

Singular Features of the *Bradyrhizobium-Lupinus* Symbiosis

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ABSTRACT

Lupinus is a legume with great agronomic potential due to the high protein content of its seeds and its positive effect on soil fertility. It is able to fix atmospheric nitrogen through the establishment of a symbiosis with soil bacteria of the genus *Bradyrhizobium*. The process is carried out in a special subclass of indeterminate nodules known as lupinoid nodules. The *Bradyrhizobium-Lupinus* symbiosis has particular characteristics, which makes it different from other *Rhizobium*-legume symbioses. The entry of the bacteria into the plant root occurs at the junction between the root hair base and the adjacent epidermic cell and only sporadic “infection threads” have been observed. The involvement of mitogen-activated protein kinases (MAPKs) and aldehyde oxidase in infection and nodule morphogenesis have been reported for the first time in this symbiosis. The presence of nitric oxide synthase activity in plants has been detected for the first time, in roots and nodules of *Lupinus albus*. The unique lupin nodule cortical structure has contributed to the visualization and elucidation of the operational mechanisms of the oxygen diffusion barrier. Nodule senescence takes place in a similar way to that of determinate nodules, starting in the central area of the nodule. This symbiosis is especially resistant to abiotic stresses such as herbicides, nitrate, salinity and heavy metals. This unusual tolerance has permitted the use of inoculated lupin plants for the re-vegetation of degraded areas and as a pioneer plant to fight soil erosion and to reclaim eroded soils. The capability to accumulate Cd, Zn and others heavy metals in the nodulated roots has resulted in the consideration of this symbiosis as a potential phytoremediator.

Keywords: abiotic-stress-tolerance, aldehyde oxidase, MAPKs, nodule-senescence, oxygen-diffusion-barrier, root-infection

Abbreviations: ALT, alanine aminotransferase; APX, ascorbate peroxidase; AO, aldehyde oxidase; AST, aspartate aminotransferase; DHAR, dehydroascorbate reductase; EPSPS, 5-enolpyruvylshikimic-3-phosphate synthase enzyme; GOGAT, glutamine-2-oxoglutarate-amino-transferase; GR, glutathione reductase; GS, glutamine synthetase; HPF, high pressure freezing; LMZ, lateral meristematic zone; L-NMMA, N-Monomethyl Arginina; MAPKs, mitogen-activated protein kinases; MAC, Monoclonal Antibody Cambridge; MBP, myelin basic protein; MDA, malondialdehyde; MDH, malate dehydrogenase; MDHAR, monodehydroascorbate reductase; NADPH, nicotinamide adenine dinucleotide phosphate reduced; NO, nitric oxide; Nod, nodulation factors; NOS, nitric oxide synthase; PCA, protocatechuic; PEPC, phosphoenolpyruvate carboxylase; Rmin, minimum resistance; ROS, reactive oxygen species; SAMK, stress-activated MAPK; SIMK, salt stress-inducible MAPK; SOD, superoxide dismutase; SS, sucrose synthase

CONTENTS

INTRODUCTION.....	2
<i>Rhizobium</i> -legume symbiosis	2
Genus <i>Lupinus</i>	3
<i>Bradyrhizobium</i> -nodulating <i>Lupinus</i>	3
THE <i>BRADYRHIZOBIUM</i> SP. (<i>LUPINUS</i>)- <i>LUPINUS</i> SYMBIOSIS.....	4
INFECTION AND NODULE DEVELOPMENT	4
Colonization and root infection	4
Nodule primordium	5
Involvement of MAPKs in the early steps of infection.....	5
Aldehyde oxidase and indol acetic acid in the nodule meristem.....	5
Nitric oxide synthase activity in roots and nodules	6
GENES AND GENE EXPRESSION IN LUPIN NODULES.....	7
Cell-cycle regulation.....	7
Nodulins.....	7
OXYGEN REGULATION.....	7
The oxygen diffusion barrier	7
Leghemoglobin.....	8
NODULE SENESCENCE	8
Ultrastructural alterations	8
Antioxidants and related proteins	8
SYMBIOSIS TOLERANCE TO ABIOTIC STRESS	9
Nitrate	9
Salinity	9
Herbicides.....	9
Heavy metals	10
AGRICULTURAL IMPORTANCE	11
Lupin cultivation	11
Lupins in agricultural systems	11
Lupins crop rotation in sustainable agriculture.....	11
ECOLOGICAL IMPORTANCE AS A PIONEER	12
<i>Lupinus nootkatiensis</i> (Nootka lupin).....	12
<i>Lupinus arboreus</i> (yellow bush lupin) and <i>L. lepidus</i>	12
CONCLUDING REMARKS	13
ACKNOWLEDGEMENTS	13
REFERENCES.....	13

INTRODUCTION

Rhizobium-legume symbiosis

The *Rhizobium*-legume symbiosis is one of the more prominent beneficial plant microbe interactions; by means of this association legume plants get nitrogen directly from the atmosphere and this nitrogen fixation has a positive effect on soil fertility. Nitrogen fixation is carried out in highly specialized organs called nodules which are formed by the interaction between nitrogen-fixing bacteria (rhizobia) and the legume root. Nodule formation on the host plant is a complex process regulated by both the plant and the bacteria (Limpen and Bisseling 2003). Cross-inoculation groups have been established depending on this specificity, i.e. *Rhizobium leguminosarum* can nodulate pea or chickpea whilst in a more extreme case *Sinorhizobium* sp. NGR234 is able to nodulate 232 legume species (Pueppke and Broughton 1999). Symbiosis establishment, nodule formation and subsequent development requires a series of complex interactions between two very different organisms which involve structural and biochemistry changes. The rhizobial microsymbiont grows actively in the rhizosphere due to exudates from the legume roots. Interaction for the recognition of the correct symbiotic partners begins with a molecular dialogue represented by multiple signal exchange between legumes (flavonoids, hormones) and rhizobia (Nod factors, exopolysaccharides and lipopolysaccharides). Flavonoids secreted by the host plants induce the rhizobial *nod* genes in the bacteria that encode the nodulation factors (Nod factors). These molecular signals are essential for initiating root morphogenetic and organogenetic changes, leading to the formation of nodules (Denarie *et al.* 1996). Furthermore, Nod factors induce the formation of nodule-specific or nodule-enhanced proteins (early nodulins), which participate in the infection and organogenesis of nodules. The late nodulins are expressed around the onset of nitrogen fixation.

The most common infection route is through root hairs by formation of infection threads. After recognition, rhizobia adhere to the surface of an epidermal cell, generally a root hair. The entry of the bacteria is afforded by enzymatic hydrolysis of the cell wall (Callahan and Torrey 1980; Martínez-Molina and Olivares 1982). The plant reacts against the invasion by developing invaginations of the cell wall and plasmalemma which enclose the rhizobia. These invaginations, now called infection threads, grow, form branches and cross the adjacent root cells liberating bacteria (Cabezas de Herrera and Fernández-Pascual 1982). At the same time, nodule primordia start to form inside the root. Rhizobia are liberated from the infection threads into the central cells of the nodule primordia then start to divide, multiply and differentiate to form bacteroids. These are separated from the cytoplasm of the host plant cells by a specialized peribacteroid membrane, forming an organelle-like structure termed a symbiosome. The peribacteroid membrane and bacteroid membranes are in close contact, allowing for an exchange of metabolites between the bacteroids and the host plant cell (de Felipe *et al.* 1997). Cells within the center of the nodule become enlarged due to the presence of numerous symbiosomes and differentiate to form an infected zone where nitrogenase is expressed within the bacteroids and leghemoglobin is expressed in the host cell tissue.

The nitrogenase enzyme requires high rates of ATP from oxidative phosphorylation, a process which requires oxygen as the final electron receptor of the respiratory chain. However, nitrogenase (component II) is inactivated by an excess of oxygen, whilst the expression of *nif* and *fix* genes requires a microaerobic environment (Soupey *et al.* 1995). This implies a very accurate control of oxygen supply to the bacteroids. This control is established in the nodule cortex by an oxygen diffusion barrier and in the infected cells by the oxygen-carrying leghemoglobin and bacteroids' respiration. The cortical oxygen diffusion barrier

(ODB) is variable and able to balance oxygen entry against consumption by metabolic activity (Witty *et al.* 1986). It permits the maintenance of very low oxygen levels in the infected zone (Hunt and Layzell 1993; Minchin 1997). The ODB is formed by layers of cells where the intercellular spaces can be filled with either water or a matrix of glycoproteins and the nodules are able to modify the thickness of the ODB depending on environmental conditions (de Lorenzo *et al.* 1993; Iannetta *et al.* 1993). In the infected cells, oxygen distribution is achieved through leghemoglobin (Bergersen 1982) which is the most abundant protein in the nodules (approximately 25% of nodule dry weight). It is a monomeric hemoprotein which is codified by a multigenic family. It is localized in the cytoplasm of the infected cells (Robertson *et al.* 1984; Vivo *et al.* 1989) and consists of a hemo prosthetic group and only one polypeptide (globin) of 16 kDa.

The first product of nitrogenase is NH_3 but this is quickly protonated to form NH_4^+ . The initial pathway of ammonium assimilation is the same for all the legumes (Schubert 1986) and involves ammonium binding to glutamic acid to form glutamine in the presence of ATP and the enzyme glutamine synthetase (GS). Glutamine can then form 2 molecules of glutamic acid in the presence of oxoglutarate and the enzyme glutamate synthase (glutamine-2-oxoglutarate-amino-transferase; GOGAT). Thus, one of the glutamates represents a net product of nitrogen fixation, and can be used to form different amino acids by transamination, whilst the other glutamate can be used to maintain the supply of substrate for further ammonium incorporations.

The type of nodule that is formed is controlled by the macrosymbiont and can be one of two types depending on whether or not there is persistence of meristematic activity (Vessey *et al.* 1990). This persistence depends on the cell layer where the nodular primordia start to form. If the primordia start to form in the inner root-cortex, the meristem is persistent and the nodule growth is continuous to form elongated indeterminate nodules. This class of nodules shows different zones with the meristematic tip followed by an infected area, where both cells harboring symbiosomes and non-infected, or interstitial cells, can be observed. In older nodules the distal end of the infected zone forms a senescent zone consisting of degenerating cells. Thus, mature indeterminate nodules have a white meristem (zone I), an infected zone which is coloured red due to the presence of leghemoglobin (zones II and III), and a senescent zone (zone IV) which is coloured green due to the breakdown products of leghemoglobin, with both infected and senescent zones being surrounded by an outer cortex. This type of nodule is typical of temperate legume species such as *Pisum*, *Vicia*, *Medicago* and *Trifolium*. Determinate nodules are produced when primordial development starts in the outer root-cortex, with meristematic activity finishing very early during nodule growth (Patriarca *et al.* 2004). These nodules are spherical in shape, with only two observable zones: the cortex and an inner infected zone, composed of infected cells interspersed with uninfected cells without a gradient of developmental zones. In addition, the connection with the subtending root is thin and the nodule is completely surrounded by a network of vascular bundles within the cortex. Legume species originating from tropical climates, such as *Phaseolus*, *Vigna* and *Glycine*, have this class of nodule. The products of nitrogen fixation are assimilated by the plant cells in the form of either amides, especially asparagine and glutamine, in indeterminate nodules, or as ureides (such as allantoin, allantoic acid and citrulline) in determinate nodules (Bergersen 1982). However there are some exceptions, such as the *Lotus-Mesorhizobium loti* symbiosis, which has determinate nodules but is an amide exporter (Streeter 1991).

When nitrogen fixation is finished the nodule starts to senesce; a process which is genetically controlled and involves reactive oxygen species (ROS), antioxidants, hormones, proteinases and also antioxidant metabolites such as ascorbate and glutathione (Puppo *et al.* 2005). The form of senescence is different depending on the type of nodule. In

determinate nodules, the process starts in the central part of the infected zone, where the oldest cells are located, and develops radially. In indeterminate nodules the process starts in zone IV and progresses towards the nodule meristem.

Genus *Lupinus*

Lupinus is a temperate legume with great agronomic potential due to the high protein content of the seeds and its positive effect on soil fertility. The genus *Lupinus* includes about 300 species of herbs and small shrubs spread over the entire world, especially the Mediterranean area and the American continent where they colonize very different environments. The genus is divided into Old World and New World species, dealing with various climates and environmental conditions (Naganowska *et al.* 2006). Old World species from the Mediterranean region and Africa consist of only 12 recognized lupin species, including the better known species, such as *L. albus*, *L. angustifolius* and *L. luteus*. These native *Lupinus* species from the Mediterranean region were introduced as grain and forage crops into Australia and South Africa at the end of the 19th and the beginning of the 20th centuries (Stepkowski *et al.* 2005). New World lupins are spread over almost all off the American continent and differ in their karyological, embryological and biochemical characters. *L. mutabilis* is the New World species which is probably most studied and utilized.

In taxonomic terms, *Lupinus* belongs to the family Leguminosae, subfamily Papilionodeae, tribe Genisteeae, subtribe Lupininae. It is a primitive genus with particular features; annual or perennial plants, with a tap root and deep side roots which are very sensitive to heavy, badly drained soils. They are calcifugal plants which do not grow well in soils with a pH of 6.8 or higher. Therefore, they are suited to poor acid soils, which probably explains the success of lupin crops in Australia, where there are large areas of acidic soils (Howieson 1995). Although *Lupinus* is a plant of temperate climate, it is very resistant to low temperatures. For example, *L. albus* is able to resist temperatures of -9°C or more, depending on the variety (Von Baer 1990). Lupins are amongst the more effective nitrogen-fixing legumes (20-200 kg N/ha; Burns and Hardy 1975), which provides sufficient nitrogen to satisfy the plant's requirements. They are also able, despite the absence of a mycorrhizal symbiosis, to mobilize P and others elements from the soil, due to the exudation of citrate from specialized proteoid roots. These features permit the recycling of soil nutrients, making the elements available for subsequent crops as well as the present one.

Lupinus is also salt tolerant, to a greater or lesser extent depending on the species (van Steveninck *et al.* 1982); for example, *L. luteus* is more tolerant than *L. angustifolius*. This salt tolerance may depend on the capacity to maintain relatively low levels of Na and Cl, especially in the stem and the foliage (van Steveninck *et al.* 1982). Lupin crops can improve the physical soil structure due to the deep penetration of the large tap root. This feature can also lead to enhanced water storage in the soil to an extent which may even benefit the subsequent crop. Another advantage over some other grain legumes is the erect nature of mature lupins, which makes it possible to directly collect the pods. As with other grain legumes, lupins are widely utilized for animal food, but can also be used for human food by its high grain protein content (López-Bellido 1994; Graham *et al.* 1995). Furthermore, a wide range of products can be produced from lupin seeds, such as flours, coffee substitutes and protein concentrates.

Lupin varieties are classed as bitter when their alkaloid content is 1-2% of seed dry weight. Sweet lupin varieties, which are specially improved to eliminate the alkaloid content, contain less than 0.05% of dry weight as alkaloids. Bitter lupin seeds can contain more than 170 known structures of quinolizidine alkaloids, which are synthesized in the leaves and translocated via the phloem to the seeds. Immunolocalization of alkaloids in lupin seeds has been per-

formed by Pozuelo *et al.* (2001) who found that they were localized in the protein bodies of the cotyledon cells. They are very strongly bound to proteins, so that protein bodies act as reservoirs of alkaloids, in a similar way to cell vacuoles. Although alkaloids are a problem when using lupin seeds for animal or human consumption they can have important roles in terms of defense against predators of seeds and plants (Pozuelo *et al.* 2001).

Lupinus plants are mainly nodulated by specific rhizobia called *Bradyrhizobium* sp. (*Lupinus*). With respect to the infection mechanism and nodule structure the symbiosis presents some exclusives features which make it different from other legumes, as shown below.

Bradyrhizobium-nodulating *Lupinus*

Lupins are nodulated mainly by slow-growing soil bacteria of the genus *Bradyrhizobium* (Jordan 1984), which belongs to the order Rhizobiales and family *Bradyrhizobiaceae*. This genus is composed of rod-shaped bacteria, which are mobile due to polar or subpolar flagella, do not form spores, and usually containing the carbon storage compound poly-β-hydroxybutyrate. *Bradyrhizobium* belong to the class of α-Proteobacteria, as do many others rhizobia which nodulate legumes. For more than a century nodulation was considered to be an exclusive capacity of microorganisms belonging to the α-Proteobacteria class. However, four non-rhizobial species, belonging to both α- and β-Proteobacteria classes, have recently been identified as legume-nodulating bacteria (Chen *et al.* 2005; Trujillo *et al.* 2005; Chen *et al.* 2006). The *Bradyrhizobium* which nodulate *Lupinus* are not recognized as a separate species but, as with some other *Bradyrhizobium*, are given the name of the genus followed by the name of the host legume, in this case *Bradyrhizobium* sp. (*Lupinus*). It has the slow-growing characteristic of other species of the *Bradyrhizobium* genus, but it is different in being acid-tolerant and able to grow in soils with high levels of free aluminum (Howieson *et al.* 1998).

Characterization of the bradyrhizobial strains has shown that they are phenotypic and genotypically diverse, and data obtained from lupin isolates from Mexico have indicated a very close relationship with *Bradyrhizobium japonicum* type strains (Barrera *et al.* 1997). Furthermore, genetic diversity in *B. sp.* (*Lupinus*) has been studied by characterization of *hup* gene clusters with the aim of defining the extent of diversity in *hup* gene composition and regulation in endosymbiotic bacteria. Genomic DNA hybridizations showed a diversity of intraspecific *hup* profiles within *B. sp.* (*Lupinus*) (Baginsky *et al.* 2002).

A great genetic diversity among the microsymbionts of *Lupinus* has been found in a relatively wide collection from different geographic locations (Jarabo-Lorenzo *et al.* 2003). In this work bradyrhizobial strains isolated mainly from *Lupinus* species (*L. luteus*, *L. angustifolius*, *L. hispanicus*, *L. consentinii*, *L. micranthus*, *L. albus* and *Lupinus* sp.) but also from *Ornithopus* (*O. compressus*, *O. sativus* and *Ornithopus* sp.) showed that *Lupinus* seems to be a promiscuous host legume nodulated by rhizobia with different genotypes that could belong to several species of *Bradyrhizobium*. Some of these isolates were closely related to *B. japonicum*, but the majority was new genotypes. It was found that *Lupinus*, a member of the Genisteeae tribe, shares the same microsymbionts with other genistoid legumes (Jarabo-Lorenzo *et al.* 2003). These legumes are nodulated by a highly diverse group of bradyrhizobial strains, with a minority of them closely related to *B. japonicum* (Jarabo-Lorenzo *et al.* 2003), including one which has been classified as *B. japonicum* biovar. *genistearum* (Vinuesa *et al.* 2005). However, most *Bradyrhizobium* nodulating lupins and other genistoid plants belong to a new, unnamed species. *B. canariense* bv. *genistearum* is the name given to the major rhizobial genotype found in these legumes (Vinuesa *et al.* 2005), but two other genospecies have been detected. These results are similar to that presented by Kalita *et al.* (2006) for rhizobia of *Genista tinctoria*. These rhizobia formed symbioses not

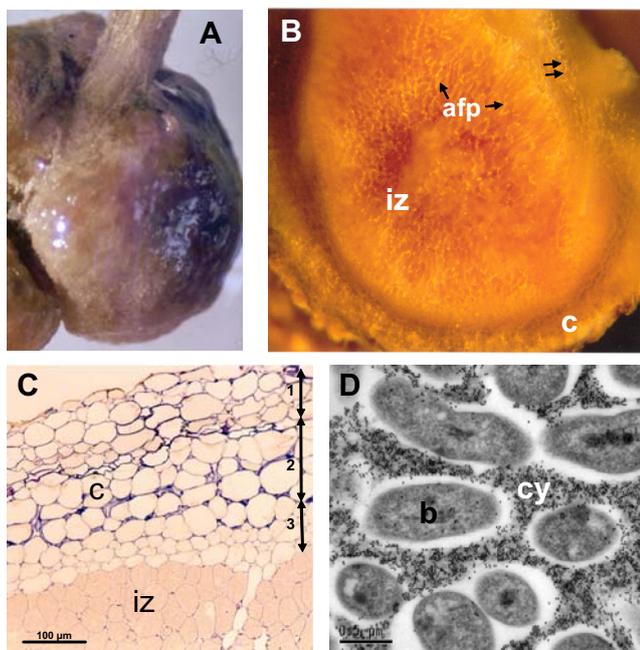


Fig. 1 *Lupinus albus* nodules. (A) Lupinoid nodule surrounding the lupin root. (B) Transversal sections obtained with light microscopy of dark field and polarized light showing the air-filled pathways in the cortex and the infected zone. (C) Light micrograph of a nodule section (stained with toluidine blue) showing the infected zone and the cortex zones (1, 2 and 3) where the oxygen diffusion barrier is localized. (D) Transmission electron microscopy micrograph showing the immuno-gold localization of leghemoglobin in the cytoplasm of the infected cells. afp, air filled pathways; b, bacteroid, c, cortex; cy, cytoplasm; iz, infected zone.

only with the native host, but also with other plants of the Genistee tribe such as *Lupinus luteus*, *Sarothamnus scoparius*, and *Chamaecytisus ratisbonensis*, and were classified as the genistoid cross-inoculation group. Genistoid microsymbionts appear together in monophyletic clusters, which suggests that their nodulation genes evolved from a common ancestor. Genetic diversity has also been showed in *Bradyrhizobium* populations infecting lupins and *Serradella* in soils of Western Australia and South Africa (Stepkowski *et al.* 2005). However, Robinson *et al.* (2000) utilized 60 bradyrhizobial strains and 80 lupin lines and observed that nodulation effectiveness was dependent on specific strain and lupin line combinations. They concluded that for the use of lupins in sustainable crop production to be successful it was necessary to identify specific combinations of lupin cultivar and bradyrhizobial strain. One of the more distinctive characteristics of *Bradyrhizobium* sp. (*Lupinus*) is that it is one of a few representatives of the *Bradyrhizobium* genus associated with legumes in temperate or Mediterranean agriculture. Furthermore, this species has dual hosts, *Lupinus* and *Ornithopus*, both of which have agricultural importance (Howieson *et al.* 1995).

THE BRADYRHIZOBIUM SP. (LUPINUS)-LUPINUS SYMBIOSIS

Lupin plants have been cultivated from ancestral times and their capacity to fix nitrogen in symbiosis with rhizobia has been known for more than one century. However, there are basic aspects of this symbiosis that are still not well understood and make it different from other, better known, symbioses. These differences could start with the production of Nod factors with monounsaturated fatty acyl groups (Howieson *et al.* 1998). The infection of lupin roots by *Bradyrhizobium* does not follow the "general" pattern of invasion through the root hair, followed by infection threads formation. Instead, cracks or openings in the root epidermis have been speculated (Howieson *et al.* 1998) and only sporadic

infections threads have been described (Tang *et al.* 1992, 1993; James *et al.* 1997). Gonzalez-Sama *et al.* (2004) have recently described the exact infection mechanism, which will be dealt with in the next section. The resulting nodule development produces a unique type of indeterminate nodule which is termed a lupinoid (Fig. 1A). This kind of nodule is formed by the continuous branched growth of the apical meristem, so that the nodule begins to surround the root, and can eventually form a complete collar around it (Bergersen 1982; Corby *et al.* 1983).

Furthermore, the meristematic divisions start in the outer cortex (as in determinate nodules) and the infected region does not show the typical zonation of indeterminate nodules. Indeed, the overall morphology is more similar to determinate nodules. The infected zone is devoid of uninfected interstitial cells, and the basal-laterally located meristematic region contains bacteroids (Golinowski *et al.* 1987). The ability of infected cells to divide is another special feature of these nodules (Fedorova *et al.* 2005). In the follow sections we will describe in detail the early events of nodulation in *Lupinus* and nodule organogenesis. In addition, the role of glycoproteins, MAP-kinases and aldehyde oxidases in the early steps of root-infection, and the special structure of lupin nodules which made possible the visualization of the oxygen diffusion barrier will be detailed.

INFECTION AND NODULE DEVELOPMENT

The morphogenesis of the lupinoid nodules has been studied in *L. luteus*, *L. angustifolius* and *L. albus* (Golinowski *et al.* 1987; Tang *et al.* 1992, 1993; Lotocka *et al.* 2000; González-Sama *et al.* 2004). Important features of this type of nodule are the presence of a meristem, which consists of both dividing infected and uninfected cells, and the absence of the typical infection threads. Both characteristics are closely related to the infection and nodule development pattern, which differs from the normal patterns for typical indeterminate and determinate nodules. Infection thread-like structures have been found in the central zone of young nodules of *L. angustifolius* (Tang *et al.* 1993) and *L. albus* (James *et al.* 1997) but never in the nodule primordium or in the meristematic zone of both developing and nitrogen-fixing nodules. Therefore these structures are too scarce and sporadic to provide a significant pathway for rhizobia invasion of the host cells.

Colonization and root infection

Four days after inoculation, rhizobial colonization is evident on the root surface of *L. albus*. Bradyrhizobia accumulate preferably in the root hair zone of the main root, and become embedded in a polysaccharide mucilaginous material containing glycoproteins with the MAC 265 epitope (de Lorenzo *et al.* 1998; González-Sama *et al.* 2004 and see section: OXYGEN REGULATION). Later, rhizobia colonize root hair and epidemic cell surfaces. Tang *et al.* (1992) reported that infections leading to nodulation in *L. angustifolius* occurred in an area of epidermal cells either lacking root hairs or with very young root hairs. Indeed, the involvement of root hairs in the early steps of lupin nodulation could be dependent of the Lupin species. In *L. luteus*, infections have been reported to occur via the curled root hair (Lotocka *et al.* 2000). The authors assumed that rhizobia penetrated the cell wall of the curled root hair in a similar way to that described for other legumes, and bacteria then multiplied inside root hairs (although an infection thread is never formed). Bacteria surrounded with a cell wall-like matrix form a protuberance within the curled root hair tip (on its inner surface) and within the root hair cell base. Similar structures were found on the inner surface of the cortex cell adjoining the infected root hair. More recently, the immunolabelling of *Bradyrhizobium* sp. (*Lupinus*) ISLU16 and the green fluorescent protein labelling of *Mesorhizobium loti* NZP2037, two strains that induce nodulation in *L. albus*, allowed us to monitor the infection and morpho-

genesis process in *L. albus* and describe the process in detail (González-Sama *et al.* 2004). In *L. albus* no curled root hairs have been observed after inoculation, similar to observations made with *L. angustifolius* (Tang *et al.* 1993). By using laser confocal microscopy it was shown that bacteria colonize the root hair surface in *L. albus* roots but they were never seen inside. Instead, bacteria enter the root intercellularly between the cell walls of the root hair base and the adjoining epidermal cell and cortical cells. They then accumulate between the cell walls of the cortical cells. At this location, rhizobia penetrate into the cytoplasm of the first host cell through structurally altered cell wall regions. This infection process seems to be similar to that reported for *L. angustifolius* (Tang *et al.* 1993). Therefore, at least in *L. albus* (but most probably in other lupin species) bradyrhizobia root infection occurs intercellularly and the invasion by rhizobia of a cortical cell immediately beneath the root hair constitutes the first step of the infected tissue development.

All the above studies on morphogenesis of lupin nodules have been performed using *B. sp.* (*Lupinus*) strains as inoculum. Nodules formed along the length of both main and secondary roots. The nodulation of lupin by fast-growing rhizobia was reported many years ago (Shlinkert-Miller and Pepper 1988) and recently a new species, named *Phyllobacterium trifolii* sp. Nov., has been reported to nodulate *L. albus*. Nodules were morphologically different to those induced by *Bradyrhizobium* sp. and were formed at the intersections of the main and secondary roots (Valverde *et al.* 2005). Similar nodule locations have been found in roots of *L. honoratus* and *L. albus* inoculated with *Ochrobactrum lupine* sp. Nov. (Trujillo *et al.* 2005). However, details of the infection process with either *P. trifolii* or *O. lupine* have yet to be reported. Because of the nodule locations it is tempting to speculate that both bacteria do not infect lupin roots as described above, but they might follow an alternative mode of infection, known as crack entry, at the emergence sites of lateral roots, which occurs in *Arachis hypogaea*, *Stylosanthes* and *Aeschynomene*.

Nodule primordium

Several days after bradyrhizobial inoculation the division of outer cortex cells of the lupin root can be detected (Tang *et al.* 1992; Lotocka *et al.* 2000; González-Sama *et al.* 2004). The initially infected cell divides rapidly and repeatedly and freshly divided cells (6-8) housing few bacteria can be observed within the initial cell wall. Bacteria then divide actively inside their host cells, whilst simultaneously, the cortical cells surrounding the initial infected cell also divide, to form the origin of the nodule cortex. Cell division in deeper layers of the root takes place later (González-Sama *et al.* 2004). Structural details of the nodule primordium formation are shown in the above cited papers. In later stages of nodule development, the central infected zone is partially surrounded by the meristem, which is located in basolateral regions and is now termed the lateral meristematic zone (LMZ). This LMZ is composed of both uninfected and infected cells (with low number of bacteria) that retain their ability to divide for several cycles. In each cell cycle, the intracellular reorganization of the rhizobia can be observed. Because bacteria appear to be equally distributed between the daughter cells, and the cytoskeleton conformation dynamics during the division of the infected cells appear relatively normal, host cells might recognize the microsymbiont as their own cell organelles (Fedorova *et al.* 2007).

Involvement of MAPKinases in the early steps of infection

Mitogen-activated protein kinase (MAPK) signalling cascades are one of the major pathways by which extracellular stimuli are transduced into intracellular responses in mammals, yeast and plants. Activation of MAPKs occurs by phosphorylation of both threonine and tyrosine residues

catalysed by an upstream MAPKK. Following activation, MAPKs translocate from the cytoplasm into the nucleus and phosphorylate a number of transcription factors leading to changes in gene expression (Whitmarsh and Davis 1998). In plants, a number of studies have demonstrated that MAPKs signals are involved in abiotic and biotic stresses, including cold and drought, wounding, hormone action and pathogen attack (Hirt 1997; Ligterink and Hirt 2001). Extensive research has been done to elucidate the role of MAPKs in plant-pathogen interactions and several members of the MAPK family have been shown to be involved in plant defence responses (Asai *et al.* 2002). So far, there is little information about MAP kinases in the *Rhizobium*-legume symbiosis. Common strategies have been found in the early plant response to infection by pathogenic and symbiotic bacteria. In this framework, it was interesting to study the involvement of MAPKs in the symbiotic interaction as good candidates to integrate a variety of signals triggered from the two partners.

Fernández-Pascual *et al.* (2006) used *L. albus* inoculated with *B. sp.* (*Lupinus*) to analyse the putative role of MAPKs in the *Rhizobium*-legume symbiosis. Results obtained by immunoblotting with polyclonal antibodies, raised against alfalfa MAPKs show the presence in lupin of the mitogen-activated protein kinases SIMK and SAMK. SIMK and SAMK can be activated by a number of diverse stimuli. SIMK is activated by high salt concentrations (Munnik *et al.* 1999) and SAMK by cold, drought and wounding (Bögre *et al.* 1997). Inoculation of seedling roots of lupin with *B. sp.* (*Lupinus*) activated both SIMK and SAMK transiently and rapidly 5 min after inoculation. Neither incubation with the dead bacteria of *B. sp.* (*Lupinus*) nor with a non-compatible bacterium like *Sinorhizobium meliloti* was able to induce activation of SIMK and SAMK. These data reveal that activation of these MAPK pathways is a specific response of the host cells to bacteria that may lead to a successful symbiotic interaction suggesting that MAPKs may take part in the recognition of compatible partners.

The maximal enhancement of MBP phosphorylation by SIMK and SAMK root immunoprecipitates of seedlings after 10 min inoculation with *B. sp.* (*Lupinus*), takes place in the region of the tip with emerging hairs or preceding hair emerge which correspond to the infection zone in the legume root. SIM and SAM kinase activation could mediate the early events of rhizobial infection in response to a bacterial signal molecule. Inhibition of SIMK and SAMK by the specific inhibitors of MAPK SB 202190 and SB 203580 showed that these two lupin root MAPKs are functional enzymes that are activated by inoculation and suggest that MAPKs might mediate the infection processes by rhizobia. To investigate whether the effect of mammalian MAPK inhibitors observed *in vitro* could affect the physiological process of nodulation of lupin roots, germinated seedlings were pre-treated with SB 202190, a MAPK inhibitor, and UO 126, a MAPKK inhibitor before inoculation with *B. sp.* (*Lupinus*). MAPK inhibitors altered the pattern of nodulation in the main root, decreasing the number and weight of nodules in the upper sites while increasing the nodule number and nodule weight in younger lower root zones. These data suggest that inhibition of MAPKs block some early events in the susceptible root zone to rhizobial infection, delaying nodulation, and supporting a role for MAPKs in the infection and nodulation of *L. albus* by *B. sp.* (*Lupinus*).

Aldehyde oxidase and indol acetic acid in the nodule meristem

Aldehyde oxidase (AO; EC 1.2.3.1) belongs to the family of molybdenum hydroxylases and catalyses the oxidation of a variety of aldehydes and nitrogen-containing heterocycles. AO has been found in animals, plants and microorganisms. In plants, AO is involved in the biosynthesis of two hormones: abscisic acid (ABA), and the auxin, indole-3-acetic acid (IAA), via the Trp-dependent indole-3-pyruvic acid pathway (Koshiba *et al.* 1996).

Phytohormones are involved in legume root nodulation as both infection and nodule organogenesis requires integration of Nod factor signalling with programmes controlled by phytohormones (Mulder *et al.* 2005). Rhizobia (via Nod factors) induce an accumulation of the auxin IAA and a local inhibition of polar auxin transport in roots, which occurs before the first cell division. During nodule development, genes mainly related to activation of the cell cycle are induced by auxin. In *L. luteus*, two mitotic B-type cyclins have been reported to be regulated by auxin (Jelenska *et al.* 2000). However, the origin of IAA in legume nodules is still not clear. Young root nodules have increased concentrations of auxin, which has been attributed mostly to polar transport. Nevertheless a microsymbiont origin for IAA in nodule cannot be leftover.

Recently, Fedorova *et al.* (2005) reported the presence of an enzyme involved in the biosynthesis of IAA in legume nodules. In this work, the enzyme AO was studied in the nodules of *Lupinus albus* and *Medicago truncatula* and the authors showed that both plant cells and bacteroids have an active AO. Nodule primordia and young nodules from lupin, plus mature *M. truncatula* nodules, showed AO activity bands after native polyacrylamide gel electrophoresis. Gel activity analyses using indole-3-aldehyde (an analog of indole-3-acetaldehyde) as substrate indicated that the nodules of white lupin and *M. truncatula* have the capability to synthesize IAA via the indole-3-pyruvic acid pathway. Bacteroids and free-living bacteria of *Bradyrhizobium* sp. (*Lupinus*) strain ISLU 16 also have AO isoforms with a preference for the above substrate and, therefore, the potential capacity to synthesize IAA.

Immunolocalization of AO by fluorescence and laser confocal microscopy, and *in situ* hybridization experiments, revealed that AO is preferentially expressed in the meristematic and the infection zones of the nodules (Fedorova *et al.* 2005). In *M. truncatula*, these locations correspond to zones I and II, respectively, whilst in *L. albus* nodules, AO was located in the lateral meristematic zone, which is composed of non-infected and infected cells that retain their ability to divide. Because AO is mostly accumulated in the meristem of *M. truncatula* (cells devoid of bacteria), nodule AO activity might be mainly attributed to the plant symbiont and not to the rhizobia.

On the other hand, the presence of the two putative products of AO activity, ABA and IAA, was analyzed in nodules by immunofluorescent labelling in the same work. ABA localization did not show any specific site of overexpression, but the highest IAA immunolocalization signal was found in the same nodule zones in which AO was detected. These results suggest that AO might be mainly involved in local IAA synthesis in nodules.

It is interesting to notice that low expression levels, and no AO activity, were detected in lupin Fix-nodules that displayed restricted growth and early senescence (Fedorova *et al.* 2005). These nodules were induced by the *Mesorhizobium loti* strain NZP2037 (González-Sama *et al.* 2004). Thus, the growth of effective nodules and the suppression of non-effective symbiotic nodules, might be controlled by the plant through the modulation of auxin synthesis. It is possible that self-autoregulation of nodule development occurs by a local control of auxin synthesis via the indole-3-pyruvic acid pathway in the nodule meristem, by means of either AO expression, activity, or both (Fedorova *et al.* 2005).

Nitric oxide synthase activity in roots and nodules

Nitric oxide (NO) is now recognized as an important signalling molecule in different animal and plant biological processes. In mammals, NO is produced by nitric oxide synthase (NOS; EC 1.14.23.39), which catalyses NO and L-citrulline formation from L-arginine, requiring NADPH as an electron donor and molecular oxygen as a co-substrate. Nitric oxide is involved in signal transduction mechanisms as a regulator, or by acting as a cytostatic or cytotoxic ef-

factor (Moncada *et al.* 1991). In mammals, two isoforms are constitutively expressed in cells, the neuronal (nNOS) and endothelial (ecNOS). By contrast, the inducible NOS (iNOS) is not normally expressed in cells, but its synthesis can be induced by cytokines or microbial wall products such as lipopolysaccharides.

The presence of NOS-like activity in plants was reported for the first time in roots and nodules of *L. albus* by Cueto *et al.* (1996), suggesting the conservation of NOS function across the animal and plant kingdoms. These authors studied the activity of nitric oxide synthase in cytosolic preparations of uninoculated roots and nodules in three ways, measuring the synthesis of NO by monitoring methemoglobin formation, quantifying the conversion of L-(¹⁴C) arginine to L-(¹⁴C) citrulline, and using a histochemical method for detection of NADPH-diaphorase activity, commonly employed as a marker for nitric oxide synthase in mammals. Nitric oxide, assayed by L-(¹⁴C) citrulline formation was synthesized in lupin root and nodules in an arginine-dependent manner. In an effort to define the potential similarities between this enzymatic system and those described in higher order organisms, the inhibitory effect of L-NMMA, an L-arginine analog, was studied. L-NMMA is an inhibitor of constitutive and inducible nitric oxide synthase in animal cells. ¹⁴C citrulline formation by roots and nodules was inhibited in a concentration-dependent manner and this inhibitory effect could be reversed by increased concentration of L-NMMA. A dependence of Ca²⁺ has been observed in the NOS-like enzymatic activity of roots. This effect was not observed in nodules. These data suggest the potential presence of a constitutive form of NOS in roots, while in nodules an inducible NO could be the predominant isoform. The presence of an inducible Ca²⁺-independent NO synthase has been described in several mammalian cells, after activation with cytokines or lipopolysaccharides (Radomski *et al.* 1990; Hortelano *et al.* 1993). It is attractive to speculate that the nodule Ca²⁺-independent NO synthase may be induced by rhizobia LPS. Cell wall rhizobia LPS are essential in the early interaction processes between plant host and bacteria, initially avoiding host cell defence reactions and also in later stages during nodule development.

The presence of NO synthase in the nodules might be of great interest since the two most important proteins, nitrogenase and leghemoglobin, are able to bind to nitric oxide, affecting their function in nitrogen fixation. On the other hand the synthesis of NO could be related to the function of hemoproteins sensors of NO, such as the Fix L proteins which sense oxygen and regulate the symbiotic expression of *nif* and *fix* genes in *S. meliloti*.

NADPH-diaphorase activity was located in the vascular bundles of both root and nodules, and in the infected cells of nodule. A significant disappearance of diaphorase activity was observed inside the cells of the infected zone and in the vascular bundles in the presence of the inhibitor NMMA. These data indicate two sites of localization for the putative plant NOS, one in the vascular bundles, common for roots and nodule since nodule vascular bundles are connected to the vascular system of the root and a second one in the inner cells of the nodule infected zone, which may be induced during symbiosis. In plants, as in animal cells, the NO released by NO synthase might be involved in signal transduction mechanisms, since phloem vessels are now recognized as a signal transport system.

In recent years the NO molecule has received considerable attention and there has been an increasing number of reports on the presence and function of nitric oxide synthase-like activities in plants. It has been reported that NO functions as a signal in plant disease resistance (Delledonne *et al.* 1998), also it has been proposed that NO regulates the expression of proteins involved in programmed cell death and plant-pathogen responses (Durner *et al.* 1998). Furthermore, MAPKs and NO seems to exert a protective function during a number of stresses in which ROS are involved (Kumar and Lessing 2000; del Rio *et al.* 2004; Teixeira da Silva 2006).

GENES AND GENE EXPRESSION IN LUPIN NODULES

The unusual features of lupin nodules are related to singular gene expression patterns in which temporal, rather than spatial gene expression patterns are frequently observed. Meristem-associated expression can be detected throughout the nodule life span in the lateral meristematic zones.

Cell-cycle regulation

The regulation of cell-cycle genes during nodule development in lupin has been investigated (Jelenska *et al.* 2000). The presence of a family of closely related cyclins, belonging to the same subgroup, may suggest their different function during plant developmental processes or their various regulations by plant-specific factors, such as phytohormones or signals released by symbiotic bacteria. The multiple variants of the same type of plant cell cycle regulators may be necessary to assure flexible reactions to internal signals and variable environment conditions. The cyclin genes belong to plant genes that are induced by lipo-oligosaccharide signals released by symbiotic bacteria during formation of nitrogen-fixing nodules. Increased levels of cyclin expression have been detected in different emerging legume nodules. In lupin, the transcripts of mitotic cyclins were present in dividing cells of the primary root cortex which subsequently formed the nodule primordium. Both cell proliferation and cyclin expression take place in the nodule cortex. When nodules are visible on the root surface, the induction of cyclin genes is restricted to the meristematic zone. The precise localisation of cyclin transcripts by *in situ* hybridisation revealed that the lupin nodule meristem is active during the whole process of nodule development, even in old organs (Jelenska *et al.* 2000).

The mitotic inhibitor *ccs52*, a plant homologue of APC (Anaphase-Promoting Complex) activators involved in mitotic cyclin degradation, is expressed in *Medicago* nodules in differentiating cells undergoing endoreduplication (Cebolla *et al.* 1999). González-Sama *et al.* (2006) have studied the implication of this gene in organogenesis of *L. albus* and *Lotus japonicus* nodules. In lupin, *ccs52A* expression was restricted to the earlier stages of nodule development, whereas *ccs52A* transcripts accumulated in *Lotus* nodule primordia and, to a lesser extent, in mature nodules. The authors concluded that *Ccs52A*-mediated endoreduplication appears to be a universal mechanism required for nodule cell differentiation during the establishment of nitrogen-fixing symbioses. Expression patterns are different in lupin, alfalfa or lotus nodules, while gene function appears to be conserved.

Nodulins

Nodulins are plant gene products appearing exclusively during nodule formation. However, the term "nodulin gene" is used in a less strict sense and refers to genes expressed in nodules, but not in uninfected roots. Several lupin nodulin genes have been identified and characterized. These include genes coding for leghemoglobins, glutamine synthetase and the early nodulins ENOD2 and ENOD40, which have also been identified in other legume nodules. The *enod2* gene is normally highly conserved among legumes and codes for proline-rich proteins. However, genomic sequences for *enod2* from lupin plants were obtained from *L. luteus* libraries (lenod2, Szczygłowski and Legocki 1990) and it was found that this gene codify for a protein two and half times longer than the *enod2* sequences from soybean and alfalfa. Furthermore, the *lenod2* gene sequence contains an OSE (organ specific element) homologous sequence which makes this gene different from others *enod2* genes and, in general, from early nodulin genes (Howieson *et al.* 1998). In *L. albus* nodules this gene seems to be implicated in oxygen diffusion regulation, because its expression increased in parallel with an increase of the oxygen diffusion resistance

when 40% oxygen was applied to *L. albus* root systems (Guasch *et al.* 1997).

Lupinus is probably the oldest genus in the legume family in which hemoglobins have been identified. Interestingly, lupin leghemoglobins show higher sequence similarity to cereal barley hemoglobin than to leghemoglobins of other legumes. At the same time, lupin hemoglobins reveal a much lower similarity to other legume hemoglobins than these hemoglobins show to one another (Strozycki and Legocki 1995).

A nodulin cDNA homologous to plant stearyl-acyl carrier protein desaturases, which contains crucial amino acids in the fatty acid binding domain which are characteristic for stearyl-ACP desaturases has been identified in lupin nodules (Swiderski *et al.* 2000). This desaturase gene is turned on at least three days earlier than leghemoglobin. Plant membranes contain a high fraction of polyenoic fatty acids, mainly linoleic and linolenic acids, both requiring desaturase activity for their biosynthesis. The increase in stearyl-ACP desaturase gene expression may be necessary to provide enough components for symbiosome membrane building and could be important in establishing an effective symbiosis.

OXYGEN REGULATION

Oxygen reaches the bacteroids from the exterior through a network of air-filled spaces (intercellular spaces) which form air filled pathways (Fig. 1B) in the cortical and infected regions. The oxygen flux is subjected to a very strict control from the exterior to the symbiosome. This oxygen regulation is carried out through the cortical oxygen diffusion barrier, the leghemoglobin and bacteroidal respiration. In lupin nodules the oxygen diffusion barrier has special features.

The oxygen diffusion barrier

The presence and functioning of the oxygen diffusion barrier had been detected by physiological techniques (Witty *et al.* 1983) and mathematical models (Hunt and Layzell 1993). However, determination of the nature, the exact localization and the operation mechanism has been mainly performed in lupin nodules (de Lorenzo *et al.* 1993; Iannetta *et al.* 1993) and has involved histochemistry, immunohistochemistry and physiological techniques. Lupin nodules were selected for these studies because the nodule cortex had a very clear structure and the oxygen diffusion barrier responds to stress conditions slower than with others legumes (Minchin *et al.* 1992). In the cortex of lupin nodules, three zones can be distinguished (Fig. 1C; Fernández-Pascual *et al.* 1992; Iannetta *et al.* 1993): an outer cortex (zone 1) formed by 5-6 layers of large, loosely packed cells with large intercellular spaces, a mid cortex (zone 2) and an inner cortex (zone 3). Zone 2 is a special feature of lupin nodules and is formed by two (occasionally three) layers of large cells with thickened wall around large intercellular spaces. Zone 3 is formed by 2-3 layers of small, closely packed, cells with very few or no intercellular spaces. Zones 2 and 3 have been regarded as the sites of the cortical regulatory mechanism related to oxygen diffusion in lupin nodules and abiotic stresses such as nitrate or high salinity produces alterations in these areas. The main alteration was the occlusion of the intercellular spaces by a glycoprotein recognized by the monoclonal antibodies MAC236 and MAC265, which were originally raised against the matrix glycoprotein in the infection threads of pea nodules (Vandenbosch *et al.* 1989). In control lupin nodules intracellular glycoprotein surrounds the intercellular spaces of the zone 2 while in lupin nodules exposed to nitrate (and to other abiotic stresses) the glycoprotein occludes the intercellular spaces producing an increase in oxygen diffusion resistance (de Lorenzo *et al.* 1993; Iannetta *et al.* 1993). The occlusion can be produced very rapidly (within 15 min), as demonstrated by Iannetta *et al.* (1995) with lupin nodules treated with different oxygen con-

centrations. The thick-walled cells of zone two are impermeable to gases and the intercellular occlusion by the glycoprotein permits a very accurate control of oxygen diffusion, depending of environmental conditions. The operational mechanism of the oxygen diffusion barrier requires both a decrease in the size of the intercellular spaces, by means of an increase in the volume of zone 2 cells, and the occlusion of these spaces with glycoprotein (de Lorenzo *et al.* 1993; Iannetta *et al.* 1993). Furthermore, diprenylated isoflavones have also been localized within the big intercellular spaces of zone 2 (Gagnon *et al.* 1995; Grandmaison and Ibrahim 1995) and appear to share the same extrusion pathways as used by glycoprotein, that is, via intracellular vesicles (de Lorenzo *et al.* 1993; James *et al.* 1995). Grandmaison and Ibrahim (1995) suggested that glycoproteins and diprenylated isoflavones interacted within the intercellular spaces to produce a "rigid gel" similarly to that produced in defence responses to pathogens. Zone 3 corresponds to an undifferentiated boundary layer plus distribution zone, as also found in soybean nodules (Parsons and Day 1990). Witty and Minchin (1994) demonstrated the existence of a continuous network of variable open pores across the soybean boundary layer, which did not occur in lupin nodules. Therefore, this zone provides the basal resistance to oxygen diffusion which is represented as the minimum resistance (R_{min}) in oxygen diffusion resistance characterization studies (Minchin *et al.* 1985; Guasch *et al.* 2001).

The expression of glycoproteins is, therefore, an essential feature of oxygen diffusion control in lupin nodules and it is produced in the very early stages of nodule development (James *et al.* 1997; de Lorenzo *et al.* 1998) in order to maintain the microaerobic conditions needed for nitrogenase gene expression and functioning. Glycoprotein was detected between 8-13 days after initial rhizobial infection whilst nitrogenase activity was not detected until 17-18 days after infection. de Lorenzo *et al.* (1998) proposed a possible pathway for the extrusion of glycoproteins to the intercellular spaces by summarizing the sites of immunolocalization of MAC 236 and 265 epitopes in lupin nodules.

With respect to the presence of an oxygen diffusion barrier in the infected zone, some mathematical models have been proposed for determinate nodules (soybean and cowpea) which include changes in shape and dimensions of infected cells (Bergersen 1994; van Cauwenberghe *et al.* 1994). In lupin nodules studies have been performed by applying different oxygen concentrations to nodulated lupin root systems and observing the effects with Low-Temperature Scanning Electron Microscopy. Preliminary results indicate that morphological modifications, such as changes in shape and size of infected cells, contributed to the maintenance of nodule functioning (Guasch *et al.*, unpublished results).

Leghemoglobin

Molecular features of leghemoglobins of lupin nodules have been described briefly in the section on "genes and gene expression in lupin nodules". Leghemoglobin is the most important late nodulin and its function is the transport and regulation of oxygen within the cytoplasm of infected cells. In lupin nodules, as in soybean, it was localized by immunocytochemistry, in the cytoplasm of infected cells and in the peribacteroid membranes (Fig. 1D; Vivo *et al.* 1989). Peribacteroid and bacteroid membranes are in close contact, as de Felipe *et al.* (1997) demonstrated with lupin nodules processed by HPF, implying that the oxygen transported by leghemoglobin is liberated to the bacteroid membranes which contain the respiratory chains.

NODULE SENESCENCE

Natural nodule senescence (ageing) can be considered as the last stage of nodule development. Nodules can undergo premature senescence under stress conditions (dark, defo-

liation, nitrate, salt, and drought) or as a reaction against non-compatible nodulating bacteria. Senescence of legume nodules is a complex and regulated process which is not completely understood, although it is known that proteinases, hormones, antioxidants and reactive oxygen species (ROS) seem to play key roles (for a review, see Puppo *et al.* 2005). Alterations in metabolism, especially those related to the control of the redox equilibrium, and ultrastructure of both symbionts have been reported for senescent nodules (Patriarca *et al.* 2004; Puppo *et al.* 2005, and references therein).

In lupin nodules, in contrast to many leguminous grain crops, senescence is not related to the phenology stage of the plant and it does not coincide with pod filling. As in determinate nodule, senescence starts in the central zone of the lupin nodule (where the oldest infected cells are located) and spreads radially toward the outside. Natural, nitrate- and dark-induced senescence have been studied in *L. albus* nodules (de Lorenzo *et al.* 1990, 1994; Lucas *et al.* 1998; Hernández-Jiménez *et al.* 2002) but here we will mainly focus on natural and dark-induced senescence.

Ultrastructural alterations

One of the first symptoms of senescence observed in lupin nodules is the altered electrodensity of the infected cell cytosol. Later, vesicles proliferate and eventually the cytosol undergoes lyses (Hernández-Jiménez *et al.* 2002). Plant organelles, especially those involved in redox metabolism, are affected during nodule senescence and peroxisomes (the main site of catalase activity in nitrogen-fixing lupin nodules) show membrane disruption and a decrease in catalase activity (de Lorenzo *et al.* 1990). It is interesting to notice that plastids and amyloplasts containing a massive accumulation of starch granules can be seen in infected cells where cytoplasm lyses have occurred and no other organelles can be recognized. This seems to be a general feature of legume nodule senescence and points to the putative importance of plastids in this process (Lucas *et al.* 1998).

Interesting features found in the senescent lupin nodule, as compared with other legume nodules, are related to the microsymbionts. During senescence, several symbiosomes fuse and then several bacteroids appear enclosed by a peribacteroid membrane. Later, lysis of the bacteroid cytosol occurs and only bacterial membranes can be seen inside symbiosomes. Eventually, the breakdown of the symbiosome membrane takes place at a very advanced stage of senescence. On the other hand, intercellular spaces occupied by a large number of bacteria can be seen in senescent lupin nodules (Hernández-Jiménez *et al.* 2002).

Antioxidants and related proteins

During nodule senescence both nitrogen-fixing activity and leghemoglobin content decrease. In the case of nitrate-induced senescence both decreases take place at the same time (de Lorenzo *et al.* 1994), but in dark-induced senescence the leghemoglobin content declines later than nitrogenase activity (Hernández-Jiménez *et al.* 2002). In any case, the level of catalytic iron (a powerful catalyst in the production of the very reactive hydroxyl radical) increases significantly and seems to precede the diminution of leghemoglobin during natural and induced- senescence (Hernández-Jiménez *et al.* 2002).

Accumulation of ferritin, a protein involved in the storage of iron, occurs during the senescence process and this may be considered as an antioxidant response (Lucas *et al.* 1998; Hernández-Jiménez *et al.* 2002). Immunolocalization studies showed that ferritin decreased in plastids of infected cells and accumulated in cortical cells (mainly in the inner cortex, zone 3), often as crystalline structures (Lucas *et al.* 1998). These results suggest that the nodule cortex plays a protective role against the spread of free iron into the plant.

Following the increase in catalytic iron, ascorbate levels decrease in both dark- and age-induced senescence, whilst

the oxidized/reduced thiol ratio is enhanced in both circumstances. There are also significant decreases in the activity of catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and increases in the peroxidases, glutathione reductase (GR; EC 1.6.4.2) and superoxide dismutases (SOD; EC 1.15.1.1) activities in senescing nodules. The dehydroascorbate reductase (DHAR; EC 1.8.5.1.) activity also increases in dark-induced senescence, but decreased during ageing. Protein carbonyl groups rise similarly at advanced stages of both dark-induced and natural senescence. However, enhanced levels of malondialdehyde (MDA) are only detected during ageing. These data support the idea that nodule senescence is related to an increase in oxidative stress (Hernández-Jiménez *et al.* 2002).

SYMBIOSIS TOLERANCE TO ABIOTIC STRESS

Nitrogen fixation in legumes is strongly related to the physiological state of the host plant, so that environmental stresses not only have a detrimental effect on metabolism, growth and development of the plant, but also affect the *Rhizobium*-legume symbiosis. The *Lupinus-Bradyrhizobium* symbiosis has been described as tolerant to several abiotic stresses that we will detail below.

Nitrate

Nitrate inhibits both nodulation and nitrogen fixation. Although it has not been conclusively demonstrated, it appears that the inhibition of nodulation is due to nitrate binding to some specific receptor of *Rhizobium* on the legume root. The mechanism of nitrogenase activity inhibition by nitrate has been studied with inoculated lupin plants. The inhibitory effect of nitrate was not apparent with concentrations of less than 10.5 mM, showing a greater tolerance of lupin nodules to nitrate. For example, an application of 10 mM nitrate to *Phaseolus* or *Vigna* induced a decrease in most of the parameters related to nitrogen fixation (Minchin *et al.* 1989). The application of 20 mM nitrate to lupin plants produced a decrease in nitrogenase activity and root respiration and an increase in the resistance of the oxygen diffusion barrier. Furthermore, nitrate produced alterations in nodule morphology (de Lorenzo *et al.* 1993; Iannetta *et al.* 1993) especially the occlusion of the mid-cortex intercellular spaces with glycoprotein. These occlusions produced an excessively low oxygen concentration in the infected zone, which inhibited nitrogenase activity (de Lorenzo *et al.* 1993). The application of 20 mM nitrate to lupin plants also produced senescence of the infected zone, inducing ultrastructural degradation of cytoplasm and subcellular organelles, especially peroxisomes (de Lorenzo *et al.* 1990). Furthermore, nitrate treatment induced a decrease in the activity of catalase and ascorbate peroxidase, the main enzymes that scavenge H₂O₂ in the nodule cytosol (de Lorenzo *et al.* 1994).

With respect to carbon metabolism, Lang *et al.* (1993) detected decreases in the specific activities of nodule cytosol phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31), aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase (ALT; EC 2.6.1.2) with the application of 20 mM of nitrate. Furthermore, the sucrose concentration decreased in the nodules, whilst concentrations of the organic acids oxaloacetate and alpha-ketoglutarate increased in bacteroids and malate accumulated in the nodule cytosol. Also in bacteroids the specific activities of bacteroidal malate dehydrogenase (MDH; EC 1.1.1.37) and AST rose transiently after nitrate treatment. These results provide evidence for anaerobic conditions in nodules under high nitrate supply, coinciding with the results obtained on nodule structure and oxygen diffusion resistance characterization.

Burity *et al.* (1999) have studied the effect of nitrate on N₂ fixation, growth and on several carbon and nitrogen metabolism enzymes in different *L. albus* genotypes. They concluded that all genotypes were affected; however deter-

mined genotypes have shown best adaptability.

Salinity

Although there are numerous studies on salt effect in some other symbiosis, the number of such works decreases dramatically when we refer to the *Lupinus-Bradyrhizobium* symbiosis. However, the number of works considering the two symbionts separately is moderately high. Raza *et al.* (2001) isolated *Bradyrhizobium* strains which are able to tolerate salt concentrations greater than 5% and are able to survive in extreme pH conditions and with 10% w/v of CaCO₃ in the liquid growth medium. The authors suggested that strains selected for to adverse conditions could be used as *Bradyrhizobium* inoculums to achieve successful growth of lupin plants.

The *Bradyrhizobium-Lupinus* symbiosis appears to be relatively salt tolerant as compared with other symbioses (Fernández-Pascual *et al.* 1996). These authors studied the effect of different NaCl concentrations on growth, photosynthesis, structure and functioning of oxygen diffusion barrier, carbohydrate metabolism and leghemoglobin content and the activity of enzymes involved in protection against active oxygen species. No effects were found after 6 d of salt exposure to 150 mM on plant growth, photosynthesis or shoot gas exchange. Nitrogenase activity decrease with salt application but this decrease was lower than in others legumes (Delgado *et al.* 1994). The mechanism involved in conferring this tolerance can be deduced from X-ray microanalysis and Scanning Electron Microscopy. Microanalysis showing very low amounts of Na and Cl in the infected area, possibly involving a K/Na interchange within the inner cortex (zone 3) of the nodules. This implies that the nodule cortex acts as a barrier to obstruct Na entry into the infected zone and prevents the detrimental effect of Na on this region. This hypothesis is supported by the small decreases in nitrogenase activity, leghemoglobin and total protein content compared to faba bean symbiosis (Cordovilla *et al.* 1994). Furthermore oxidative damage was also lower in lupin nodules as demonstrated by the activity of antioxidant enzymes. Catalase and peroxidase were not affected even with the exposure to 150 mM NaCl for 6 d and superoxide peroxidase was only affected after 6 d with 100 mM NaCl. Another component of the salt tolerance of this symbiosis could be the increase in sucrose concentration which may result from lower starch mobilization, which decreased in parallel with the increased NaCl applied. Carbohydrate changes were correlated with a decline in sucrose synthase (SS, 2.4.1.13) activity and as this enzyme controls the flux of carbon and energy for the bacteroids this could be the cause of the nitrogen fixation decline.

Tolerance of lupin plants to others salts, such as calcium sulphate, calcium chloride and potassium sulphate is not as high as tolerance to sodium chloride (McLay 1997). This author showed the effects of calcium sulphate and the presence of gypsum in field trials as well as others salts in green-house and field experiments using *Bradyrhizobium*-inoculated lupin seeds. Lupin growth was poor in the presence of these salts although there was no evidence that calcium, potassium or sulphate or chloride directly caused toxicity to the plants.

According to these data, the tolerance of the bradyrhizobia-lupin symbiosis to NaCl could be used in the colonization of stresses areas affected by intermittent drought and increasing salinity.

Herbicides

There are some evidences that the *Bradyrhizobium-Lupinus* symbiosis is less sensitive to herbicides than other legume-rhizobia symbiosis. It is known that these compounds interfere directly with plant metabolism, and indirectly, they exert a negative effect on the microsymbionts. In addition, they produce adverse effects on rhizobia in the rhizosphere, thus disturbing nodule formation (Zharan 1999). However

inoculation of lupin plants avoids some of the problems caused by herbicides. Field experiments by de Felipe *et al.* (1987) investigated the effect of the commercial Lindex[®] (20% cyanazine and 30% of linuron) and Simazine[®] on nitrogen fixation activity, the ultrastructure of both photosynthetic apparatus and nodules and grain yield. Using the commercial at herbicides recommended doses they found that nitrogenase activity decreased with herbicide application. However the decrease was smaller with inoculated seeds. The effects of Si-mazine[®] on the photosynthetic apparatus were more toxic than those caused by Lindex[®], with Simazine[®] producing chloroplast size reduction and damaged to the grana. Lindex[®] and Simazine[®] application on *B. sp* (*Lupinus*) cultures did not affect growth, so the nitrogenase activity decrease could be caused indirectly by a loss of photosynthates supply, due to damage to the photosynthetic apparatus. With respect to the ultrastructural changes in nodules, Simazine[®] altered nodule cells by causing vesicle formation, bacteroid degeneration, and decreasing the number of bacteroid with respect to inoculated plants without herbicides. In contrast, nodules from Lindex[®] treated plants appeared to have a very similar ultrastructure to the control nodules. The grain yield decreased with both herbicides, but the decrease was lower in the inoculated plants and the decrease in Lindex[®] treated plants was lower than in Simazine[®] treated plant. With this data the authors concluded that Lindex[®] was suitable for lupin crops.

The effects of the independently application of the herbicides that compose Lindex[®] (Cyanazine and Linuron) have been also studied (Fernández-Pascual *et al.* 1988). Both herbicides produced a decrease in the growth of nodules, roots and shoots. Nitrogen fixation and ultrastructure of photosynthetic apparatus and the nodules were more affected by Cyanazine than by Linuron. Leaf protein metabolism was also affected by the appearance of a new polypeptide of 17 kDa.

de Maria *et al.* (2005) investigated the effects of glyphosate on the structure and ultrastructure of the nodules and leaves of *L. albus* plants. The adverse effects of glyphosate on ultrastructure were progressive and differed depending on time of exposure and concentration of the herbicide. Although glyphosate produced similar effects on lupin nodule structure to those showed by other abiotic stresses, a major difference was detected: the oxygen diffusion barrier remained open. Glyphosate produced a decrease in glycoprotein content, indicating that this herbicide did not produce an inhibition of oxygen diffusion by increased glycoprotein production. The lack of adjustment of the oxygen diffusion barrier when the nodules are subjected to glyphosate is the inverse of what happens in other abiotic stresses such as nitrate (de Lorenzo *et al.* 1993), dark stress (Gorgocena *et al.* 1997; Hernández-Jiménez *et al.* 2002), drought (Verdoy *et al.* 2004) and heavy metals (Carpena *et al.* 2003).

With regard to carbon metabolism it was observed that, as in the case of salt application, glyphosate concentrations decreased nodule starch content and sucrose synthase activity (SS; EC 2.4.1.13). In contrast, sucrose content showed increases with time in the presence of glyphosate (de Maria *et al.* 2006). These effects were accompanied by a major inhibition of the activity of PEPC, which could be caused by the large increases in levels of shikimic and protocatechuic (PCA) acids in nodules and leaves after herbicide application (de Maria *et al.* 2006). This study suggested an additional mechanism mediated by the endogenous PEPC inhibitors, shikimate and PCA to explain the toxic effects of glyphosate in nodules. Glyphosate specifically inhibits the enzyme 5-enolpyruvylshikimic-3-phosphate synthase enzyme (EPSPS), causing a reduction in the biosynthesis of aromatic amino acids (tryptophan, tyrosine and phenylalanine), and the accumulation of shikimic acid and some hydroxybenzoic acids in leaves and other organs (Becerril *et al.* 1989; Hernández *et al.* 1999). The initial inhibition of EPSPS by glyphosate produces a deregulation of eritrose-4-phosphate and phosphoenolpyruvate sugges-

ting that shikimic and PCA acids are accumulated in the nodules, where the herbicide is also translocated. These acids could inhibit phosphoenolpyruvate carboxylase activity, thus decreasing malic and oxalic acids production and consequently bacteroid availability.

Pozuelo *et al.* (1989) studied the effects of 8 herbicides belonging to 5 different chemical groups on the nitrogen fixation and grain yield of lupin plants. Using half of the commercial recommended dose a beneficial effect was observed by 3 herbicides (Cyanazine, Trifluraline and Simazine[®]) on nitrogen fixation and grain yield. This implies that for every herbicide the appropriate dose rate should be established for each crop.

Heavy metals

The aim of most of the studies in this area was to investigate the tolerance, uptake and accumulation in different lupin plant organs of several metals of environmental interest, with the overall objective of using lupin plants for phytoremediation or bioremediation. The selection of lupin was essentially based on its ecophysiological characteristics related to its tolerance to salt (Fernández-Pascual *et al.* 1996), excess of nitrate (de Lorenzo *et al.* 1993) and the ability to solubilize and absorb elements through an extensive proteoidal root system. Furthermore, lupin has advantages such as high biomass production and easy growth thus providing a low cost crop in areas where lupin is commonly used and practices for its management are well established. Lupin is able to accumulate Mn and Al (Reay and Waugh 1981), Ni and Co (Page *et al.* 2006), Cd (Ximenez-Embrun *et al.* 2001; Zornoza *et al.* 2002; Carpena 2003; Vazquez and Carpena 2005; Page *et al.* 2006), Hg (Vera *et al.* 2002; Page *et al.* 2006), Zn (Pastor *et al.* 2003; Page *et al.* 2006), Cr and Pb (Gwozdz *et al.* 1997; Ximenez-Embrun *et al.* 2001; Page *et al.* 2006). There are also some works on Pb absorption by lupin plants from chemical amendments (Castaldi *et al.* 2005).

As with salt tolerance there are numerous works dealing with lupin and heavy metals, but this number decreases considerably when considering studies with bradyrhizobia-inoculated plants. Most of the studies correspond to hydroponic cultures and experiments related to the growth in contaminated soils are scarce (Pastor *et al.* 2003).

Studies with inoculated plants have looked at Cd and Zn and the absorption of these and other heavy metals from sewage sludge. With respect to Cd, Zornoza *et al.* (2002) showed the effect on growth, nodulation and nodule ultrastructure, absorption and distribution of nutrients and changes in organic and amino acids, proteins, nutrients and some stress indicators. Growth parameters and nitrogenase activity decreased with increased Cd concentration. It was also observed that *Lupinus* plants retained around 90% of the total Cd absorbed with most remaining in the cell walls of roots cells, although some was translocated to the aerial part. Furthermore, total thiols showed a high increase in roots, suggesting that lupins have developed some defence strategies against Cd, such as retention by cell walls and chelation by thiols. These two processes decrease the levels of free Cd. In addition, the high accumulation of Mn in lupin plants prevents Cd damage to the photosynthetic apparatus. Nevertheless, Cd applications produce structural alterations in both the nodule cortex and the infected zone. Alterations in the nodule cortex were similar to those produced by others stress; an increase in glycoprotein content and occlusions of the intercellular spaces of zone 2. In the infected zone a decrease in leghemoglobin content and damage to the symbiosome was observed (Carpena *et al.* 2003).

Inoculated lupin plants have also been used for phytoremediation in sealed landfills of urban and industrial waste where the major contaminant was Zn (Pastor *et al.* 2003). Zn produced decreases in plant growth and nitrogenase activity. However nodulated lupin plants are able to accumulate high amount of Zn in roots and shoots (4650 and 3605 ppm respectively) suggest that lupins should be considered as a

potential phytoremediator of Zn-contaminated areas.

Inoculated lupin plants have also been used to remediate desert soils (Abd-Alla *et al.* 1999). These authors studied the effects of sewage sludge application on nodulation, nitrogen fixation, and plant growth of different legumes including lupin. Plant analysis indicated that the inhibitory effect of sewage sludge at high application rates was most probably due to toxic (noxious) effect of heavy metals (Cu and Zn) on the microsymbiont rather than on host plants. They demonstrated that sewage sludge at low application rates may significantly improve legume growth on desert soils.

AGRICULTURAL IMPORTANCE

Lupinus is widely cultivated in north and central Europe, South America, South Africa and Australia, where the area cultivated with lupins is about one million ha, making the latter country the world leader for lupin seed production (Stepkowski *et al.* 2005). The introduced lupins in Australia were species native of the Mediterranean region. In Europe, the biggest producer of lupin is Russia where it is used mainly as a green-fertilizer. In South America, grain production is concentrated in the Andean countries, with Chile being the highest producer. World production of lupin crops is mainly from only three major species: *L. angustifolius*, *L. luteus* and *L. albus* (Howieson *et al.* 1995).

Lupin cultivation

The advantages of lupin in cropping systems, and also its importance in sustainable agriculture and animal nutrition, has been emphasized by the International Lupin Conference organized by the International Lupin Association, which have been held every two years from 1980 (Temprano *et al.* 1990). In relation to other legumes, however, the development of lupin-cropping in most countries of the world has been slower, with the exceptions of Poland, Australia and Chile.

The type of lupin variety suitable for conventional and sustainable agriculture has been discussed for several years. A comparison between sweet and bitter lines indicated that bitter lines had a protein content similar to the sweet lines (35% each) but greater values for plant height and yield. However, the bitter varieties have the disadvantage of a high alkaloid content in leaves and seeds (Pozuelo *et al.* 2001) and it would be necessary to eliminate this from the seeds before using them in human and animal nutrition. Then again the alkaloids protect the bitter varieties from pests and herbivory.

L. albus is one of the four economically important species of the genus *Lupinus* (Hondelmann 1984). This species is receiving increasing attention due to its high potential in sustainable production systems. Within the different cultivars of *L. albus*, it is worth mentioning the variety Multolupa obtained by Von Bauer in 1977, which is able to grow at low temperatures, even down to -6°C .

The culture of white lupin was introduced by the Greeks and Romans who used the aerial part as green manure and the seeds for human consumption. The main early focus of genetic seed diversity was the Aegean and Adriatic seas and more recently south Spain and Portugal where lupin varieties are well adapted, as confirmed by the archeological remains collected in these areas. Remains of white lupin species of balkanic origin (*L. albus* sp. *graecus*) and from Egypt and Turkey (*L. termis* Forskal) have also been found in the south of the Iberian Peninsula (Hondelmann 1984).

From an agronomic point of view, the more ancient species are represented by *L. albus* and *L. mutabilis* (Hondelmann 1984). *L. albus*, better known by its subspecies *termis*, was evidently grown in ancient Greece and possibly also in the Nile valley. *L. mutabilis* proceeds from the pre-Columbian civilizations of the American Andes and has been considered as a prehistoric agricultural species.

L. angustifolius, the narrow-leaved lupin, seems to have appeared in Europe at the same time as the yellow lupin. This species is very rich in alkaloids and when the seeds are used for sheep or cattle feed, pathologic symptoms appear after long administration periods. This illness, called "lupinosis", produces liver degeneration, leading to the death of sheep in almost all cases (Hill and Arnold 1975).

Lupins in agricultural systems

Although the genus *Lupinus* comprises a large number of species, only some of them have importance in agriculture. These are the "Old world" species: *L. albus*, *L. luteus* and *L. angustifolius*, all of them from the Mediterranean area, plus the Andean lupin, *L. mutabilis*, a "New World species". The culture of *L. albus* started in the XVI century, when the *L. luteus* and *L. angustifolius* were still wild species.

New varieties, which are better adapted to different soil types, and therefore of greater interest for agriculture, have appeared in the last few years, due mainly to the efforts of plant breeders to improve grain yields. In this way, sweet varieties of lupin have been obtained, and others are in initial stages of production. Also wild lupins, such as *L. consentini*, *L. atlanticus*, *L. digitatus*, *L. pilosus* are now being domesticated. The main effort is always directed towards obtaining varieties with a low content of alkaloids, which are able to be used in animal and human feeding.

Significant *Bradyrhizobium* strain lupin genotype interactions have been found to influence nodulation score, and shoot and root dry weights. This has led to the evaluation of potential interactions between selected *Bradyrhizobium* strains and lupin lines. Indeed, specific bradyrhizobial strains and lupin genotypes will need to be identified if lupin is to be a successful component of sustainable crop production systems (Robinson *et al.* 2000). Inoculated lupin plants differ in morphological parameters when compared to the non-inoculated plants, which seem to be weaker and stunted. However, applying N fertilizer to the preceding wheat crop increased legume seed yield and total dry matter (López-Bellido *et al.* 2003). Lupins yield also increased when the plants were grown in the presence of inorganic nitrogen fertilizer (López-Bellido and Fuentes 1990).

There is little information about rhizobia inoculation on lupin seed yield and often it is contradictory. It has been found in field studies, that lupin yield and seed protein content increased by 50% and 30% respectively, when the roots were inoculated with *Bradyrhizobium* (Ayisi *et al.* 1992). Also Temprano *et al.* (1990) obtained yield increases of 100% in *L. albus* and 200% in *L. angustifolius* which were inoculated with *Bradyrhizobium*. However, the yield experiments carried out by Larson *et al.* (1989) on inoculated and non inoculated plants did not show significant differences between treatments, even though the total dry matter and N accumulation were higher in the inoculated plants. Root inoculation also benefits sustainable agriculture by adding significant amounts of N to the soil and reducing energy requirements (Entz *et al.* 2002).

Lupins crop rotation in sustainable agriculture

Legumes are commonly used in agricultural systems as a source of N for subsequent crops and for maintaining soil N levels (Glasener *et al.* 2002). Lupins have developed a special role as a rotation crop with cereals; their contribution to farming systems is much greater than that of either crop alone. The role of lupin in crop rotation has been studied by several workers who concluded that it is very beneficial, economically valuable, improves the physico-chemical characteristics of the soil and increases soil fertility. Also it has been shown to be a good soil cover in winter and its residual value is considerable.

Nelson and Delane (1991) summarized the beneficial use of lupins in crop rotation as follow: protection by the stubble against wind effects and erosion in sandy soils, reduction of diseases and increases in control, the subsequent

cereal crop benefits from lupin nitrogen fixation and bio-control effects on disease, the lupin roots extract nutrients from soil layers which are below the reach of the cereal crop roots and there is evidence that lupin can act as “biological plows”, permitting root penetration by the following crop through compacted soil layers.

Sustainable inter-cropping systems are essential for agronomic, economic and environmental reasons (Camara *et al.* 2003). However, yields may also decrease with intensification and diversification of crop rotations due to nutrient availability. Lupin crop rotation can also have other disadvantages (Loss *et al.* 1993): the extensive lupin long roots can reduce the level of water available for the following crop, it can cause soil acidification and the high rate of K extraction may require K to be added to low K soils.

One of the main benefits of crop rotation with lupins is the increased yield of the following cereal grain, which has been estimated to be about 45% in the case of wheat (López-Bellido and Fuentes 1990). Also the lupin stubble and seed drop can act as cattle feed in summer when grass is scarce. But precise information about lupin inoculation on grain yield is not yet clearly resolved.

ECOLOGICAL IMPORTANCE AS A PIONEER

Lupinus nootkatensis (Nootka lupin)

Lupinus plants have been utilized as a pioneer in very different climatic conditions such as Iceland and California. To be a pioneer plant means that it disappears, after the establishment of a vegetative cover and also contributes to increase soil fertility (Arnalds and Runolfsson 2002).

Iceland is a country where more than a third of its surface is barren land (Arnalds and Runolfsson 2002). This land degradation was provoked mainly by the interaction of human and climatic actions, such as tree cutting and grass grazing combined with a harsh climate and volcanic activity. Thus the needed for land reclamation is well established and the huge areas of sterile land involved makes it necessary to look for economical methods. Furthermore, major problems in revegetating Iceland are the low soil fertility, with nitrogen and phosphorous being the main limiting factors, plus the seasonal cycles of freezing and thawing. Legumes, with their capacity to fix nitrogen, could be a good solution, but there are only a few native legumes in Iceland (Orradottir and Helgadóttir 1997) and none have been developed for large scale use in reclamation (Arnalds and Runolfsson 2002). *Lupinus nootkatensis* (Nootka lupin), a native of Alaska, was able to withstand the harsh climatic conditions and had a high yield without fertilization. It was introduced from Alaska in 1945 and by 1975 the ability of this lupin to colonize eroded areas with low levels of nutrients was recognized. Nootka lupin was used for revegetation in poor soils, mainly in deforested areas where they spread vigorously (Magnusson *et al.* 2002). Nowadays it is found throughout all the country and a visitor to Iceland is pleasantly surprised by the high number of hectares which contain lupins plants. The introduction of inoculated *Nootka lupin* introduces nitrogen into the soil and, as with others lupins, the plants are able to mobilize P from the soil due to the exudation of citrate from specialized proteoid roots (Wang *et al.* 2006). Furthermore Nootka lupin is able to form a persistent seed bank, which is a prerequisite to use in land reclamation (Sigurdsson and Magnusson 2002).

Strains of *Bradyrhizobium* sp. (*Lupinus*) adapted to harsh conditions have been selected and developed (Sverrison 1977). However, in order to extend the area of the Nootka lupin it was necessary to produce a robust, low-cost bacterial inoculant (Einarsson *et al.* 1993). Therefore, volcanic pumice was utilized as a supporting medium for the selected *Bradyrhizobium* sp. (*Lupinus*) strains. This is a naturally expanded mineral which is available in vast amounts in Iceland and proved to be very economical for the inoculation of large amount of lupin seeds. The viability of the strains was very high in this material, even after 35 weeks

of storage and the success of the nodulation was more than 56%.

The increase in soil fertility produced by nitrogen fixation and leaf litter makes the lupin sown areas especially attractive for soil fauna and microorganism and also indirectly increases the biodiversity of the biota especially the number of birds (Sigurdardottir 2002). This author studied the role of earthworm activity in the decomposition of lupin litter and soil fertility and found that earthworm activity increased the release of nutrients making them available to plants and microorganism. The author found a great abundance of earthworms in lupin patches of 12-15 years-old lupin areas, whilst no earthworms were found outside of the lupin patches. The decomposition of lupin litter by earthworms participates together with lupin effect in soil fertility in the initial colonization of barren lands. Thus, planting of Nootka lupins facilitated the colonization of native birch in Iceland, especially after cutting of the established lupin plants (Aradóttir 2002). Indeed, one of the main beneficial effects of Nootka lupin was the prevention of frosting of the birch seeds due to the insulating layer formed by the lupin litter and mosses.

After the success of the distribution of Nootka lupin inoculated with *Bradyrhizobium* and the improvement in soil fertility, Magnusson *et al.* (2002) considered a series of ecological questions regarding with vegetation and soil changes which might occur after a lupin invasion. They also wondered if Nootka lupin would invade other plant communities and if the lupin plants would persist or degenerate after such events. They concluded that native plant diversity could be reduced and that lupin invades vegetated areas such as heathland or meadow communities and, in consequence, the area of extent Nootka lupin areas should be controlled.

Lupinus arboreus (yellow bush lupin) and *L. lepidus*

For many years, perennial shrub lupins have been introduced as pioneer plants countries for mobile dune stabilization; a practice that has occurred in northern California, Britain, Australia and New Zealand (Pickart 2002). The main introduced species has been *Lupinus arboreus* (yellow bush lupin), a large, fast-growing, short-lived shrub native to southern and Central California (Pickart 2002). Furthermore, its size (up to 2 m in height), the large seed size, the vigorous seedlings establishment, the ability to fix nitrogen in a low-nutrient environment, and a long tap root to access water, made it easy to establish this plant amongst the original dune vegetation (dune mat).

The introduction of *Lupinus arboreus* in northern California was first performed around 1900 when the native dune mat consisted of low-growing, herbaceous plants that formed a mat-like layer of vegetation which was variable in cover and frequently contained large amounts of open sand areas (Pickart *et al.* 1998). Once yellow bush lupin was introduced, it rapidly invaded both the herbaceous dune vegetation and the open sand areas provoking profound changes in dune vegetation structure and composition and also an enrichment of the soil (Maron and Connor 1996). This generated colonization by other species, especially annual grasses, such as *Baccharis pilularis* (coyote brush). *Lupinus arboreus*, together with *Baccharis pilularis* create a practically continuous canopy which is able to stabilize dunes but it displaces the native vegetation. This ecological effect of the invasion of coastal dunes by *Lupinus arboreus* was studied by Pickart *et al.* (1998) and in order to eliminate the invasion, they proposed a protocol for the restoration of dunes invaded by yellow bush lupin. In areas with young plants, manual removal is effective using volunteer labour, but in areas heavily infested areas, they suggest that the mechanical removal of non-native vegetation and prevention of its re-establishment would allow that native species to increase significantly. However, in some cases revegetation with native species is required to establish again native dune mat

(Pickart 2002).

Another species of *Lupinus*, *L. lepidus*, has been considered as a pioneer to recuperate areas after volcanic eruptions, such as Mount St. Helens (Washington, USA) where a volcanic eruption occurred in 1980 (del Moral 1993). In this case *L. lepidus* was not deliberately introduced but it was part of the native vegetation and after the eruption, it tended to promote other species though its nitrogen fixation activity and appeared to accelerate succession by enhancing the rate of biomass accumulation (del Moral 2002). Twenty five years after, *L. lepidus* and mosses have become widely distributed over Mount St. Helens and others species have become common (del Moral and Lacher 2005).

In conclusion, the utilization of lupin as a pioneer has produced good results in terms of the revegetation of eroded areas, the improvement of soil reclamation and dunes stabilization in very different climatic conditions. However, some negative ecological impacts has been induced, such as displacement of native vegetation and alteration in dune soil structure and chemistry by the accumulation of litter and the release of nitrogen.

CONCLUDING REMARKS

Lupins plants have been cultivated from ancestral times, and their capacity to fix nitrogen in symbiosis with rhizobia has been known from more than a century, but there are still basic aspects of this symbiosis that are not well understood.

Over the last few years, *Medicago truncatula*, which has indeterminate nodules, and *Lotus japonicum*, which has determinate nodules, have been considered as two representative models of the rhizobia-legume symbiosis. Lupin nodules share characteristics of both indeterminate and determinate nodules and have been utilized as a useful tool to study novel aspects of the legume nodules functioning; infection and nodule development, operational mechanisms and structure of the oxygen diffusion barrier, presence of nitric oxide synthase in nodules and the role of aldehyde oxidase in the nodule morphogenesis. In addition, Legocki's group has been also used lupin nodules as a model to study cellular and molecular components of the symbiotic nitrogen fixation process (Strozycki and Legocki 1995) whilst, in the last year, morphological features of nodules from wild legumes (Genistea) have been published (Kalita et al. 2006) which share features with the *Bradyrhizobium-Lupinus* symbiosis described in this review. Therefore, we propose that the *Bradyrhizobium-Lupinus* symbiosis be considered as a new nodule model for future studies of nodulation in legumes plants. Furthermore, this symbiosis is tolerant of stress conditions such as high soil acidity, salinity or heavy metals, has been successful in the recuperation of very poor soils and the revegetation of degraded areas and has potential in the sustainable agriculture. These factors make this symbiosis very important not only for basic research but also at the level of environmental protection and the improvement of production in arid and semiarid areas with nutrients-poor soils. Finally, considering that the *Bradyrhizobium-Lupinus* symbiosis is relatively primitive it could help in understanding the beneficial interactions of less evolved microorganisms-plant partnerships which have a future potential in very important agricultural crops such as corn and sugar cane.

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