been compared to rhizobial strains currently used for field inoculation of *L. tenuis* in this region [216]. A considerable variation in the infective ability was evident and some native strains were more efficient than the current commercial inoculants. Although salinity decreased the symbiotic performance of all the strains analyzed in this work, one of the native strains exhibited a considerably higher symbiotic performance under this stress condition. This finding suggests that the formulation of inoculants for *L. tenuis* based on native strains can increase forage production.

4.3. Arbuscular mycorrhizae

Arbuscular mycorrhizae (AM) are widespread symbiotic associations between plant roots and fungi belonging to the Glomeromycota [217–219]. This symbiosis is obligate for the fungal partner which is unable to complete its life cycle in the absence of a host root. The most distinctive structure of this symbiotic association is the arbuscule, a short hyphal ramification that branches profusely within parenchimatic root cells. Phosphate, nitrate and several minerals are absorbed by fungal hyphae that develop outside the root. Then, ions are transported towards the intra-radical hyphae and delivered to the host-fungus interphase at the arbuscule. In return, the fungus receives a considerable part of the photosynthesis products from the host [220,221].

4.3.1. Occurrence of AM fungi in Lotus species

The occurrence of AM fungi in root or rhizosphere has been analyzed in a limited number of *Lotus* species. At least eight colonization patterns of AM fungi were found in roots of *L. tenuis* plants growing in the Salado River basin in Argentine [161]. In this survey, the most abundant AM fungus was *Glomus intraradices* followed by a morphotype, possibly assignable to *G. tenue*. In grasslands dominated by this same *Lotus* species, spore density was significantly correlated with root colonization registered 3 months before, suggesting that high colonization in one season precedes high sporulation in the next season [222]. Seasonality in AM diversity and root colonization was also found in *Lotus bryantii* and *Lotus distichus* in coastal sand dunes of Baja California [223]. One *Acaulospora* spp. and three *Glomus* spp. nucleotide sequence types were discovered in *L. corniculatus* plants that were growing in a dune grassland [224]. A high level of AM fungi diversity was observed in the rhizosphere of *Lotus pedunculatus* and *Lotus australis*, which was correlated with the level of AM root colonization and frequency of extraradical hyphae [225]. Heavily AM colonization was observed in *L. creticus*, the dominant plant species at a revegetated foredune of the Valencian Devesa [141].

4.3.2. Influence of AM fungi on Lotus species plant growth

Improved mineral nutrition derived from the establishment of the AM symbiosis leads to the most acknowledged effect of this interaction, which is plant growth promotion [226]. Only few *Lotus* species were examined with regard to the effect of the inoculation with AM fungi on plant growth. In a microcosm experiment, in which the composition of the AM fungi community was manipulated, *L. corniculatus* showed to be almost completely dependent on the presence of AM fungi for biomass production [227].

Later, mycorrhizal inoculation was used for efficiently supporting plant growth of *L. corniculatus* during direct reclamation of spoil banks generated during coal mining [228]. The last observation, along with the high level of AM root colonization observed in *L. creticus* at a revegetated foredune of the Valencian Devesa [141] constitute a hint of the relevance that AM symbiosis would have on the potential that some *Lotus* species show as pioneers for plant restoration in native ecosystems. *L. tenuis* plants mycorrhized with *G. intraradices* showed variable growth effects depending on the *L. tenuis* genotype [229,230]. In plants of this species grown in a P-deficient soil, growth response and characteristics of mycorrhizal root infection varied according to the level of added P [231]. Moreover, the greatest arbuscular colonization in *L. tenuis* (besides two other grasses), was associated with higher P (and N) concentrations in plant tissue, suggesting a correspondence with increases in the rate of nutrient transfer between the symbiotic partners [232]. In fact, P metabolism is a key factor in AM-induced plant growth enhancement. The simultaneous observation in *L. japonicus* of polyphosphates (polyp) and alkaline phosphatase (ALP) activity signals by a dual-labelling method put forward that there may be some relationships between polyP metabolism and ALP activity in arbuscules, and that these are, in part, controlled by Pi uptake by plants via an AM-inducible Pi-transporter [233]. The dynamics of polyP, total P and Pi was investigated in *L. japonicus* plants inoculated with *Glomus* sp. HR1, using a dual mesh bag culture system, under P-starvation conditions [234]. Results showed that under P-starvation conditions, polyphosphate accumulation in extraradical hyphae is rapid and may reach >60% of total cellular P, implying that the potential pool size of polyP in the fungal cell was much larger than previously considered. In addition, massive polyP accumulation was also found within *L. japonicus* mycorrhizal roots.

Since as reward for inducing improved mineral nutrition, AM fungi receive photosynthesis-derived carbohydrates from the plant, photosynthesis may regulate levels of AM root colonization. There are few reports on this subject in legumes. As example, colonization of soybean roots by the AM fungus *Glomus fasciculatum* was little affected by photosynthate stress as a result of defoliation, and the intensity of colonization increased with increasing stress [235]. As far as we know, the single report on such trade-off between *Lotus* species and AM fungi shows that root AM colonization responds to *L. wrangelianus* grown in elevated CO_2, as a function of soil depth [236].

4.3.3. Influence of AM fungi on Lotus species stress tolerance

Besides growth, the association with AM fungi increases plant tolerance to water, saline and biotic stresses (revised by Augé [237], Evelin et al. [238] and Pozo et al. [239], respectively. A small number of *Lotus* species occurring under stressing soil conditions have been found to be associated with high levels of AM root colonization. In natural grasslands of the Buenos Aires (Argentina) characterized by high levels of soil salinity and sodicity (pH 9.2; exchangeable sodium percentage = 65%), *L. tenuis* presented high levels of root AM colonization and diversity of AM fungi [161,240]. Salt-stress alleviation of *L. tenuis* plants inoculated by *G. intraradices* was studied in laboratory experiments [230,241]. Under the saline condition, mycorrhizal *L. tenuis* plants showed enhanced K+/Na+ ratio, root K+ content, protein concentrations and higher chlorophyll levels compared with non-AM ones. On other hand, this *Lotus* species showed ability to grow, nodulate and maintain a symbiotic association with AM fungi even under drought or excess water conditions [232].

4.3.4. Rhizobial–AM interaction in Lotus

Several environmental variables like soil pH [242], salinity [243] and temperature [244,245,198] may hamper legume-rhizobial symbiosis. In salt-sensitive legume crops, diminished symbiotic development of root nodules [246] and lower nitrogen-fixation capacity [247] have been related with reduced plant yield. In *L. japonicus* and *Medicago truncatula*, NaCl treatment decreased nitrogenase activity, although nodule carbon metabolism in the first species proved to be less sensitive to salinity than that of the second [248]. It has been suggested that the improvement of plant growth could be attained through synergistic interactions between nitrogen fixing bacteria (rhizobia) and AM fungi [249]. Within the *Lotus* genus particularly, *L. pedunculatus* (big trefoil) simultaneously inoculated with rhizobia and *Gigaspora margarita* resulted in a greater biomass production at 100 kg N ha^{-1} m [250]. The nodular response of *L. corniculatus* in a non-sterile soil was increased by the combined inoculation of an acid-tolerant strain of *G. intraradices* and the plant growth-promoting bacterium *Pseudomonas putida* R-20 [251]. In *L. tenuis*, salinity reduced the number of nodules per root but only in non-mycorrhizal plants of both genotypes [229].

AM root colonization also induces different effects on plant herbivores [252,253] and inversely, plant herbivores affect root mycorrhizal development [254], *L. corniculatus* was employed to test the hypothesis that arbuscular mycorrhizal fungi influence life history traits of a lepidopteran herbivore [255]. The survival and lar-

val weight of third instar larvae of *Polyommatus icarus* (Lycaenidae), fed with sprigs of mycorrhizal *L. corniculatus* (Fabaceae), were greater than those of larvae fed with non-mycorrhizal plants [255]. In *L. japonicus* plants, the aboveground herbivory by spider mites (*Tetranychus urticae*) increased the colonization and activity of the AMF fungus *Gi. margarita*, but this effect was transient, since both parameters returned to the initial levels in the absence of mite herbivory after a few days, suggesting that the change in AM association in response to mite herbivory is a short-term response [256]. The fact that all these *Lotus* species presented high levels of AM root colonization, puts forward that this association is a key factor in their establishment under stressing soil conditions.

4.3.5. Basic studies of AM symbiosis in the model plant L. japonicus

Basic research on root symbiosis was initially focused on agriculturally important species like *G. max*, *P. vulgaris*, *Pisum sativum* and *Vicia* spp. However, the study of physiological, biochemical and molecular aspects of symbiosis in these species has been hampered by their size, ploidy, genome characteristics and breeding behaviour. This, in addition to the fact that the first sequenced plant model *Arabidopsis thaliana* does not form symbiotic associations with arbuscular mycorrhizae (neither with rhizobia), encouraged the search of legume model candidates. In 1990 two legume species emerged as new model plants: *M. truncatula* [257] and *L. japonicus* [258]. The latter was proposed as an amenable plant species for classical genetics techniques well as for mutant analysis, gene cloning and transformation procedures. Techniques for AM infection and visualization of AM structures in roots have been adapted to the specific requirements of *L. japonicus* [259]. A great deal of studies have been conducted having *L. japonicus* as experimental system for the elucidation of the mechanisms involved in the recognition between the host and the AM fungus (signaling), the systemic regulation of the colonization process, the nutrient exchange between the AM fungus and its host and the AM-induced plant tolerance to biotic stress. We will not go into details of these works since they have been extensively reviewed by other authors [260–277].

5. Conclusions and future directions

As stated in the chapters above, the enormous ecological and agronomic importance of several Lotus species is unquestionable. Many of these species constitute perhaps the best alternative to alleviate serious threats imposed to formerly cultivated areas. In addition, their use could be implemented as an approach to incorporate to the agricultural landscape great extensions that never were sought as cultivable soils. Nevertheless, a considerable improvement of the adaptability and forage capabilities of these cultivars is still needed and would only be attained after a deeper understanding of the mechanisms that regulate those processes.

Fortunately, over the last few years we have witnessed tremendous advances in the knowledge on legume genomics and physiology. This has been promoted by works focused on the physiological events that occur in *Lotus spp*. during stress adaptability. Particularly, these advances have been achieved thanks to the optimization of several genetic approaches in the model legume *L. japonicus*. Transcriptomic analysis by means of DNA microarrays has shown a fast progress on this species, and even though more research needs still be conducted in order to comprehend in depth the regulatory responses identified in these studies, its contribution to the field is undeniable. Most of all, these genetic analyses have been focused on nitrogen-fixing nodule development and growth under saline stress conditions [277–282]. However, the genetic picture is growing considerably since new profiles are under current evaluation, as the responses occurring in the interaction with pathogenic bacteria and the effect of several abiotic streses (own unpublished research). Other "omic" strategies, namely proteomics, metabolomics and ionomics, are also destined to fulfill unavoidable gaps left behind by genomic approaches. Even though these types of analyses have not kept pace with regard to transcriptomic, the literature concerning this subject is currently increasing [137,283–285]. In addition, these analyses are being expanded to several *Lotus* species which makes an outstanding contribution to the field [281]. That means that we are still making the first steps to a thorough understanding of the legume physiology and, most of all, that the potential of *L. japonicus* as a model system has not been fully exploited. In fact, several technical advances in this model species promise big leaps in a short time with regard to genetics and physiology. The sequencing of the *L. japonicus* genome is under process (current progress can be followed at http://kazusa.or.jp/lotus/), and the full data package is expected to be released in the near future. This knowledge constitutes a milestone in legume research and is now making possible the comparison of the genetic repertoire among several members of the family. Perhaps the best example of the benefits of this comparative analysis is the construction of the LegumeTFBD [286], an interesting database of putative transcription factors in *L. japonicus*, *G. max* and *M. truncatula*, that would facilitate the evaluation of the functionality of transcription factors by classical mutant-generation approaches. In addition, we can rely today on proper genetic linkage maps that proved to facilitate loci positioning [287,288]. These maps have been successfully used to characterize intraspecific as well as interspecific populations generated between *L. japonicus* and *L. filicaulis*, and two accessions of *L. japonicus* (Gifu and Miyacojima MG-20), respectively [287,289]. Identification of gene loci associated to a desirable phenotype in these populations opens the possibility to unravel the mechanisms associated to important traits sought by breeding programs. It is fundamental to keep in mind that, because of the high degree of synteny among legumes, any results coming from studies on *L. japonicus* could be extrapolated not only to additional *Lotus* species, but also to other important crops from this family.

At last, any task facing legume improvement should be carried out along with more efforts put on the identification, selection and evaluation of natural fungal and bacterial species establishing symbiotic interactions of the *Lotus* genus. Integrating a good repertoire of symbiotic material with a substantial improvement in *Lotus* will provide us with a wonderful tool to assure the best ecological/agronomic performance under any environmental condition.

Acknowledgments

This research was supported by: Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina); Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT, Argentina); EU-INCO Lotassa Project; Comisión de Investigación Científica (CIC); San Martín University (Argentina) and the Iberoamerican network for biofertilizers – Biofag- (CYTED). FJE and NLC are doctoral CONICET fellows, MJE is a member of the researcher Career of CIC (BsAs), and ABM, FLP, AG and OAR are CONICET researchers.

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