Legumes in the reclamation of marginal soils, from cultivar and inoculant selection to transgenic approaches

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REVIEW PAPER

Legumes in the reclamation of marginal soils, from cultivar and inoculant selection to transgenic approaches

Teodoro Coba de la Peña · José J. Pueyo

Abstract Mineral nitrogen deficiency is a frequent characteristic of arid and semi-arid soils. Biological nitrogen fixation by legumes is a sustainable and environmental-friendly alternative to chemical fertilization. Therefore, legumes have a high potential for the reclamation of marginal soils. Such issue is becoming more urgent due to the ever-rising requirement for food and feed, and the increasing extension of salinized and degraded lands, both as a consequence of global change and irrigation practices. This manuscript reviews current research on physiological and molecular mechanisms involved in the response and tolerance to environmental stresses of the *Rhizobium*–legume symbiosis. We report in particular recent advances on the isolation, characterization, and selection of tolerant rhizobial strains and legume varieties, both by traditional methods and through biotechnological approaches. The major points are the following. (1) Understanding mechanisms involved in stress tolerance is advancing fast, thus providing a solid basis for the selection and engineering of rhizobia and legumes with enhanced tolerance to environmental constraints. (2) The considerable efforts to select locally adapted legume varieties and rhizobial inocula that can fix nitrogen under conditions of drought or salinity are generating competitive crop yields in affected soils. (3) Biotechnological approaches are used to obtain improved legumes and rhizobia with enhanced tolerance to abiotic stresses, paying particular attention to the sensitive nitrogen-fixing activity. Those biotechnologies are yielding transgenic crops and inocula with unquestionable potential.

In conclusion, the role of legumes in sustainable agriculture, and particularly, their use in the reclamation of marginal lands, certainly has a very promising future.

Keywords Legume · *Rhizobium* · Soil · Nitrogen fixation · Nodule · Stress · Salinity · Drought

Contents

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1 Introduction

Over recent decades, anthropogenic activities such us urban development, road construction, industrial processes, mining, and inadequate agricultural practices have resulted in the eutrophication and pollution of soils and fresh water resources, soil degradation, loss of soil fertility, and desertification (McLauchlan 2006; Spiertz 2010; Gordon et al. 2010). Agriculture management, including activities such as tillage, fertilization, and biomass alteration, has a critical impact on food and environmental quality (McLauchlan 2006). In addition, arid and semi-arid regions are particularly prone to salinization, which might occur naturally or as a consequence of mismanaged irrigation, a process that has a profoundly negative effect on crop production (Szabolcs 1994). In fact, almost 40% of the world's land surface is affected by salinity-related problems (Zahran 1999). Nutrient depletion and soil acidification are two other common consequences of inadequate soil management (Hungria and Vargas 2000). Nitrogen is an essential nutrient for plant growth, and its availability is one of the most limiting factors in agricultural systems. For decades, nitrogen fertilizers have been continuously used to improve crop yield. However, the use of nitrogen fertilizers accelerates the depletion of large amounts of fossil, non-renewable energy resources, and it contributes substantially to environmental pollution through atmospheric emission and leaching of nitrogenated compounds to ground or surface water (Bohlool et al. 1992; Peoples and Crasswell 1992; Velthof et al. 2009).

Sustainable agriculture involves ecological management, which implies considering elements, such as biodiversity, nutrient cycling, and energy flux, in order to avoid the loss of nutrients and soil and to prevent the attack of pests and diseases (Spiertz 2010). Biological nitrogen fixation, that is, the assimilation of atmospheric nitrogen in form of organic compounds, is a sustainable source of nitrogen in cropping systems, as fixed nitrogen can be used directly by the plant, and it is less susceptible to volatilization, denitrification, and leaching (Jensen and Hauggaard-Nielsen 2003; Garg and Geetanjali 2007). Thus, biological nitrogen fixation in agrosystems can mitigate the use of fertilizers and consequently reduce global warming and water contamination (Bohlool et al. 1992). Nitrogen fixation (diazotrophy) is an exclusive property of prokaryotic organisms containing the nitrogenase enzyme complex. Diazotrophs include some archaea and within the eubacteria, members of proteobacteria, cyanobacteria, actinobacteria, and others. Eukaryotic organisms cannot fix nitrogen, and as such, different types of symbiotic interactions have been established between certain eukaryotes and diazotrophic bacteria. These associations range from loose interactions to highly regulated intracellular symbioses, whereby eukaryotic organisms supply nutrients and energy to the diazotrophs in exchange for fixed nitrogen (Kneip et al. 2007).

The most important nitrogen-fixing agents in agricultural systems are the symbiotic associations between legumes and the group of soil bacteria collectively designated as rhizobia. Rhizobia are a group of diazotrophs, most of them belonging to the α-proteobacteria, that include the genera *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, *Azorhizobium*, *Methylobacterium*, *Devosia*, *Ochrobactrum*, *Phyllobacterium*, and *Shinella*. Some nodulating bacteria within the genera *Burkholderia*, *Cupriavidus*, and *Herbaspirillum* included in the β-proteobacteria class have also been described (Masson-Boivin et al. 2009 and references therein; Weir 2009). In conditions of limited nitrogen availability in the soil, specific recognition between the plant and the micro-symbiont takes place by signal molecule exchange: legume plants secrete flavonoids and other compounds to the rhizosphere, inducing the rhizobial lipo-chito-oligosaccharides, the so-called Nod factors (Cooper 2007). These molecules act as mitogens and the cell division they induce in the root cortex leads to the formation of a new organ, the root nodule, involving the progressive differentiation of specialized cells and tissues. Concomitant with the development of the nodule primordium, bacteria enter the root cortex and in most cases, they form intracellular infection threads in root hair cells that grow inwardly until reaching the nodule primordium cells. The rhizobia infect cells of the nodule primordium entering the nodule host cell by an endocytosis-like process (González-Sama et al. 2004), becoming surrounded by a host-derived membrane. The intracellular bacteria and enveloping membrane becomes known as the symbiosome (Brewin 1991; Jones et al. 2007), which is the basic nitrogen-fixing unit of the nodule. Subsequently, transport of reduced carbon compounds from the plant to the nodule and of fixed nitrogen from the bacteroids to the host plant cytoplasm occurs. Many other metabolites are also exchanged (Udvardi and Day 1997; Hinde and Trautman 2002).

Legumes are second to grasses in their importance to humans and among grain crops, legumes rank third behind cereals and oilseeds in world production. It is estimated that about 88% of legume species examined to date can form nitrogen-fixing nodules with rhizobia, being responsible for up to 80% of the biological nitrogen fixation that takes place in agricultural settings. Owing to this capacity, legumes are a major source of food, fodder, timber, phytochemicals, phytomedicines, nutriceuticals, and nitrogen fertility in agrosystems (Graham and Vance 2000, 2003). Thus, legumes may play a key role in sustainable agriculture. Most importantly, mineral nitrogen deficiency is an important limiting factor for plant growth in arid and semi-arid regions, and rhizobia–legume symbioses are the

primary source of fixed nitrogen in such areas (Zahran 1999). Nitrogen rhizodeposition by legumes occurs by death and decomposition of nodules and roots and by exudation of soluble nitrogen compounds. Rhizodeposition of nitrogen, as a percentage of total plant nitrogen, can vary from 4% to 71% (Fustec et al. 2010, and references therein). Exudation of amino acids into the rhizosphere helps prevent loss of organic matter from the soil (Brophy and Heichel 1989). Legume-based pastures can rehabilitate degraded land by improving soil aggregation and by stimulating the activity of different soil organisms that modify soil structure (Parker 1986; Karlen et al. 1994). Legume cover crops also help prevent erosion during the establishment of the plantation (Giller and Wilson 1991). Some legumes, such as alfalfa, have deep roots able to absorb nutrients and water that are not available to other crop plants (Karlen et al. 1994). Phosphorus availability is another limiting factor in many agrosystems. Symbiotic nitrogen fixation and ammonium assimilation induce soil acidification (Raven 1986), which may benefit alkaline soils by solubilizing phosphorus from rock phosphates (Israel and Jackson 1978). Some legumes, such as lupin, can secrete organic anions into the soil, hereby inducing phosphorus solubilization in phosphorus-deficient soils (Weisskopf et al. 2009).

It has long been known that cereals, oilseeds, grasses, and herbs produce higher protein grains and higher yields when grown after or in conjunction with legumes (Dakora and Keya 1997; Jensen and Hauggaard-Nielsen 2003; Danga et al. 2009). Accordingly, legumes are frequently rotated with non-legume crops such as cereals. Intercropping legumes and cereals annually may be beneficial to the environment because it permits more efficient exploitation of the available nutrients, leading to a better use of nitrogen in the agrosystem and reducing post-harvest nitrogen availability and nitrate leaching. Intercropping can also reduce pests and diseases as well as the use of fertilizers and pesticides (Jensen and Hauggaard-Nielsen 2003).

The benefits of nitrogen fixation by legumes are summarized in Fig. 1. In conclusion, the introduction of legumes and their nodulating rhizobia can have an
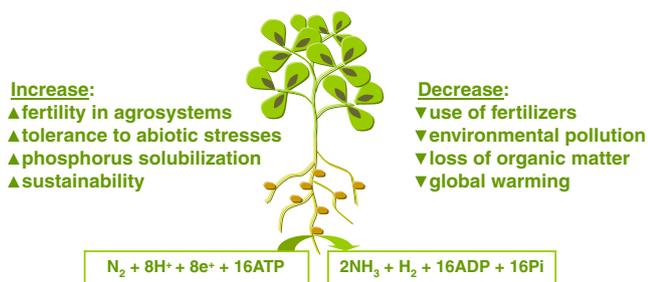
important effect on the reclamation of degraded and marginal soils for sustainable agriculture. Such recovery is becoming an urgent matter due to the increasing extension of salinized land and the ever-rising requirements for food and feed. Both rhizobia and legumes have a low or moderate tolerance to abiotic stresses. Moreover, symbiosis and nodule functions are very sensitive to abiotic stresses, more so than the host legume or the rhizobia (Delgado et al. 1994; Serraj et al. 1997). Thus, it is of interest to obtain bacterial inocula and legume varieties with enhanced tolerance to abiotic stresses for use in soil reclamation, which can be achieved by traditional trait selection or by biotechnological procedures. We will review here the current research into the physiological and molecular events involved in the response and tolerance to environmental stresses in legumes, rhizobia, and the *Rhizobium*–legume symbiotic system. Elucidating the physiological and molecular mechanisms that determine sensitivity or tolerance to abiotic stress in this biological system can help establish different strategies to obtain rhizobia, legumes, and nodulated legumes with enhanced tolerance to environmental stresses that act efficiently in reclaiming marginal soils. In the subsequent sections, we will report on recent research concerning the isolation, characterization, and selection of tolerant rhizobial strains and legumes both by traditional methods and biotechnological approaches.

2 The *Rhizobium*–legume symbiosis under environmental constraints

2.1 Effects of abiotic stresses on free-living rhizobia

The sensitivity of different rhizobial species and strains to abiotic stress varies considerably (Hungria and Vargas 2000; Vriezen et al. 2007). Salt stress affects both bacteria and plants in two ways: it induces ionic stress due to the high concentration of ions and also osmotic stress through the change in the solute concentration around the cells, producing water deficit and desiccation. It has been reported that fast growing rhizobia are more salt-tolerant than slow-growing rhizobia (Zahran 1999) and that bacteria subjected to salt stress may undergo morphological alterations. Such alterations include cell expansion and distortion of the cell envelope (Busse and Bottomley 1989). Surface components, some of which are necessary to establish symbiosis, can also suffer changes in a saline environment. Decreasing exopolysaccharide synthesis (Breedveld et al. 1991; Lloret et al. 1998; Vanderlinde et al. 2010), structural alterations, and changes in side chain length of lipopolysaccharides (Lloret et al. 1995; Soussi et al. 2001; Bhattacharya and Das 2003; Campbell et al. 2003; Vanderlinde et al. 2009), as well as the suppression or

Increase:
▲fertility in agrosystems
▲tolerance to abiotic stresses
▲phosphorus solubilization
▲sustainability

Decrease:
▼use of fertilizers
▼environmental pollution
▼loss of organic matter
▼global warming

$$N_2 + 8H^+ + 8e^- + 16ATP \qquad 2NH_3 + H_2 + 16ADP + 16Pi$$

Fig. 1 Some benefits of nitrogen fixation by legumes

alteration of periplasmic oligosaccharides that are involved in bacterial osmotic adaptation (Miller et al. 1986; Ghittoni and Bueno 1995) have been reported. Under osmotic stress, the general metabolism of the *Rhizobium* slows down, and there is a repression of genes involved in the tricarboxylic acid cycle, in the uptake of carbon supply, in glycogen metabolism, in the respiratory chains, and in ribosomal genes (Dominguez-Ferreras et al. 2006). The expression of genes encoding chaperones and elongation factors, as well as genes involved in cell division, are also altered during salt stress (Wei et al. 2004; Miller-Williams et al. 2006).

Compatible solute (osmolyte) intracellular accumulation has been observed in some species of rhizobia when subjected to salt stress. Higher intracellular concentrations of carbohydrates such as sucrose, trehalose, and maltose (Breedveld et al. 1991, 1993; Gouffi et al. 1999), amino acids such as glutamate and proline (Hua et al. 1982; Botsford and Lewis 1990; Ruberg et al. 2003; Dominguez-Ferreras et al. 2006), and imino acids such as glycine betaine (Talibart et al. 1997; Boncompagni et al. 1999), ectoine (Talibart et al. 1994, 1997), and *N*-acetylgluta-minyl-glutamine amide (Smith and Smith 1989; Fougère and Le Rudulier 1990) have been reported. It is thought that these substances and others like proline betaine (Alloing et al. 2006) act as genuine osmoprotectants. In fact, betaine, trehalose, and sucrose accumulation increase rhizobial survival during desiccation (Sauvage et al. 1983; Leslie et al. 1995; Streeter 2003). It was postulated that trehalose and glycine betaine are accumulated to prevent starvation rather than to function as osmotic stabilizers (Oren 1999). Moreover, intracellular accumulation of potassium and some polyamines has also been reported (Zahran 1999; Vriezen et al. 2007 and references therein).

High soil temperatures in tropical and semi-arid regions can also stress soil rhizobia, decreasing rhizobial survival (Hungria and Vargas 2000; Rahmani et al. 2009). High-temperature stress induces physiological and genetic modifications in rhizobia, such as plasmid deletion (Trevors 1986) and genomic rearrangements (Soberón-Chavez et al. 1986). Rhizobial cell surface components such as exopoly-saccharides and lipopolysaccharides are also altered under high temperature conditions (Nandal et al. 2005), and overproduction of heat-shock proteins has also been observed (Nandal et al. 2005; Vriezen et al. 2007).

Concerning soil acidity stress, the rhizobial microsymbiont is usually the most pH-sensitive partner. The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0, and relatively few rhizobia grow well at a pH below 5.0, although exceptions have been found (Jordan 1984; Graham et al. 1994). It appears that acid pH tolerance in rhizobia depends on the ability to maintain an intracellular pH between 7.2 and 7.5, even at an acidic external pH (Graham et al. 1994). Rhizobia exhibit an adaptive acid

tolerance response that is influenced by calcium (Glenn et al. 1999). Indeed, low pH soils are often associated with increased aluminium and manganese toxicity and reduced calcium availability (Hungria and Vargas 2000 and references therein). A range of genes are essential to regulate intracellular pH and the growth of rhizobia at low pH, including a two-component sensor regulatory system that is essential to induce the adaptive acid tolerance response (Glenn et al. 1999). Acidity can affect the size and morphology of rhizobia and their potassium content (Watkin et al. 2003). Furthermore, the activation of glutathione synthesis might be essential for tolerance to acid stress (Muglia et al. 2007).

To define the general stress response, gene characterization and expression studies have been performed in rhizobia. In *Sinorhizobium meliloti*, osmotic stress induces genes involved in the transport of small molecules and polysaccharide biosynthesis, whereas genes related to motility and chemotaxis, amino acid biosynthesis, and iron uptake are repressed (Ruberg et al. 2003). Some genes related to low pH tolerance were identified in rhizobial strains that nodulate French beans under arid and semi-arid conditions (Priefer et al. 2001). Moreover, several gene loci were identified in *Rhizobium tropici* that are required for adaptation to high pH (Nogales et al. 2002). Gene expression during osmotic stress has also been characterized by microarray technology (Ruberg et al. 2003), and the expression patterns of up- and down-regulated genes in response to acidic pH shifts have been studied in *S. meliloti* (Hellweg et al. 2009). In addition, a sigma factor was identified and characterized that acts as a general stress response regulator in *S. meliloti* (Sauviac et al.; 2007; Bastiat et al. 2010).

2.2 Effects of environmental stress on legumes and their symbiotic performance

2.2.1 Plant growth and photosynthesis

Legumes have long been recognized as either sensitive or only moderately resistant to salinity (Zahran 1991). Legume seed germination is affected by salinity and other stresses, like alkalinity or temperature (Guan et al. 2009). Most legumes respond to moderate salinity by decreasing their growth, both by inhibiting plant cell expansion due to osmotic stress and through cell injury in transpiring leaves and other plant tissues due to the accumulation of Na^+ and Cl^- ions (Delgado et al. 1994; Soussi et al. 1998; Gholipoor et al. 2000; Bayuelo-Jimenez et al. 2003; Ahmad and Jhon 2005; Manchada and Garg 2008). Ion toxicity induces alterations in the acquisition and homeostasis of essential nutrients, such as K^+, Ca^{2+}, and Mg^{2+}, and even in nutrient transport (Jeschke et al. 1992; Manchada and Garg 2008).

Legumes usually respond to salinity by excluding Na^+ and/or Cl^- ions and in fact, it is thought that salt tolerance in legumes is related to their ability to avoid salt ion accumulation in the leaves (Winter and Läuchli 1982; Läuchli 1984; Lauter et al. 1988; Teakle et al. 2006; Cabot et al. 2009). A vacuolar Na^+/H^+ antiporter was recently isolated and characterized that plays an important role in salt tolerance and ion homeostasis in legume *Trifolium repens*, probably by inducing Na^+ accumulation into the vacuole (Tang et al. 2010). Almost all metabolic processes are affected by salt and water stress. For example, complex carbohydrates and proteins are broken down by enzymes into simpler sugars and amino acids, respectively (Pandey et al. 1984). Moreover, photosynthesis and the export of photoassimilates from leaves declines upon salt or water stress due to reduced stomatal conductance, reducing transpiration and carbon dioxide entry (Nilsen 1992; Soussi et al. 1998; Iturbe-Ormaetxe et al. 1998; Ohashi et al. 2000; Reddy et al. 2003; Nunes et al. 2009). Disorganization of thylakoid and other cellular structures has also been observed during water and heat stress (Matos et al. 2002).

2.2.2 Nodule formation, structure, and physiology

Symbiosis and nodule functions are very sensitive to salt stress, more so than the host legume or the rhizobia (Delgado et al. 1994; Serraj et al. 1997; Zahran 1999 and references therein). Salinity clearly affects nodule formation and nitrogen fixation earlier and more severely than plant growth (Verdoy et al. 2004; Fig. 2). Salt stress reduces the number of rhizobia that colonize the root (Tu 1981), as well as the number and shape of root hairs, and it interferes with infection thread formation (Zahran and Sprent 1986). The initial phases of nodulation are more sensitive to salt or acid stress than later phases (Singleton and Bohlool 1984; Ikeda 1994; Miransari et al. 2006). Under conditions of acidity and high temperature, the release of Nod factors by rhizobia and/or the release of nod-gene inducers from the legume roots decreases (Hungria 1995; Hungria and Stacey 1997; Hungria and Vargas 2000). High temperature also inhibits root hair formation, adherence of bacteria to root hairs, the formation of the nodule primordium, and release from the infection thread and bacteroid development (Hungria and Vargas 2000 and references therein). Salt stress induces a decrease in nodule weight and number (El-Sheikh and Wood 1990; Cordovilla et al. 1999).

Salt stress induces morphological and ultrastructural alterations in the nodule, including cytoplasm disintegration and loss of cell wall rigidity, a decrease in the packaging of inner cortex cells, the appearance of lobulated nuclei, variations in chromatin condensation, a decrease in the volume of intercellular spaces, and an increase in the
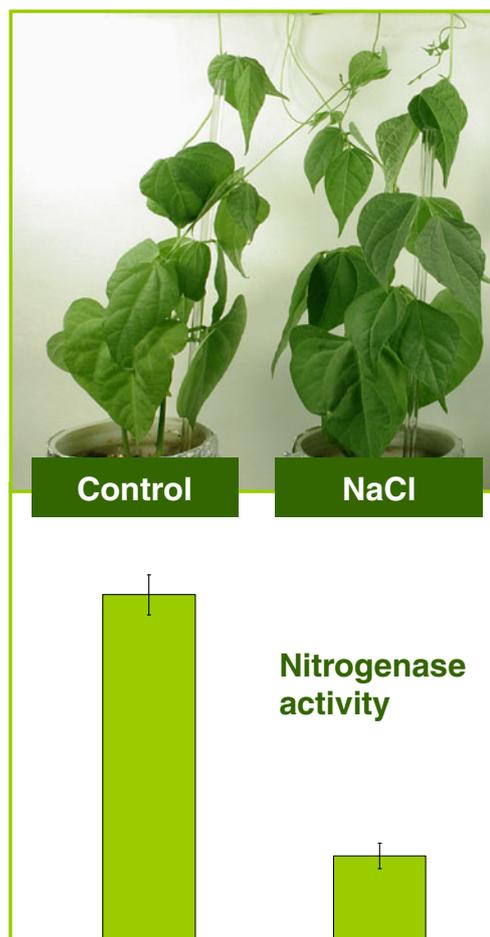
Fig. 2 The nitrogen-fixing activity is more sensitive to salt stress than the legume plant

epidermis and cortex membrane surface due to increased numbers of vesicles (Swaraj and Bishnoi 1999 and references therein; Coba de la Peña et al. 2003 and references therein; Borucki and Sujkowska 2008).

Nodule function is affected by mild stress earlier and more severely than photosynthesis (Djekoun and Planchon 1991). Nitrogenase catalyzes the fixation of atmospheric dinitrogen into ammonia, and its enzymatic activity is inhibited by free oxygen. A microaerobic environment is present in the nodule due to the combined action of the oxygen diffusion barrier in the nodular inner cortex and leghemoglobin, which transports and supplies oxygen to bacteroids for respiration. A decrease in nitrogen fixation, nitrogenase activity, and respiration, and an increase in fermentative activity have been observed in nodules subjected to salt and drought stress. It is thought that this effect is principally due to the decrease in oxygen permeability at the oxygen diffusion barrier in stressed nodules (Swaraj and Bishnoi 1999) and to a decrease in leghemoglobin levels (Delgado et al. 1994). In fact, salt tolerance of different legume varieties has been associated

with stability in nodule conductance (Aydi et al. 2004; L'taief et al. 2007). High temperature also decreases the synthesis of leghemoglobin and nitrogenase activity (Hungria and Vargas 2000 and references therein). Simulation models to estimate nitrogen fixation rates with several biotic and abiotic factors, including drought and high temperature, have been developed (Liu et al. 2010). The mechanisms by which the diffusion barrier regulates and changes the conductivity of oxygen under different environmental conditions have not been clearly elucidated. It seems that there is a correlation between those changes and cellular elongation and glycoprotein extrusion in the extracellular spaces of the inner cortex (Hunt and Layzell 1993; Iannetta et al. 1995). Ultrastructural modifications induced by salt or chilling stress have also been observed in the nodule cortex and infected zone (Serraj et al. 1995; Van Heerden et al. 2008).

Plant adaptation to saline stress involves a transient increase in the level of hormone abscisic acid (ABA). In conditions of drought, salinity, and cold, ABA mediates the closure of stomatal pores to limit water loss by transpiration, and it confers tolerance to desiccation (Montero et al. 1997; Leung and Giraudat 1998). Auxins, such as indole-3-acetic acid (IAA), are other hormones involved in the early steps of nodule organogenesis (Ferguson and Mathesius 2003). Aldehyde oxidases (AOs) are enzymes involved in the last steps of ABA and IAA synthesis and therefore, they might regulate many aspects of plant growth, development, and adaptation to a variety of stresses. Recently, several AO genes were identified in *Pisum sativum*, and some of them are induced by salt and/or ammonium stress (Zdunek-Zastocka 2008). AO transcript accumulation and enzymatic activity has been determined in nodules of *Medicago truncatula* and *Lupinus albus* (Fedorova et al. 2005). Cytokinins are another hormone type involved in the establishment of legume–*Rhizobium* symbiosis and the early steps of nodule organogenesis. Together with auxins and nodulins (legume genes involved in nodulation), cytokinins induce mitotic activity in root cortical cells, leading to the formation of the nodule primordium (Ferguson and Mathesius 2003). Cytokinins are thought to have a protective effect on plants under stress (Chernyad'ev 2009). Cytokinins are sensed by hybrid-type histidine kinases and transduced by a two-component signaling system that involves a His-Asp phosphorelay (Kakimoto 2003 and references therein). In legumes, some members of the cytokinin receptor multigenic family are essential for nodulation (Murray et al. 2007), and cytokinin receptors are involved in nodule morphogenesis, senescence, and stress response (Coba de la Peña et al. 2008a, b). In *Arabidopsis*, cytokinin receptors act as negative regulators during abiotic stress and in ABA signaling, and they probably participate in signaling cross-talk involving cytokinins, ABA, and

stress-signaling pathways (Tran et al. 2007; Coba de la Peña et al. 2008c and references therein).

Sucrose availability may also be an important factor involved in the inhibition of nitrogen fixation, as nitrogenase activity has been correlated with a decrease in sucrose synthase activity and transcript levels in nodules under conditions of drought and other environmental stress (Gordon et al. 1997; Arrese-Igor et al. 1999). Nitrogen assimilation is also affected by reducing glutamine synthetase and glutamate synthase activities (Hungria and Vargas 2000). Ascorbate and ascorbate peroxidase activities also seem to be important in regulating nitrogenase activity and dinitrogen fixation in legumes (Ross et al. 1999). Indeed, nodule nitrogen fixation appears to be inhibited by feedback regulation of ureide accumulation in the shoot during drought stress (Serraj et al. 1999), and a decrease in the activity of the enzymes involved in ammonium assimilation and in photosynthate influx into the nodule have also been observed in nodules subjected to salt stress (Coba de la Peña et al. 2003 and references therein).

Plants, like rhizobia, accumulate compatible solutes as an adaptive mechanism to salt stress. These include non-structural carbohydrates (sucrose, hexose), sugar alcohols (mannitol, ononitol, and sorbitol), amino acids (proline, alanine, arginine, glycine, and others), betaine and betaine-derived molecules (glycine betaine), and amides (glutamine, asparagine) (Keller and Ludlow 1993; Ashraf and Waheed 1993; Ashraf 1994; Schubert et al. 1995; Márquez et al. 2005; Manchada and Garg 2008). Proline is probably the most widely distributed osmolyte (Delauney and Verma 1993), and a strong correlation between osmolyte accumulation and osmotic stress tolerance has been widely reported (Zhu 2002). Legume symbiotic nodules subjected to salt or drought stress accumulate proline, other amino acids, sugars, and other osmolytes (Fougère et al. 1991; Fernández-Pascual et al. 1996; Swaraj and Bishnoi 1999; Larrainzar et al. 2009). Proline accumulation in the symbiosome has also been reported (Pedersen et al. 1996), and the accumulation of transcripts for proline-rich proteins has been observed in salt-stressed nodules (Verdoy et al. 2004). In order to explain the effect of compatible solutes, it is suggested that these molecules induce an osmotic adjustment, inducing a decrease in osmotic potential to allow an increase in water absorption and a re-establishment of intracellular salt concentration (Chen and Murata 2002). Other mechanisms against stress that have been proposed for these solutes, particularly for proline, include protection of plasma membrane integrity, providing an energy sink or reducing power, a source of carbon and nitrogen, protection of enzymes against denaturation by direct molecular interaction, heavy metal detoxification, and a signaling/regulatory role that might activate multiple responses (Rai 2002 and references therein).

The generation of toxic reactive oxygen species (ROS) is an unavoidable consequence of aerobic metabolism. ROS include, among others, hydrogen peroxide, superoxide ion, the strongly toxic hydroxyl radical, and nitric oxide. Oxygen species are principally generated in mitochondria and chloroplasts as a consequence of the electron transport associated with respiration and photosynthesis, respectively. During stress, increases in ROS generation occur due to altered metabolism within these organelles. It has been proposed that ROS induce oxidative damage in several cellular components, affecting proteins, nucleic acids, and membrane lipids. ROS play also a role in orchestrating plant gene expression and regulation (Foyer and Graham 2009; Chang et al. 2009). The legume nodule is very sensitive to ROS, where oxidative stress by ROS induces an array of toxic effects and triggers nodule senescence. Salt stress induces nodule senescence through the enhanced production of toxic ROS and the lowering of antioxidant defenses (Gogorcena et al. 1995; Sheokand et al. 1995; Swaraj and Bishnoi 1999). Nodule natural senescence also involves an increase in ROS and a decrease in antioxidant levels (Evans et al. 1999; Hernández-Jiménez et al. 2002; Puppo et al. 2005). Nodules are rich in leghemoglobin, which can undergo autoxidation and generate ROS (Puppo et al. 1981; Dalton et al. 1986). ROS induce leghemoglobin and protein degradation, originating protein radicals and catalytic iron (Davies and Puppo 1992) that induce lipid peroxidation, as well as generating hydroxyl radicals and glutathione oxidation, which in turn produce superoxide and oxygen peroxide (Puppo and Halliwell 1988; Becana et al. 1998). Hydroxyl radicals can damage sugars, lipids, proteins, and DNA. The presence of specific proteins related to nitrogen fixation in the nodule that are susceptible to autoxidation, such as ferredoxin, uricase, and hydrogenase, can also induce ROS generation (Dalton et al. 1991). Plants have developed several antioxidant systems to scavenge ROS that are also active in symbiotic nodules (Dalton et al. 1986, 1993; Evans et al. 1999; Becana et al. 2000; Matamoros et al. 2003; Puppo et al. 2005). These include the enzymes catalase, superoxide dismutase (SOD) and peroxidase, as well as the ascorbate-glutathione pathway, which includes ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase (Dalton et al. 1986, 1992; Noctor and Foyer 1998; Becana et al. 2000). Low-molecular weight antioxidants ascorbate and reduced glutathione in this pathway can also scavenge superoxide and hydrogen peroxide (Becana et al. 2000; Matamoros et al. 2006; Loscos et al. 2008). In some legume species and tissues, glutathione is partially or completely replaced by homoglutathione, which presumably fulfils analogous functions (Evans et al. 1999; Loscos et al. 2008).

Several recent studies have used molecular techniques, including transcriptomics, proteomics, and metabolomics, to define new genes, proteins, and metabolites involved in abiotic stress in legumes. In this way, a set of differentially expressed genes was identified during heat-shock treatment of cowpea nodules (Simões-Araújo et al. 2002). Similarly, expressed sequence tags (ESTs) have been analyzed in drought-tolerant horsegram under various abiotic stress conditions (Reddy et al. 2008), and a transcriptome study was performed on alfalfa subjected to drought stress (Chen et al. 2008). ESTs have also been characterized in pigeon pea subjected to water deficit conditions (Priyanka et al. 2010), as have drought and salinity responsive ESTs in chickpea (Varshney et al. 2009; Jain and Chattopadhyay 2010). Drought-induced genes were studied in peanut (Govind et al. 2009) and microRNAs were identified in *Phaseolus vulgaris* subjected to drought (Arenas-Huertero et al. 2009). A gene expression atlas of *M. truncatula* was obtained by comparative transcriptome analysis as a resource for legume functional genomics (Benedito et al. 2008), and gene expression was analyzed in the roots of *M. truncatula* subjected to salt stress (Merchan et al. 2007; Li et al. 2009). The model legume *Lotus japonicus* has been exposed to salt stress, and profiles were obtained at the ionomic, transcriptomic, and metabolomic levels, in order to perform integrative functional genomics (Sanchez et al. 2008). The characterization of genes and regulatory pathways involved in legume tolerance to abiotic stresses will allow the mechanisms of stress tolerance in legumes to be elucidated. These results hold promise for the future engineering of legume crop plants with enhanced tolerance to major abiotic stresses.

3 Rhizobial inoculants in the reclamation of marginal soils

The fact that nitrogen fixation is an essential aspect of agricultural sustainability has led to the development of different strategies to maximize legume-derived nitrogen fixation in agriculture: field inoculation with selected rhizobial strains; growth of selected legume species and varieties; and the selection and joint field application of both bacteria and legumes. In this section, we will review the current research concerning the identification and selection of rhizobial strains with suitable characteristics for field inoculation to induce legume nodulation and nitrogen fixation under environmental constraints. We will also review the existing knowledge on the role of rhizobia as endophytes and plant growth-promoting rhizobacteria, both in leguminous and non-leguminous plants. Finally, research concerning the development of genetically engineered stress-tolerant rhizobia will be examined.

3.1 Selection of rhizobial inoculants that nodulate legumes in stressed environments

Nodulation, nitrogen fixation, and growth can be improved in legumes by inoculating plants with competitive and stress-tolerant rhizobia. This is an economically feasible way to increase legume production in stress-limited environments, particularly when local rhizobia strains are absent from soils or ineffective (Zahran 1999 and references therein). An inoculant strain must display enhanced competitive ability to successfully colonize plant roots, form nodules, and subsequently perform nitrogen fixation. Thus, complex interactions between the edaphic environment with genotypes of both the legume and its micro-symbiont must be taken into account, and different strategies have been developed to select quality inoculant rhizobial strains to enhance legume-derived nitrogen fixation in agriculture (Sessitsch et al. 2002 and references therein). Different legume inoculation techniques have been developed, such as slurry, lime or phosphate pelleting, vacuum impregnation for seed inoculation, and liquid and granular inoculation for soil inoculation (Brockwell and Bottomley 1995; Deaker et al. 2004).

Molecular and phylogenetic studies have been performed in order to classify and evaluate the molecular diversity of rhizobia (Wang and Martínez-Romero 2000; Lloret and Martínez-Romero 2005; Mouhsine et al. 2007; Elboutahiri et al. 2009; Binde et al. 2009), providing a suitable means to identify and manage rhizobial isolates. Evaluating rhizobial competition in the rhizosphere must also be performed, and several specific determinants of competitive nodulation have been identified. One such factor is the secretion of rhizopines, myo-inositol derivatives by various *S. meliloti* and *Rhizobium leguminosarum* bv. *viciae* strains. Nodule bacteroids from these strains can synthesize rhizopines, which are subsequently catabolized by the corresponding free-living rhizobial strain and a few other bacteria. Studies of competition have shown that in the presence of a rhizopine-producing strain, the strain that can catabolize the rhizopine occupies a higher percentage of the nodules (Murphy et al. 1995 and references therein). However, to date, the mechanism and biological functions of rhizopines remain unclear. Another determinant of competitive nodulation is the production of trifolitoxin, a potent antirhizobial peptide that is produced by some *R. leguminosarum* strains (Triplett and Barta 1987). Similarly, *R. leguminosarum* strains can also produce bacteriocins, antibiotics that are active against closely related strains or species (Oresnik et al. 1999; Yajima et al. 2008). As explained above, the physiology of the bacterial surface may also be a competitive factor, and the competitive capacity of rhizobial mutants with altered lipopolysaccharides or exopolysaccharides may be altered (Fraysse et al. 2003; Ormeño-Orrillo

2005). For example, the production of exopolysaccharides is influenced by a complex network of environmental factors such as phosphate, nitrogen, or sulphur (Skorupska et al. 2006). Finally, genetic exchange among rhizobia in the soil and the often reported genome instability of *Rhizobium* may affect competitiveness (Ding and Hynes 2009; Orozco-Mosqueda et al. 2009). Understanding these and other factors might facilitate the development of different approaches to manipulate rhizobial competition (Sessitsch et al. 2002 and references therein).

We shall now focus on some of the recent research aimed at identifying rhizobial strains of interest and/or that has focused on their use as inocula for legume nodulation in marginal soils. Numerous studies have characterized the effectiveness of different inoculants, and their competition with indigenous strains has been determined. As such, field inoculation of the common bean with selected *R. tropici* strains induced nodulation and improved nitrogen fixation rates, even in presence of high indigenous rhizobial populations (Hungria et al. 2003). Moreover, the composition of the indigenous bacterial community was not clearly affected by massive inoculation of selected strains. Several indigenous rhizobial isolates with higher nitrogen fixation effectiveness than some commercial inoculants have been isolated and characterized (Zengeni and Giller 2007), some of which are proposed to be new potential commercial inoculants. A *Rhizobium gallicum* strain selected for its competitiveness and symbiotic effectiveness was used to nodulate common bean, inducing an increase in nodule number and plant shoot dry weight, even in a soil with a high population density of indigenous *R. gallicum* (Mnasri et al. 2007). It is also interesting that this selected inoculum produces antimicrobial activity against indigenous common bean rhizobia. Studies of competition between introduced and native *R. leguminosarum* strains have been performed for nodulation of *T. repens* and *Trifolium pratense* in Uruguay (Blanco et al. 2010). Interestingly, pre-treatment of red clover seeds with specific Nod factors significantly enhanced clover nodulation by an inoculant strain and the subsequent growth of plants in the soil (Maj et al. 2009).

Improved legume production, particularly in arid and semi-arid areas, requires the selection of effective rhizobial strains. *P. vulgaris* and other food legumes are frequently grown in rotation with cereals. Up to 30 osmotolerant strains of *Rhizobiaceae* have been isolated and characterized from Moroccan saline soils in order to use them as inoculants to improve common bean production in these soils (Bouhmouch et al. 2001). In greenhouse experiments, a comparative analysis of inoculation of *P. vulgaris* cultivars was performed with reference rhizobial strains and local isolates (Aouani et al. 1997), identifying certain improvements following inoculation with selected strains. Salt-tolerant rhizobia that were able to nodulate chickpea

(*Cicer arietinum*) were characterized and selected in conditions of water deficiency (Romdhane et al. 2008; 2009) and inoculation with selected salt-tolerant rhizobia significantly increased nodule number and grain yield of this pulse legume in the field. In some cases, improvement in plant production was equal to or better than that of nitrogen fertilization. Rhizobial species and strains isolated from wild herbs and tree legumes from arid regions often have a wide host range and are very tolerant of salinity, high temperature, and desiccation. Such species hold potential to inoculate wild and crop legumes cultivated in reclaimed arid lands (Zahran 2001 and references therein).

The genetic diversity and symbiotic efficiency of different rhizobial isolates obtained from nodules of peanut (*Arachis hypogaea*) in Morocco has also been characterized (El-Akhal et al. 2008). Isolates were grouped with species belonging to both the *Bradyrhizobium* and *Rhizobium* genera, and their phenotypic and genotypic characterization allowed them to be classified in two groups: alkali- and salt-sensitive slow growers and alkali- and salt-tolerant fast growers (El-Akhal et al. 2009). Both groups exhibited variability in their tolerance of heavy metals, and wide genotypic diversity was observed at the strain level. In arid and semi-arid regions of Morocco, strains of *S. meliloti* and *Sinorhizobium medicae* were isolated that were tolerant to salinity, water stress, high temperature, acidity, and heavy metals, and the genetic diversity of these strains was studied (Elboutahiri et al. 2010). This kind of characterization provides a basis for selecting nodulating rhizobia that may have applications in formulating appropriate inocula to improve legume crop yield in stress-affected soils, including marginal saline and acidic areas. Rhizobial strains were also isolated from leguminous shrubs in semi-arid soils of Central Spain (Ruiz-Díez et al. 2009), several of which displayed salinity, acidity, alkalinity, and cadmium tolerance. The diversity and phylogeny of rhizobial bacteria associated to nodules of the shrub legume *Ononis tridentata* have also been characterized in Spanish soils (Rincón et al. 2008). This shrub legume is highly prized for the revegetation of gypsum soils in semi-arid Mediterranean areas and thus, this molecular characterization has implications for formulating suitable bacterial inocula to recover gypsum ecosystems. The persistence of two *Rhizobium etli* strains that nodulate beans in clay soil and silty loam soil from Egypt was also established (Moawad et al. 2005).

In Brazil, numerous efficient nitrogen-fixing rhizobia have been selected to nodulate legume trees in order to revegetate poor or depleted soils and to restore their fertility (Franco and De Faria 1997). In Australia, extensive clearing of native vegetation has contributed to major environmental problems, including land degradation, dryland salinity, soil erosion, and loss of biodiversity. A low-cost revegetation approach has been to inoculate different native leguminous shrubs and trees with elite strains of native rhizobia in order to re-establish plant–soil interactions in degraded lands (Thrall et al. 2005). The diversity and salt tolerance of native isolated rhizobia nodulating *Acacia* has also been characterized in saline and non-saline Australian soils (Thrall et al. 2009), and rhizobial populations derived from saline soils had higher salt tolerance and grew better. A *Mesorhizobium* sp. strain with high tolerance to salinity, pH, and temperature and that displayed a wide host range of nodulation, was isolated and characterized from a semi-arid region of northwestern China (Wei et al. 2008).

Acidity is an increasing problem in tropical and arid soils, and it was seen that rhizobia isolated from acid soils were significantly more tolerant to acidity than isolates from neutral soils (Gemmell and Roughley 1993). Indeed, an acid-tolerant *R. leguminosarum* strain has been selected that had higher nodule occupancy at low pH in different bean cultivars than an acid-sensitive strain (Vargas and Graham 1989). *Mesorhizobium* isolated from Portuguese soils that were able to grow at acid pH could be used to develop highly effective inoculants for chickpea in acid soils (Brigido et al. 2007). The genetic diversity of alfalfa rhizobia isolated from volcanic soils in southern Chile was evaluated, and several acid-tolerant *S. meliloti* strains with high symbiotic effectiveness under acidic conditions were identified and characterized (Langer et al. 2008). In field experiments to evaluate the potential *Rhizobium* inoculants suitable for pulse legume production in acidic soils of South-East Australia storage, temperature and humidity conditions were established to maintain the viability of the inoculant (Evans 2005). Field competition experiments have been carried out in Southern Spain and different inoculant strains of *Sinorhizobium fredii* were selected that can nodulate soybean in alkaline or acid soils (Albareda et al. 2009). Halotolerant and desiccation-resistant rhizobial strains nodulating *Acacia* were isolated from Moroccan desert soil, and tolerant and sensitive strains accumulated different types of endogenous osmolytes (Essendoubi et al. 2007). Novel strains of the β-proteobacteria *Burkholderia* capable of nodulating an herbaceous legume, *Rhynchosia ferulifolia*, and with a potential role in nitrogen fixation adapted to acid, infertile soils were characterized (Garau et al. 2009).

High temperatures diminish rhizobial survival and establishment in tropical soils. Repeated inoculation of soybean compensates the detrimental effects of high temperatures and increases nodulation, nitrogen fixation rates, and crop yield in Brazil (Vargas and Hungria 1997). In tropical Brazilian soils, nodulation of common bean and other legumes is usually poor, this failure being attributed to high soil temperature, and rhizobial temperature tolerance is not always closely related to the

geographical origin of a strain (Hungria and Vargas 2000 and references therein). Different studies have shown that *R. tropici* tolerates significantly higher temperatures than other rhizobia like *R. leguminosarum* and *R. etli*. Moreover, *R. tropici* is genetically more stable, maintaining symbiotic properties under stress (Martínez-Romero et al. 1991; Hungria et al. 1993), which makes it very interesting for inoculation programs. Thermotolerant bradyrhizobial strains have been isolated to perform nodulation of soybean in semi-arid regions of Iran (Rahmani et al. 2009) and in the greenhouse, some of the thermotolerant isolates displayed good nitrogen-fixing activity even at 38°C.

In terms of reclamation or bioremediation of different types of contaminated soils, efficient strains of *Bradyrhizobium* sp. were isolated from mine spoils and used to inoculate seeds of the legume *Albizia lebbeck* grown in both gypsum and limestone mine spoils (Rao and Tak 2001). Improved nodulation and nitrogen-fixing activity was observed, and therefore, this system may be useful for revegetation of mine spoils. Indigenous toluate-tolerant bacteria were isolated from the oil-contaminated rhizosphere of *Galega orientalis*, and they constitute a potential system for bioremediation of oil-contaminated soil (Lindström et al. 2003). It has also been reported that wastewater sludge, a worldwide recyclable waste, has good potential for rhizobial inoculant production, both as a growth medium and as a carrier source for inoculant production (Ben Rebah et al. 2007).

3.2 Rhizobia as endophytes and plant growth promoting rhizobacteria in legumes and non-legumes

In non-nodulating legumes, rhizobia have been encountered inside roots without forming nodules (Allen and Allen 1991). Indeed, rhizobia have also been found as common rhizosphere colonizers of a wide range of plants and also as endophytic bacteria in legumes and non-leguminous plants such as rice, sugarcane, wheat, and maize (Sessitsch et al. 2002 and references therein). It has been shown that rhizobia can successfully colonize the mainstream roots, stems, and leaves of non-leguminous plants, and in many cases, these endophytic rhizobia benefit the host through plant growth promotion (PGP), producing a marked increase in plant growth, vigor and yield, and stress tolerance (Sessitsch et al. 2002; Hossain and Martensson 2008; Bhattacharjee et al. 2008 and references therein; Bano and Fatima 2009). In exchange, endophytes enjoy a more favorable environment than rhizospheric bacteria, being protected from stress and enjoying the direct provision of nutrition within the host (Sevilla and Kennedy 2000). The traditional legume (berseem clover)–cereal (rice) association in Egypt involves an endophytic activity

of *R. leguminosarum*, which induces PGP in rice. Moreover, inoculation of *R. trifolii* to a rice variety significantly increases its total nitrogen content and grain yield (Yanni et al. 1997, 2001; Biswas et al. 2000a, b). Examples of endophytic or rhizospheric rhizobia associations with non-leguminous plants are reviewed and listed in Sessitsch et al. (2002) and Bhattacharjee et al. (2008).

The entry of endophytic rhizobia takes place through root tips, lateral root cracks, sites of injury, and damaged stomata. Infection thread-like structures have also been observed in inoculated plants. It seems that bacteria colonize intercellular spaces of root cortex and extend to the xylem vessel for further propagation, and they can propagate to the next plant generation through the seeds (Bhattacharjee et al. 2008 and references therein). There are several propositions to explain the PGP effects of rhizobia on the non-legume host plant. Endophytic rhizobia have been reported to induce nitrogen accumulation in the host plant, which may be due to nitrogen fixation by rhizobia (Oliveira et al. 2002) or an increase in nitrogen uptake from the soil (Yanni et al. 1997). In fact, the expression of nitrogenase was detected in epidermal cells, in the intercellular region of the root cortex and in vascular tissue of the roots of rice and other plants (Egener et al. 1999). Alternatively, the legume or non-legume plant growth stimulation might be due to the production and secretion of plant growth regulators by rhizobia, such as indole-3-acetic acid (IAA), gibberellins, and cytokinins (Yanni et al. 2001; Verma et al. 2001; Boiero et al. 2007; Vargas et al. 2009). Endophytic or rhizospheric rhizobia also favor the solubility of phosphorus through the production of phosphate-solubilizing enzymes (Verma et al. 2001). Bacteria also secrete 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which diminishes ACC levels, a precursor of ethylene, thereby reducing the levels of ethylene (Glick 2005; Duan et al. 2009). Rhizobia can also produce strain-specific siderophores that can help overcome iron starvation (Jin et al. 2006; Vargas et al. 2009). In some cases, rhizobia-produced LPS can induce systemic resistance which buffers the deleterious effects of phytopathogens (Reitz et al. 2001).

An increasing number of bacteria other than rhizobia have been identified as rhizospheric and/or endophytic nitrogen fixers in a variety of cereal crops and pasture grasses (Franche et al. 2009, and references therein). Sugar cane is a good example of a crop that can benefit from nitrogen fixation (Boddey et al. 2001, 2003). Other associations with Gramineae and grasses have also been described (Boddey and Döbereiner 1982; Reis et al. 2001). These discoveries suggest that it is possible to increase biological nitrogen fixation in cereal crops by inoculation with wild-type or genetically modified nitrogen-fixing bacteria (Franche et al. 2009).

3.3 Genetically modified stress-tolerant rhizobia

One aim of the genetic engineering of rhizobia (Table 1) is to develop stress-tolerant strains that can be used as inoculants in marginal soils. However, the release of genetically improved strains is often restricted by National regulations. Several studies have been performed on field inoculation with genetically modified rhizobia carrying chromosomally located antibiotic resistance markers in order to study the population dynamics of indigenous and genetically modified rhizobia over a period of several years (Hirsch and Spokes 1994; Hirsch 1996). The luciferase gene-tagged *S. meliloti* L33 strain has also been used as inoculant in greenhouse and field experiments to evaluate the efficacy of different strategies to eliminate an established population of inoculated *Rhizobium* when its function is completed (Miethling and Tebbe 2004).

Acid-tolerant strains of *R. leguminosarum* have been constructed with enhanced capacity for nitrogen fixation by replacing the symbiotic plasmid pSym (Chen et al. 1991). Transfer of pSym to a genetically modified strain of *R. leguminosarum* conferred advantages in the rhizosphere by increasing host fitness in the field (Clark et al. 2002). An enhanced salt-tolerant *R. leguminosarum* strain was also obtained by transformation with DNA from a salt tolerant *Bacillus* species (El-Saidi and Ali 1993), and the plant yield of host legumes nodulated by this strain increased, as did the nitrogen content in arid soils. Transformed *R. trifolii* and *R. leguminosarum* were obtained that constitutively expressed a chimeric *nodD* gene, involved in the regulation of Nod factor synthesis (Spaink et al. 1989). Legumes nodulated by these transformed rhizobia displayed significantly higher nitrogen fixation. Moreover, in a field experiment, a genetically modified *S. meliloti* strain overexpressing the *putA* gene, which codes for proline dehydrogenase (an enzyme that catalyzes the conversion of proline present in root exudates to glutamate), has a competitive advantage over natural rhizobial populations with regards to infecting and nodulating alfalfa roots subjected to drought stress (Van Dillewijn et al. 2001, 2002).

A *S. meliloti* recombinant strain was produced that contained additional copies of *nifA* and *dctABD* genes to increase nitrogen fixation, and thus, the yield of alfalfa (Bosworth et al. 1994). *nifA* is involved in regulating the expression of nitrogen fixation genes, and *dctABD* genes are involved in the transport of dicarboxylic acids, which are required as a carbon energy source. This recombinant strain has been commercialized and tested in the field, inducing an improvement in biomass yield where soil nitrogen and organic matter content were low. A hemoglobin gene from *Vitreoscilla* sp. was introduced in *R. etli*, and the recombinant bacteria obtained had greater respiratory activity, chemical energy, and expression of the nitrogen fixation gene *nifH*. Plants inoculated with this recombinant strain had enhanced nitrogenase activity and total nitrogen content when compared with plants inoculated with the wild-type strain (Ramírez et al. 1999). A *Rhizobium tropici* mutant with enhanced respiration and symbiotic performance was obtained by mutating the glycogen synthase gene (Marroquí et al. 2001).

Symbiotic nitrogen fixation is an energy-consuming process that takes place under microaerobic conditions, since nitrogenase activity is inhibited by oxygen. Terminal oxidase cbb_3 has an extremely high oxygen affinity, and it is efficiently coupled to ATP production and is essential for nitrogen-fixing endosymbiosis. However, due to the presence of oxygen, the cbb_3 gene is not usually expressed in the free-living state. NtrC, a transcriptional activator that modulates gene expression in response to nitrogen, represses the free-living cell production of this terminal oxidase. Overexpression of the *Bradyrhizobium japonicum* terminal oxidase cbb_3 in a *R. etli ntrC* mutant increased nitrogen fixation (Soberón et al. 1999). *S. meliloti* strains have been constructed that contain different copy numbers of a symbiotic gene region covering a regulatory gene (*nodD1*), the common nodulation genes (*nodABC*), and a gene essential for nitrogen fixation (*nifN*). The strains with a moderate increase in copy number of this symbiotic gene region were associated with significantly improved nodulation, nitrogenase activity, plant nitrogen content, and plant growth (Castillo et al. 1999).

Inoculated strains must compete with native soil bacteria for root nodulation. As indicated above, a strategy to alleviate this problem is to produce the anti-rhizobial peptide trifolitoxin. Under field conditions, inoculation of a genetically engineered *R. etli* strain that produces this peptide increased nodule occupancy (Robleto et al. 1997, 1998). Several rhizobial strains of the genus *Bradyrhizobium* possess a hydrogen uptake (Hup) system able to recycle the hydrogen evolved by nitrogenase, resulting in more energy efficient nitrogen fixation. The Hup gene cluster has been introduced into various *Rhizobium* strains (Báscones et al. 2000), and in some strains, high levels of hydrogenase activity were achieved, leading to nodules that release no hydrogen. In a field experiment, the yield of *P. vulgaris* inoculated with a trifolitoxin-producing Hup$^+$ *R. leguminosarum* strain was significantly increased (Iniguez et al. 2004).

S. meliloti possesses several betaine transporters. Bacteroids overexpressing a high-affinity betaine transporter accumulated high concentrations of proline betaine, the endogenous betaine synthesized by alfalfa plants, in nodulated alfalfa plants subjected to salt stress. Moreover, nitrogen fixation was better maintained in nodulated plants (Boscari et al. 2006). A novel pathway for IAA synthesis was introduced into *R. leguminosarum* using a construct containing the *iaaM* gene from *Pseudomonas syringae* and

Table 1 Genetic engineering of rhizobia to obtain abiotic stress tolerance and enhanced nitrogen fixation

Gene	Origin	Host *Rhizobium*	Enhanced trait or effect	Proposed mechanism	Reference
Chimeric *nodD*	*S. meliloti* and *R. trifolii*	*R. trifolii* and *R. leguminosarum*	Enhanced nitrogen fixation in *Vicia sativa* and *Trifolium repens*	Enhanced production of Nod factors	Spaink et al. (1989)
Sym plasmid	*R. leguminosarum*	*R. leguminosarum*	Acid tolerance in free-living rhizobia. Enhanced nitrogen fixation in *Trifolium subterraneum* at low pH	Enhanced efficiency of nodulation genes located in the plasmid	Chen et al. (1991)
Chromosomal DNA	Salt tolerant *Bacillus sp.*	*R. leguminosarum*	Salt tolerance in free-living rhizobia. Plant yield and nitrogen content of nodulated host lentil in arid soils	Transfer of genes determining salt tolerance	El-Saidi and Ali (1993)
nifA and *dctABD*	*S. meliloti*	*S. meliloti*	Enhanced nitrogen fixation and biomass yield of alfalfa nodulated plants in soils with low nitrogen and organic matter content	Enhanced regulation of nitrogen fixation and increased dicaboxylic acid import to bacteroids	Bosworth et al. (1994)
Trifolitoxin	*R. leguminosarum*	*R. etli*	Enhanced nodule occupancy in host *Phaseolus vulgaris*	Antibiotic activity	Robleto et al. (1997, 1998)
Haemoglobin *VHb*	*Vitreoscilla sp.*	*R. etli*	Nitrogenase activity and total nitrogen content in nodulated bean plants	Stimulated respiratory efficiency in free-living rhizobia and bacteroids	Ramírez et al. (1999)
Terminal oxidase *cbb*₃	*Bradyrhizobium japonicum*	*R. etli ntrC* mutant	Nitrogen fixation in nodulated *Phaseolus vulgaris* plants	Enhanced ATP supply to nitrogenase	Soberón et al. (1999)
Symbiotic gene region containing *nodD1, nodABC* and *nifN*	*S. meliloti*	*S. meliloti*	Nodulation, nitrogenase activity, plant nitrogen content and growth in alfalfa	Increase in Nod factor production and in regulation of nitrogen fixation	Castillo et al. (1999)
Glycogen synthase *GlgA*	*R. tropici*	*R. tropici*	Symbiotic performance and dry weight of *Phaseolus vulgaris* nodulated plants	Block of glycogen synthesis and putative pleiotropic effects	Marroquí et al. (2001)
Proline dehydrogenase *putA*	*S. meliloti*	*S. meliloti*	Advantage over natural rhizobial populations to infect and nodulate alfalfa under drought stress	Oxidation of proline from alfalfa root exudates to glutamate as energy source for rhizobia	Van Dillewijn et al. (2001, 2002)
Trifolitoxin and *Hup* gene cluster	*R. leguminosarum* and *Bradyrhizobium* sp.	*R. leguminosarum*	Dry seed weight yield in nodulated *Phaseolus vulgaris* plants	Antibiotic activity and recycling and oxidation of hydrogen derived from nitrogenase activity	Iniguez et al. (2004)
Betaine transporter *BetS*	*S. meliloti*	*S. meliloti*	Nitrogen fixation in nodulated alfalfa subjected to salt stress	Accumulation of osmolytes proline betaine and glycine betaine in bacteroids under osmotic stress	Boscari et al. (2006)
iaaM and *tms 2*	*Pseudomonas syringae* and *Agrobacterium tumefaciens*	*R. leguminosarum* and *S. meliloti*	Nitrogen fixation in *Vicia hirsuta* nodulated plants	Enhanced IAA synthesis	Camerini et al. (2008)
			Tolerance to heat, cold, salt and UV-irradiation stresses in free-living rhizobia	General hormone re-modulation in the plant	Imperlini et al. (2009)
			Nitrogen-fixing activity, stem dry weight and salt tolerance in *M. truncatula* nodulated plants.		Bianco and Defez (2009)
Flavodoxin	*Anabaena variabilis*	*S. meliloti*	Delayed nodule senescence in alfalfa nodulated plants	Enhanced ROS detoxification	Redondo et al. (2009)
			Nitrogen fixation with enhanced cadmium tolerance in alfalfa nodulated plants.	Changes in antioxidant metabolism	Shvaleva et al. (2010)

ROS Reactive oxygen species, *IAA* indole-3-acetic acid

the *tms2* gene from *Agrobacterium tumefaciens* under the control of a stationary phase-induced promoter from *Agrobacterium rhizogenes* (Camerini et al. 2008). The *iaaM* gene codes for an indolacetamide hydrolase that converts tryptophan into indolacetamide (IAM), and the *tms2* gene encodes a tryptophan monooxigenase that converts IAM into IAA. Free-living bacteria harboring this construct release 14-fold more IAA into the growth medium than wild-type strains. When vetch (*Vicia hirsuta*) roots were nodulated with the transformed rhizobia, nodules contained up to 60-fold more IAA than nodules infected by the wild-type strain. These nodules were heavier in terms of dry weight, with enlarged and more active meristems, significantly augmenting nitrogen fixation (Camerini et al. 2008). Free-living *S. meliloti* transformed with the same construct displayed increased activities for some enzymes in the tricarboxylic acid cycle, enhanced acetyl-CoA, and poly-beta-hydroxybutyrate content; they accumulated more endogenous osmolyte trehalose and survived better (Imperlini et al. 2009). The transformed bacteria also better tolerated heat, cold, and salt stress, as well as UV-irradiation (Imperlini et al. 2009; Bianco and Defez 2009). *M. truncatula* plants nodulated by the IAA-overexpressing *S. meliloti* strain showed an induction of nitrogen-fixing activity, increased stem dry weight production (Imperlini et al. 2009), and enhanced salt tolerance (Imperlini et al. 2009; Bianco and Defez 2009). Similarly, these *M. truncatula* plants had a higher proline content, almost unchanged hydrogen peroxide levels, enhanced activities of several antioxidant enzymes, reduced foliar senescence, higher nitrogenase activity, and lower expression of ethylene signaling genes than plants nodulated by the wild-type strain. These effects seem to be due to general phytohormone re-modulation in the plant (Bianco and Defez 2009).

Flavodoxins are electron carrier flavoproteins present in prokaryotes and some eukaryotic algae (Erdner et al. 1999). They contain a flavin mononucleotide (FMN) group acting as a redox center that transfers electrons at low potentials (Pueyo et al. 1991; Pueyo and Gómez-Moreno 1991). In cyanobacteria and enterobacteria, flavodoxin levels increase several-fold upon exposure to oxidative stress (Zheng et al. 1999; Yousef et al. 2003). Transformation of *S. meliloti* with the *A. variabilis* flavodoxin gene provided enhanced tolerance to stresses involving ROS generation in free-living bacteria. Flavodoxin-overexpressing *S. meliloti* was used to nodulate alfalfa plants and bacteroids overexpressing flavodoxin induced changes in antioxidant metabolism, affecting antioxidant enzymes, ascorbate-glutathione cycle enzymes and their metabolites, and inducing delayed senescence and starch accumulation in alfalfa root nodules (Redondo et al. 2009). Moreover, the nitrogen-fixing activity of alfalfa nodules elicited by the flavodoxin-

overexpressing *S. meliloti* had enhanced tolerance to cadmium stress (Shvaleva et al. 2010). These results suggest that rhizobia overexpressing flavodoxin may serve as biotechnological tools to improve the symbiotic performance of legumes subjected to environmental stress involving damage by ROS generation.

4 Legume plants for the reclamation of marginal soils

There are numerous recent studies aimed at identifying legume species with the potential to enhance the cultivation and revegetation of marginal areas. These will be considered along with the current efforts to select stress-tolerant legume varieties by classical methods or transgenic approaches.

4.1 Selection of new legumes and the breeding of old traits

Genetic variability within a plant species causes variations in plant responses to abiotic stress and nutrient imbalances, and it allows crops to be selected that are tolerant to abiotic stresses. For example, legumes that grow in saline soils are considered to be naturally selected salt-tolerant genotypes with potential value as genetic resources for saline areas. In fact, the best strategy is to select a tolerant legume in combination with a tolerant *Rhizobium* (Zahran 1991 and references therein; Herridge and Danso 1995; Howieson and Ballard 2004). However, to a large extent, breeding to improve nitrogen fixation has not been fully successful, maybe due to the difficulty in combining different traits and agricultural management techniques and the lack of screening technologies (Herridge et al. 2001).

Different studies have been performed to identify and select tolerant legume genotypes that can be employed in sustainable agriculture. For example, several studies aimed at identifying salt-tolerant genotypes and to evaluate genotype variation in response to salinity have been carried out in different species and lines of legumes, such as chickpea, *Lotus* sp. or *Trifolium* sp. (Rogers et al. 1997; Sadiki and Rabih 2001; Nichols et al. 2008; Teakle et al. 2010). Different drought-tolerant and sensitive cultivars of the important oilseed crop legume, groundnut (*Arachis hypogea*), have been analyzed to elucidate drought tolerance traits that might enable appropriate genetic enhancement strategies to be developed to increase yield in drought environments (Reddy et al. 2003). The yield of the lentil (*Lens culinaris* Medik.), a food legume crop, is markedly affected by drought, heat, and salt stress, as well as by iron deficiency. Comparative genomics and a consensus genetic map for the lentil are under development to identify and select resistant germplasm (Muehlbauer et al. 2006 and references therein). Different lines of annual and perennial

legumes have been tested to evaluate their agronomic potential in semi-arid climates (Small 2003; Caravaca et al. 2003; Merou and Papanastasis 2009). Different legume cultivars have also been tested for drought (Acuna et al. 2010; Kostopoulou et al. 2010), frost (Ratinam et al. 1994; Meyer and Badaruddin 2001), flooding (Vignolio et al. 1999), cold (Gan et al. 2009) and acid (Howieson et al. 1995) tolerance, as well as for winter survival (Annicchiarico and Iannucci 2007). Different perennial legumes that can use phosphorus efficiently were screened for their potential use in revegetating Australian soils with phosphorus deficiency (Pang et al. 2010). The influence of the legume cultivar to improve performance in rotation systems is also being evaluated (Cock 1992; Adjei-Nsiah et al. 2007; Soon and Lupwayi 2008).

There is great interest in rapidly improving both plant and microbial germplasms (Sprent et al. 2010). In Mediterranean areas, shrub legumes and their corresponding rhizobia microsymbionts have a strong potential to enhance nitrogen and carbon levels in arid and acidic soils, making them suitable candidates for revegetation of these areas (Alegre et al. 2004; Rodríguez-Echevarría and Pérez-Fernández 2005; Villar-Salvador et al. 2008). This potential has even been observed in soils containing composted sewage sludge (de Andrés et al. 2007). A new species of the annual arrowleaf clover (*Trifolium vesiculosum* Savi) has been identified and characterized recently with improved pastoral production in dryland areas in the central-southern region of Chile (Ovalle et al. 2010). Numerous legume tree species have also been tested for their capacity to help revegetate degraded soils in Brazil and Australia (Franco and De Faria 1997). *Lupinus argenteus* (Pursh.), a legume native to sagebrush steppes, is also of interest for revegetation as it favors nitrogen availability and cycling under conditions of water stress (Goergen et al. 2009). In India, little known wild legumes are being characterized as potential germplasms for use in the reclamation of marginal soils. One example is the biochemical characterization of some *Canavalia* spp., which are naturally distributed in sand dunes and mangrove areas. The stress-tolerant rhizobia of these plants are also being characterized (Seena and Sridhar 2006). Tropical *Stylosanthes* species from Australia, Colombia, Brazil, and Ethiopia have been introduced in India, and they constitute a new germplasm to improve soil fertility and provide nutritive forage in rain-fed situations of heavy clay and cracking soil types (Chandra 2009). Different halophytes, including several legumes, were described within the desert flora of Central Asia, and they are very attractive to reclaim degraded and drought-affected soils (Toderich et al. 2009).

Africa has a vast array of indigenous legumes that are adapted to soil and climatic conditions, in particular drought and low nutrients, ranging from large rainforest trees to small annual herbs. These legumes and their rhizobial microsymbionts have so far been a poorly exploited source of germplasms for the revegetation of marginal soils (Sprent et al. 2010). There is a wide diversity of food legumes adapted to drought-prone, low nutrient environments, and they are used in rotation or as intercrops with cereals in Bostwana. Moreover, African tree and shrub legumes are a major source of phytopharmaceuticals (Pule-Meuelenberg and Dakora 2007). Malawian smallholder farmers have adopted legumes, mainly edible legume intercrops like pigeon pea and groundnut, to improve both human nutrition and soil fertility (Kerr et al. 2007).

Classical heritability studies of selected traits are also ongoing (Pimratch et al. 2009) and new molecular techniques, such as microsatellite characterization (Lazrek et al. 2009) and transcriptome and proteome studies, will help to better classify and characterize tolerant and sensitive legume cultivars.

4.2 Transgenic legumes with enhanced stress tolerance

Several biotechnological approaches are available to enhance biotic and abiotic stress tolerance in legumes, including molecular marker-assisted (MAS) breeding, tissue culture, and gene transformation (Dita et al. 2006 and references therein). Here, we will focus on research developed to obtain abiotic stress-tolerant legumes by gene transformation and expression. A gene transfer method used in legumes involves the use of *A. tumefaciens* as a vector for transformation (Fig. 3), although *A. rhizogenes* is also used to transform some species to produce composite plants with hairy roots or hairy root cultures. Another method used involves transformation by particle gun bombardment (Eapen 2008 and references therein). The distinct protocols for genetic transformation of the main legumes are in constant development to ensure food and environmental safety (Popelka et al. 2004 and references therein; Eapen 2008 and references therein).

Biotic stresses often involve monogenic traits and several legume cultivars have been transformed in order to enhance resistance to biotic stress (Dita et al. 2006 and references therein). Abiotic stresses generally involve perturbation of several cellular activities and the activation of complex metabolic pathways, as explained above. Tolerance to different abiotic stresses is considered polygenic traits, and therefore, successful genetic transformation to enhance tolerance in legumes requires a thorough physiological and molecular understanding of these stresses. Recent advances in understanding the physiological and molecular events and the pathways involved in abiotic stress and tolerance in legumes, together with progress in developing gene transfer protocols, have helped to generate transgenic legumes with enhanced tolerance to abiotic stresses (Table 2).

Fig. 3 *Agrobacterium*-mediated transformation of *Medicago truncatula*
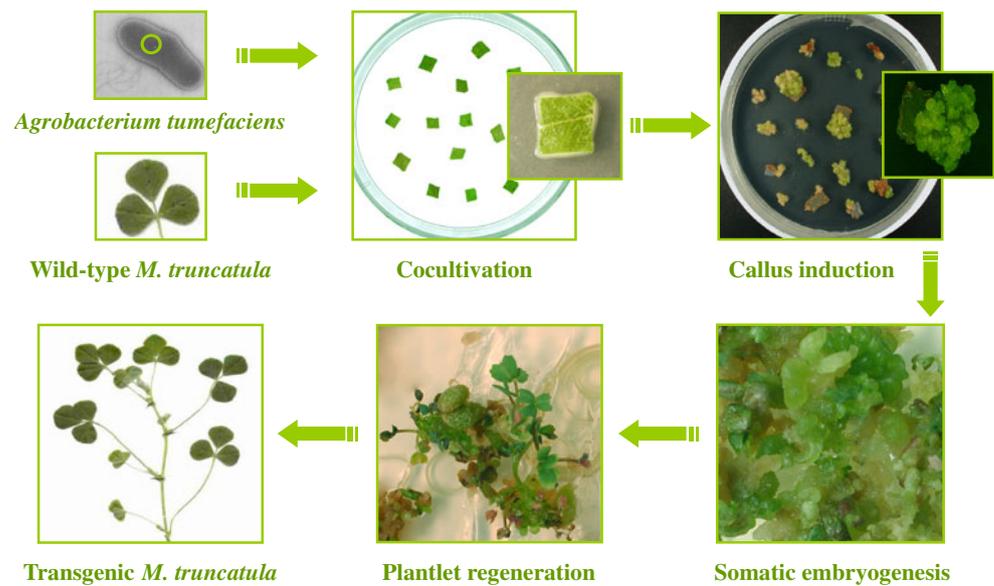
Overexpression of the *WXP1*, a putative *M. truncatula* AP2 domain-containing transcription factor gene, increases cuticular wax production and accumulation, and it enhances drought tolerance in transgenic alfalfa (Zhang et al. 2005). Indeed, transgenic white clover (*T. repens*) overexpressing the same *WXP1* gene displays improved tolerance to drought stress (Jiang et al. 2010). Alfalfa has been transformed with the *AVP1* gene, a vacuolar H^+-pyrophosphatase (H^+-PPase) from *Arabidopsis thaliana* (Bao et al. 2009). These transgenic plants accumulate more Na^+, K^+,

Table 2 Transgenic approaches to engineer abiotic stress tolerance and enhanced nitrogen fixation in legumes

Gene	Origin	Host legume	Enhanced trait or effect	Proposed mechanism	Reference
Superoxide dismutase	*Nicotiana tabacum*	*Medicago sativa*	Drought and freezing stress tolerance, and winter survival	Enhanced overall defense system induced by SOD-induced production of H_2O_2. Reduction in secondary ROS injury symptoms	McKersie et al. (1993, 1996, 1999, 2000)
Putative transcription factor *Alfin1*	*M. sativa*	*M. sativa*	Salt tolerance	Enhanced expression of proline-rich protein *MsPRP2* and probably regulation of the expression of other genes	Winicov and Bastola (1999)
Superoxide dismutase	*Nicotiana plumbaginifolia and Arabidopsis thaliana*	*M. sativa*	Mild water stress tolerance	Enhanced ROS detoxification	Rubio et al. (2002)
Transcription factor *WPX1*	*M. truncatula*	*M. sativa*	Drought tolerance	Increased cuticular wax accumulation in leaves	Zhang et al. (2005)
Δ^1-pyrroline-5-carboxylate synthetase *P5CS*	*Vigna aconitifolia*	*M. truncatula*	Nitrogen fixation under osmotic stress	Proline accumulation	Verdoy et al. (2006)
Antisense beta-1,3-glucanase gene *LjGlu1*	*Lotus japonicus*	*L. japonicus*	Nodule number and nitrogen fixation	Unknown mechanism mediated by suppressing *LjGlu1* expression	Suzuki et al. (2008)
Vacuolar H^+-pyrophosphatase *AVP1*	*A. thaliana*	*M. sativa*	Salt and drought tolerance	Enhanced electrochemical gradient of H^+ across the tonoplast, favouring Na^+ compartmentalization and rhizosphere acidification	Bao et al. (2009)
Phytase *MtPHY1* and purple acid phosphatase *MtPAP1*	*M. truncatula*	*Trifolium repens*	Tolerance to phosphate deficiency	Accumulation of total phosphorus	Ma et al. (2009)
Transcription factor *WPX1*	*M. truncatula*	*T. repens*	Drought tolerance	Unknown. Putative activation or suppression of target genes	Jiang et al. (2010)
Flavodoxin	*Anabaena variabilis*	*M. truncatula*	Nitrogen fixation under salt stress	Enhanced ROS detoxification Changes in antioxidant metabolism	Coba de la Peña et al. (2010)

SOD Superoxide Dismutase, *ROS* Reactive Oxygen Species

and Ca^{2+} in leaves and roots under salt and drought stress conditions, and they retain more water during drought stress. Thus, transgenic plants overexpressing *AVP1* display enhanced tolerance to salt and drought stress. Increased K^+ uptake and root activity in transgenic alfalfa may be a consequence of rhizosphere acidification resulting from expression of *AVP1*. Transgenic expression of *Alfin1*, a transcription factor that binds to promoter elements in genes regulated by salt or drought stress, improves tolerance to salinity in transgenic alfalfa plants (Winicov and Bastola 1999).

Phosphate is one of the most limiting macronutrients, restricting crop production in many ecosystems. Phytases are enzymes that catalyze the hydrolysis of phytic acid, and a product of this hydrolysis is inorganic phosphate. Purple acid phosphatases are enzymes that hydrolyze phosphate esters and anhydrides under acidic conditions. A phytase gene and a purple acid phosphatase gene, both isolated from the model legume *M. truncatula*, have been introduced into white clover. Transgenic expression of both these genes in white clover enhanced phytase and acid phosphatase activities in root apoplasts, and transgenic plants were more capable of utilizing organic phosphorus in response to phosphorus deficiency (Ma et al. 2009). It was hypothesized that root nodule formation would be promoted by weaker expression of a beta-1,3-glucanase gene, since expression of this gene is increased in transgenic *L. japonicus* which has reduced nodulation (Suzuki et al. 2008). When the expression of this gene was suppressed by introducing an antisense gene into *L. japonicus*, transgenic plants harbored more root nodules after inoculation with *Mesorhizobium loti*, and the nitrogen fixation of transgenic plants was enhanced.

As explained above, plants accumulate proline and other osmolytes under osmotic stress. Transgenic plants that accumulate high levels of proline better tolerate salt and osmotic stress, as well as cold and frost (Kishor et al. 2005 and references therein). Transgenic *M. truncatula* plants overexpressing the Δ^1-pyrroline-5-carboxylate-synthetase (*P5CS*) gene from *Vigna aconitifolia*, an enzyme involved in the first two steps of proline biosynthesis, accumulate more proline, resulting in stronger tolerance to salt and osmotic stress when compared with control plants (Verdoy et al. 2006). Transgenic *M. truncatula* accumulate more proline in leaves, roots and nodules, and the proline levels increase in transgenic plants following salt treatment. When compared to wild-type plants, when subjected to salt stress, the transgenic plants have significantly higher nitrogen-fixing activity and no significant ultrastructural alterations to nodules (Verdoy et al. 2006). To our knowledge, these are the first transgenic legumes that displayed nitrogen-fixing activity with enhanced tolerance to osmotic stress.

A correlation has been suggested between antioxidant levels and tolerance to several abiotic stresses. Transformed plants overexpressing antioxidant enzymes often show increased tolerance to salt and other abiotic stresses, such as drought, cold and heavy metal stress (Ashraf 2009 and references therein). Transgenic alfalfa plants overexpressing a superoxide dismutase (SOD) displayed enhanced tolerance to water deficit, freezing stress and winter survival (McKersie et al. 1993, 1996, 1999, 2000; Rubio et al. 2002). As explained above, flavodoxin expression in *S. meliloti* led to oxidative stress tolerance in alfalfa nodules. Indeed, transgenic tobacco plants expressing a cyanobacterial flavodoxin displayed increased tolerance to multiple sources of stress (Tognetti et al. 2006, 2007a, b). These effects seem to be due to the capacity of flavodoxin to mediate electron transfer and to react with ROS, facilitating ROS detoxification and protecting against oxidative damage. Expression of flavodoxin in transgenic *M. truncatula* plants does not confer saline tolerance to the whole plant, although the sensitive nitrogen-fixing activity was maintained under salt stress in this transgenic legume (Coba de la Peña et al. 2010). Small but significant flavodoxin-induced changes in enzymatic activity associated with the nodule redox balance might be responsible for the positive effect on nitrogen fixation.

5 Conclusions and prospects

The studies reviewed here have provided clear evidence of the considerable effort being made to select legume varieties and rhizobial inocula that can fix nitrogen and generate competitive crop yields in degraded and marginal stress-affected soils. Furthermore, better understanding the physiological and molecular mechanisms involved in the tolerance to environmental stresses is giving rise to numerous biotechnological approaches aimed at obtaining improved legumes and rhizobia with enhanced tolerance to abiotic stress, paying particular attention to the sensitive nitrogen-fixing activity.

In a context of global and climate change, with a growing population and an increasing demand for food and feed, the importance of legumes in sustainable agriculture, and particularly in the reclamation of marginal lands, seems beyond any doubt. Needs might diverge in different parts of the globe, often in close connection with the regional development, the natural and acquired richness of the countries and the level of wellbeing, environmental awareness or unacceptable poverty of their peoples. For different and often cumulative reasons, sustainable agriculture is an ever increasing and universal need. While developing countries focus on providing sufficient food to their population, which requires both increasing yields and

reclaiming yet-unexploited marginal lands, developed states are increasingly aware of the importance of the quality of their food and of the need to restore degraded environments. In either case, improved legumes are equally advantageous. High yields without the added costs of nitrogen fertilization are becoming a must for precarious economies (Chianu et al. 2010, and references therein), whereas chemical-free, organic foods and feed are in increasing demand in advanced societies.

Given that research in the field of legume and inoculant stress tolerance is increasingly necessary, it appears that field experimentation is still insufficient, and laboratory and greenhouse results must be contrasted in cultivation. Despite some astounding success stories, much still remains to be achieved in this respect, which is necessarily associated with technology transfer and the spread of knowledge to farmers, including the acquisition of agricultural management practices to maximize biological nitrogen fixation, such as intercropping or limited tillage. Selecting locally adapted legumes and rhizobia proves to be essential and as such, small scale approaches are indispensable. Regarding transgenic approaches, the perspectives are extremely promising as our understanding of the mechanisms involved in stress tolerance is advancing in great bounds. Transcriptomic approaches are being complemented by proteomics, metabolomics and gene regulation studies, and new genes and traits are being made available to engineer tolerance in the extremely complex legume–*Rhizobium*–soil–climate system. Thus, research is underway, and unprecedented local and global success is expected.

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