Saad Sulieman · Lam-Son Phan Tran
*Editors*

# Legume Nitrogen Fixation in a Changing Environment

## Achievements and Challenges

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# Preface

Climate change is a topic that increasingly occupies the attention of the world. The study of plant responses to various environmental stresses has been a central feature of plant biologists' efforts to understand how plants function in a changing environment. In recent years, much progress has been made in understanding how environmental stresses affect legumes performance. As a consequence, we considered that it is timely to re-examine the physiological, biochemical, and molecular responses of plants to a variety of stresses with a view of identifying the major principles. In this approach, the present volume aims to identify the existing gaps in scientific understanding and fill the niche. In particular, we were interested to examine whether we are now in a position to provide plant breeders with certain useful strategies for development of elite adapted cultivars through conventional breeding and sophisticated techniques of genetic engineering.

The "*Legume Nitrogen Fixation in a Changing Environment—Achievements and Challenges*" volume brings together the state-of-the-art overview of legume growth and production as influenced by environmentally imposed stresses. As editors, we favored a collection of specialized chapters rather than a textbook style. The present volume was edited with contribution from distinguished experts who have worked diligently in compiling information and knowledge in their respective areas of research. This volume consisting of five chapters opens with an Introduction and Overview that sets the scene, outlines essential background, and considers the impact of different stresses as limiting factors for legume production in the context of a changing global environment. The chapters assembled here provide an up-to-date account of our knowledge about the responses of $N_2$-fixing leguminous symbioses to drought (Chap. 2), salinity (Chap. 3), heat stress (Chap. 4), elevated carbon dioxide concentrations (Chap. 5), and soil acidity (Chap. 6) as well as discuss the potential approaches for the improvement of $N_2$ fixation under stresses. Categorically, these stresses represent the major environmental factors that are directly related to climate variability and account for major limitations to legume production worldwide. The authors have provided latest insights in their research areas. Numerous figures and tables have been supplied to facilitate the comprehension of the presented materials.

Thus, “*Legume Nitrogen Fixation in a Changing Environment—Achievements and Challenges*” is a new volume with focus on how symbiotic legumes adapt to abiotic stress and how molecular and biotechnological approaches could allow us to mitigate the climate variability on the productivity of legume crops, thereby improving food supply in a sustainable agricultural system. We hope that this volume will serve as a major source of information and knowledge to senior undergraduates and research students seeking an introduction to the area of plant stress physiology and biological nitrogen fixation. We also trust that it will be of interest to a wide range of plant scientists, including agronomists, physiologists, biotechnologists, molecular biologists, and plant breeders, who have concern about biological nitrogen fixation of legume crops under stressful environments.

As editors of this volume, we feel pleasure to extend our heartfelt thanks to all the invited authors for their efforts and cooperation in bringing out this volume in time. We are very grateful to the support provided by the Japan Society for the Promotion of Science (JSPS) and RIKEN Center for Sustainable Resource Science, Japan, for our academic research. We are indebted to Mr. Kenneth Teng and Mr. Joseph Quatela, the editorial staff of Springer, New York, who have been of invaluable help in the production of this endeavor. Finally, it is a profound pleasure to thank Springer New York for publishing this volume. We hope that the final result will be worth the effort and will make a sound contribution to this fascinating research area.

Khartoum, Sudan Saad Sulieman
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# Contents

# Introduction

**Saad Sulieman and Lam-Son Phan Tran**

There is a strong awareness that the world population will grow more rapidly during the few coming years. The world population of 6.91 billion in 2010 will increase to 8.43 billion in 2030 and 9.55 billion in 2050, which will be probably stabilized at around 10–11 billion by the end of the century (http://en.wikipedia.org/wiki/World_population). This awareness must be translated into a parallel increase in the agricultural production to secure adequate food for the additional 3–4 billion inhabitants. If such an increase in production should be realized with current agricultural management that would similarly require the double use of fossil fuel energy for fertilizer production. This would cause economic hardship and surpassing damage to the environment. The intensive application of chemical nitrogen (N) fertilizers has led to an unprecedented perturbation of the N cycle, illustrated by the growing accumulation of nitrates in soils and waters and of nitrogen oxides in the atmosphere. Sustainable agriculture has mandated that alternatives to chemical N fertilizers must be urgently sought. Biological dinitrogen ($N_2$) fixation, a microbiological process that converts atmospheric $N_2$ into a plant-usable form, offers this alternative. Among these renewable sources, $N_2$-fixing legumes offer an economically attractive and ecologically sound means of reducing external inputs and improving internal resources.

Legumes (*Leguminosae* or *Fabaceae*) represent the second major crop of agricultural importance worldwide and cover about 14 % of total land under cultivation. In many regions of the world, legumes contribute a number of function and ecosystem services with great impact to the sustainability of various agricultural systems.

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These plants provide important sources of oils, fiber, micronutrients, minerals, and vegetable proteins suitable for livestock feed and human consumption while supplying N to agro-ecosystems via their unique ability to fix atmospheric $N_2$ in symbiosis with the soil rhizobia. Legumes therefore possess great potential for use in soil stabilization, reforestation, and agricultural practices. The symbiotic relationship between legumes and their rhizobial partners takes place in the root nodules and provides access to atmospheric $N_2$. Nevertheless, the contribution of the symbiotic process to legume production is recognized to be highly sensitive to adverse environmental conditions, such as edaphic and environmental constraints. Therefore, understanding how symbiotic $N_2$ fixation responds to the surrounding environmental conditions is particularly important for both agriculture and preservation of the ecosystems.

As leguminous plants are inextricably linked to the surrounding environment, their mere inclusion in various cropping systems does not always ensure the attainment of the estimated optimal levels of symbiotic $N_2$ fixation in the field. Day by day, the cycle of climate on earth is changing and according to the predictions of the Intergovernmental Panel on Climate Change (IPCC), most climatic scenarios are expected to be affected by climate change. Expectations of impending climates specify immense alterations in temperature, rainfall pattern, humidity, and soil moisture regimes. Changes in climate not only influence the entire cropping system but also affect the performance of cultivars of different field crops, including legumes. Thus, climate change has become a major concern in agricultural development. Several environmental factors, such as drought, elevated temperature, salinity, soil acidity, and rising $CO_2$, are known to dramatically affect the symbiotic process and thus play a part in determining the actual amount of N fixed by a given legume in the field. Accordingly, environmental stress factors adversely affect $N_2$-fixing legume growth and pose a growing threat to sustainable agriculture. This has become a hot issue due to concerns about the effects of climate change on plant resources, biodiversity, and global food security. Understanding the responses of $N_2$ fixation and legume performance to global environmental change is crucial for improving legume production and maintaining agricultural sustainability in the context of global change. In this thoughtful and provocative new volume, we provide critical information on how current and projected future changes in the environment will affect legume growth and their symbiotic $N_2$-fixing capabilities.

To cope with various abiotic constraints, legumes have evolved a number of strategies at both morphological and physiological levels. With the advances in physiological methodology and molecular biotechnology, diverse arrays of biochemical, physiological, and molecular mechanisms underlying those adaptive strategies have been well studied in a broad range of plants, both model and crop species. Some particular strategies have been observed for the adaptation of nodulated leguminous plants to drought, elevated temperature, salinity, soil acidity, and rising $CO_2$. The application of omic technologies, i.e., transcriptomics, proteomics, metabolomics, and comparative genomics, reveals complex internal reactions and acclimations of plant organs to unfavorable conditions. Significant research efforts in the genomics of various stresses have shown that many genes regulated in a coordinated fashion are involved in plant acclimation to abiotic stresses. Being the most important model legumes, the sequenced genomes of *Medicago truncatula* Gaertn., *Lotus japonicus* (Regel) K. Larsen, and more recently soybean (*Glycine max* (L.) Merr.) have provided valuable resources for dissecting the molecular events regulating physiological and biochemical responses of legumes to abiotic stresses. Thus, a better understanding of the mechanisms of crop adaptations under variable climatic conditions will remarkably assist in breeding or engineering of future stress-tolerant crop plants. The development of such improved leguminous plants with more efficient symbiotic capabilities is a necessity for sustainable farming practices as well as mitigation of the negative impact of climatic changes. The challenge now is the translation of knowledge gained in model systems to cash crops grown in open-field conditions which are facing with the simultaneous occurrence of extreme events, such as drought, heat stress, salinity, soil acidity, and elevated levels of $CO_2$.

Evidence about the changing climate and its negative impact on plants and development is increasing. As biologists, we cannot ignore this reality; and if we want our work to be relevant, we then need to consider carefully whom and how we are targeting with our knowledge. In this book volume, we are honored to have five distinguished research groups to review the main effects of environmental changes on the legume performance, of which we are most concerned about those of drought, elevated temperature, salinity, rising $CO_2$, and soil acidity. These adverse environmental factors currently represent the greatest challenge affecting the symbiotic $N_2$-fixing process, and thus are expected to have tremendous effects particularly at long-term scale. Each chapter will review the current state of knowledge of nodule performance to one of these major stress factors, including the perspectives on the molecular approaches used for the analyses of stress responses in legumes and the possible biotechnological strategies to overcome their detrimental effects. Achievements as well as challenges are discussed across the chapters. Several perspectives regarding new approaches for screening, breeding, or engineering legumes with desirable abiotic stress-tolerant traits are anticipated. Graduate students and researchers of various disciplines relating to crop productivity and global change will find the perspectives and analyses offered by this volume an exciting contribution to the development of our understanding of these ongoing environmental changes for present and future legume production as a means to enhance food security for a rapidly expanding population.

# Physiological Responses of $N_2$-Fixing Legumes to Water Limitation

**Esther M. González, Estíbaliz Larrainzar, Daniel Marino, Stefanie Wienkoop, Erena Gil-Quintana, and César Arrese-Igor**

**Abstract** A significant decline in the content of water in soils provokes a water deficit at the plant level. In plant physiology, water deficit can be defined as the water content of a tissue or cell below the highest water content under the optimum hydrated state. The basis of the fundamental mechanism involved in stress tolerance, although intensively explored, is still matter of debate. Cell growth is the physiological process first affected as cell water content decreases when plants encounter mild water-deficit levels, followed by an inhibition of cell wall and protein biosynthesis. Although stomatal conductance and photosynthesis are affected in more intense water-deficit stages, most research efforts have focused on the study of these processes. In legume plants grown under symbiotic conditions, one of the primary effects of water deficit is a decline in the rates of symbiotic nitrogen fixation (SNF). The causes of this inhibition, which occurs even before a measurable decline in the rates of photosynthesis, have been explored in detail in the last decades, although the molecular mechanism involved are yet not fully understood. In the present chapter, we summarize our current understanding of the factors involved in the regulation of SNF in different legume species, including crops such as soybean (*Glycine max*), alfalfa (*Medicago sativa*), bean (*Phaseolus vulgaris*), and pea (*Pisum sativum*) but also model legumes like *Medicago truncatula*. Finally, an overview of the available resources and applications of molecular system-based approaches for understanding the complex responses of legumes to drought stress is provided.

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## 1 An Introduction to Drought and Symbiotic $N_2$ Fixation

Anthropogenic climate change is having a significant impact on biological systems globally (Rosenzweig et al. 2008). In this context, more intense drought periods are some of the events predicted as a consequence of climate change. Although it is not easily demonstrated, Herring et al. (2014) concluded that this type of events are unlikely to occur due exclusively to variability of natural systems after examining several recent extreme events from a worldwide climate perspective. The Intergovernmental Panel on Climate Change report (IPCC 2012) concluded that drought events will intensify in the twenty-first century in some areas due to reduced precipitation and/or increased evapotranspiration. Drought can be defined as an extended period of deficient precipitation compared to the multiyear average for a region, resulting in water shortage for some activity, group, or environmental sector (National Drought Mitigation Center, drought.unl.edu). Attending to agricultural criteria, drought is defined as insufficient soil moisture to meet the needs of a certain crop at a particular time with further socioeconomic impact in human activities. In contrast to drought, which is a temporal anomaly, aridity is a permanent drought feature of a region climate (Wilhite and Glantz 1985). In both cases, other factors such as evaporation rates, temperature, wind speed, soil type, ground water supply, and vegetation play a relevant role in the severity of the stress perceived by the plant (Maliva and Missimer 2012). Periodic drought is considered the major limiting factor of crop production in agricultural systems, whilst aridity hinders the establishment of agricultural systems.

Grain and forage legumes are grown on around 15 % of the arable surface of the Earth, being the second more important crops after cereals attending to world primary crop production (Graham and Vance 2003). The potential of legume crops is evident considering their multiple applications for human use, as a protein source for animal feed, human nutrition and health (MacCracken 2008; Edgerton et al. 2008), as well as useful raw material in industry. Legume crops have important agronomical and environmental advantages due to their capacity to reduce atmospheric $N_2$ to ammonium via symbiotic $N_2$ fixation (SNF). In terms of energy use, SNF provides a source of reduced nitrogen ultimately derived from solar energy, while nitrogen (N) fertilizers require significant amounts of fossil fuels, which makes the cultivation of legumes a sustainable practice in agricultural systems (Crews and Peoples 2004). However, one of the limitations to their widespread cultivation is their limited production under environmental constraints. This limitation can be partially explained by the sensitivity of SNF process to abiotic stresses such as drought. The fact that under symbiotic conditions N assimilation occurs mostly in roots, whereas in non-symbiotic plants N is mainly assimilated in leaves, has led to the generalized misconception that legume plants grown under symbiotic conditions are less tolerant to water deficit than their N-fertilized counterparts. Several lines of experimental evidence actually prove quite the opposite (Lodeiro et al. 2000; Frechilla et al. 2000, 2001; Antolín et al. 1995; Kirova et al. 2008). Depending on the N source under which the plant grows (nitrate, ammonium or a combination of both), specific responses are triggered at the transcript level (Ruffel et al. 2008). Nevertheless, the exact mechanism(s) behind this tolerance remains largely unknown. One of the hypotheses suggested relates this increased tolerance to the beneficial effects that symbiotic microorganisms have on modulating the root system physiology through hormonal balance (Dimkpa et al. 2009; Redman et al. 2011). A symbiotic priming effect was observed in $N_2$-fixing *Medicago truncatula* plants, in which higher levels of stress responsive metabolites and proteins were induced compared to N-fed plants (Staudinger et al. 2012). In soybean (*Glycine max*), for instance, higher rates of photosynthesis and delayed senescence have been attributed to larger carbon (C) sink during efficient SNF (Kaschuk et al. 2010). Additionally, non-pathogenic endophytic interactions have been shown to trigger plant stress protection mechanisms (Redman et al. 1999).

Common bean (*Phaseolus vulgaris*), broad bean (*Vicia faba*), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), groundnut (*Arachis hypogaea*), and soybean are some of the most commonly cultivated food legumes worldwide, while cowpea (*Vigna unguiculata*), pigeon pea (*Cajanus cajan*), lentil (*Lens culinaris*), and grass pea (*Lathyrus sativus*) are less extended but equally important for particular regions of the world. Species belonging to the *Medicago*, *Trifolium*, and *Lotus* genera are probably the most extended forage legumes for livestock production. Depending on the species and location, different legumes may be grown in rain-fed or irrigated fields during the winter or summer seasons in self-sufficiency agriculture. However, in intensive crop-based agricultural systems worldwide, legumes are commonly grown under rain-fed conditions. This is the case in the USA, Brazil, and Argentina, the three countries that are responsible for approximately 87 % of the soybean production worldwide (FAOSTAT 2013), as well as for 90 % of the chickpea grown in Asia (Kumar and Abbo 2001). Similarly, in the Mediterranean basin, legume crops traditionally depend on the incoming rainfall during the last part of their crop cycle, which usually implies periods of terminal drought around the late vegetative to reproductive phases. Additionally, legumes are often grown in rotation systems after cereal harvest towards the end of the growing season when environmental conditions are more limiting. Despite this shift towards harsher periods and/or regions, breeding for the improvement of legume crops has not kept pace with those of cereals for which higher yielding modern varieties have been developed (Jeuffroy and Ney 1997). Given the predicted expansion of arid areas in the world along with the increase in population (Postel 2000), there is a clear need to obtain legume varieties with improved tolerance to drought, as well as to implement agricultural practices that potentiate SNF while maintaining high yields.

In the case of $N_2$-fixing legumes, the tolerance of the microsymbiont to drought conditions needs to be considered. Drought has an important impact on soil microbiology, since it reduces the water availability around soil particles and increases the salt concentration in the soil solution, which has negative effects in bacterial growth and persistence, limits their movement and, ultimately, the diversity of the bacterial populations in the soil (Postma et al. 1989; Wadisirisuk et al. 1989; Mnasri et al. 2007; Orchard and Cook 1983) and the general distribution of soil biota (Tate 1995). Under water-limiting conditions reduced viability of almost all the rhizobial species able to establish symbiosis with crop legumes has been reported (Miller and Pepper 1988; Boonkerd and Weaver 1982; Danso and Alexander 1974), being fast growing rhizobia more susceptible to soil dehydration than slow growing ones (Sprent 1971). Nevertheless, genetic potential to improve drought tolerance has been shown in several rhizobial species (Athar and Johnson 1997; Athar 1998; Mnasri et al. 2007; Waldon et al. 1989; Elboutahiri et al. 2010). For instance, Gehlot et al. (2012) found several herbaceous drought-tolerant legumes hosting fast- and slow-growing rhizobia of different genera, although a particular preference for *Sinorhizobium* was observed. Similarly, Guerrouj et al. (2013) identified several rhizobial symbionts of the desert legume *Astragalus gombiformis* in sandy soils in Morocco, although these strains did not exhibit drought tolerance under free-living conditions. On the other hand, it should be noted that rhizobium survival under drought is conditioned by the possibility of establishing a symbiotic interaction, even if it is not a highly effective symbiosis (Sprent 1994). In this regard, under stressful conditions it has been reported that bacteria tend to lose their DNA (Stouthamer and Kooijman 1993) and suffer morphological changes leading to a reduction in infection and nodulation rates (Busse and Bottomley 1989; Hunt et al. 1981).

The first steps towards potential improvement of drought tolerance in legumes have been taken in some legume crops such as chickpea (Varshney et al. 2013, 2014), cowpea (Fatokun et al. 2012), soybean (Silvente et al. 2012; Deshmukh et al. 2014; Seversike et al. 2014), and common bean (Beebe et al. 2008). Additionally, genetic variation in the response of SNF to water deficit has been also reported in soybean (Chen et al. 2007; Ladrera et al. 2007; Serraj et al. 1999a) and peanut (Devi et al. 2010, 2013). However, we yet lack a complete understanding of the mechanisms regulating SNF and further studies are needed in order to provide the tools to ultimately improve SNF tolerance to drought through selection and breeding. This chapter aims to summarize our knowledge on the regulation of SNF under drought stress both at the nodule and whole plant level.

## 2 Metabolic Changes in Nodules Under Water Stress Conditions: Key Factors Regulating SNF

The effects of drought on SNF occur at different steps of the symbiotic interaction, namely: at the early infection stages, during nodule development and function. Under water-limiting conditions both formation of new root hairs and elongation of previously differentiated ones are limited and, as a consequence, the development of new plant-bacterium interactions and infection threads is greatly reduced (Worrall and Roughley 1976). Under salt stress, the biosynthesis and secretion of flavonoids and Nod factors in different legume–rhizobium interactions is altered (Estévez et al. 2009;

Dardanelli et al. 2009). The complex biochemical, genetic, and morphological changes taking place in legume root hairs during the symbiotic interaction with rhizobia (reviewed in Oldroyd and Downie 2008) are severely affected under water-limited conditions. In addition to the decrease in biomass observed in $N_2$-fixing legume plants under drought, the process of SNF is one of the physiological processes to first show stress responses in nodulated legumes. This decline cannot be explained by the relatively slow decline in photosynthetic rates (Durand et al. 1987) and, therefore, alternative causes have been analyzed aiming to understand the key factors regulating SNF under drought. Three major factors have been proposed to be involved in this inhibition, namely: oxygen ($O_2$) limitation, C shortage, and N feedback. In the last few decades, evidence for the involvement of oxidative stress (Marino et al. 2006, 2013; Naya et al. 2007) and sulfur (S) metabolism (Larrainzar et al. 2014a; Irar et al. 2014) as part of the nodule response to water deficit have also been described. The following sections review our current understanding on the role of these factors in the regulation of SNF under drought.

## 2.1 $O_2$: Is It a Limiting Factor Under Drought?

Legume nodules need to maintain a microaerobic environment within the $N_2$ fixation zone. Nitrogenases are sensitive to the presence of $O_2$, being irreversibly inhibited when surpassing an optimum $O_2$ level (Burris 1991). To counteract this situation, nodule permeability is self-regulatory to certain extent. Since $O_2$ diffusion in air is $0.176\ cm^2\ s^{-1}$ but it is dramatically reduced to $2.1\ 10^{-5}\ cm^2\ s^{-1}$ in water (Cussler 1997), nodule permeability might be exerted by the collapse and reduction of interconnected air space at the inner cortex level (James et al. 1991; De Lorenzo et al. 1993; Vandenbosch et al. 1994; Verdoy et al. 2004). Microscopic structural changes in drought-stressed soybean nodules, including dehydration of the cell wall, disruption of plasma membrane and organelle disorganization, were first reported by Sprent (1971). Subsequent work showed a reduction of the air space in cortical cells and collapse of the inner cortex in drought-stressed bean (Guerin et al. 1990; Verdoy et al. 2004; Ramos et al. 2003). In this context, a variable $O_2$-diffusion barrier has been described in nodules, which would be responsible of regulating $O_2$ flow to the infected cells so as to match the needs associated to maintain the high respiration rates measured (Witty et al. 1987; Minchin et al. 2008). The closure of the $O_2$ diffusion barrier implies a decrease in $O_2$ availability for bacteroid respiration and, therefore, a lack of energy to support the highly demanding SNF process. A number of evidences of this response have been obtained for different types of abiotic stresses other than drought such as salt stress (Serraj et al. 1994), nitrate (Vessey et al. 1998) and dark/chilling (van Heerden et al. 2008). In fact, the modulation of nodule $O_2$ permeability was proposed to be a universal stress response in legume root nodules and a key factor in the regulation of SNF (Denison 1998).

On the other hand, leghemoglobin (Lb), a plant protein that can reach concentrations of 2–3 mM in the cytoplasm of the infected cells (Bergersen 1982), contributes to maintain a low $O_2$ concentration within the infected cells providing a continuous flux of $O_2$ to the high $O_2$-affinity bacteroid cytochromes. Although changes in the level of Lb have been documented in drought-stressed nodules of different legumes using different experimental approaches, a consistent conclusion has not been reached. For instance, the levels of Lb transcript were found to decline at moderate drought stress in soybean (Gordon et al. 1997), while they were increased during severe drought in the same legume in a later study (Clement et al. 2008) and no changes were detected in alfalfa (*Medicago sativa*) nodules (Naya et al. 2007). At the protein level, a decline in the content of Lb has been reported in broad bean, pigeon pea, and common bean (Guerin et al. 1990; Nandwal et al. 1991; Talbi et al. 2012), while it was found unaffected in soybean, pea, and chickpea exposed to moderate-severe drought stress (González et al. 1995, 1998; Nasr Esfahani et al. 2014), occurring in all cases concomitantly with reduced nitrogenase activity. Thus, it seems clear that the observed responses depend on the legume species under study and the severity of the imposed stress. In any case, since nitrogenase activity is impaired regardless of the levels of Lb, it appears that other factors are additionally involved in the regulation of SNF.

Regardless whether $O_2$ levels decline due to a reduced permeability and/or to a limited $O_2$ flux mediated by Lb, it is clear than an $O_2$ limitation to the bacteroid would impair SNF by limiting the stock of ATP to be used for respiration. However, Del Castillo et al. (1994, 1995) showed that the decline in respiration and SNF during drought stress preceded the reduction of nodule $O_2$. Additionally, the increase of $O_2$ concentration in the rhizosphere of drought-stressed nodules, leading to an increase of the $O_2$ concentration within the infected zone, did not contribute to fully restore $N_2$ fixation rates (Del Castillo et al. 1994; Del Castillo and Layzell 1995), which indicates that other regulatory factors are involved in SNF regulation under drought stress. In summary, although drought stress affects $O_2$ availability in $N_2$-fixing nodules, $O_2$ limitation does not seem to play a key regulatory role in the regulation of SNF under this abiotic constrain.

## *2.2 C Metabolism in the Regulation of SNF*

Sucrose is the C source supplied to nodules from the shoot to fuel the energy-consuming process of SNF (Fig. 1). Before C is used into the bacteroid respiratory chain, sucrose has to be hydrolyzed to hexoses through the plant activities of sucrose synthase (SuSy) or alkaline invertase (AI) (Day and Copeland, 1991). SuSy has been described to be the main responsible for the hydrolysis of sucrose in nodule tissue (Craig et al. 1999; Gordon et al. 1999). Hexoses are then catabolized to phosphoenolpyruvate (PEP) through the glycolytic pathway and further converted to oxaloacetate by PEP carboxylase (PEPC). Finally, oxaloacetate (OAA) is reduced to malate thanks to the enzymatic activity of malate dehydrogenase (MDH) regenerating $NAD^+$ (Vance and Gantt 1992). Increasing evidence for the key role of malate to fuel SNF has been provided using $CO_2$-labelling experiments (Rosendahl et al. 1992; Salminen and Streeter 1992).

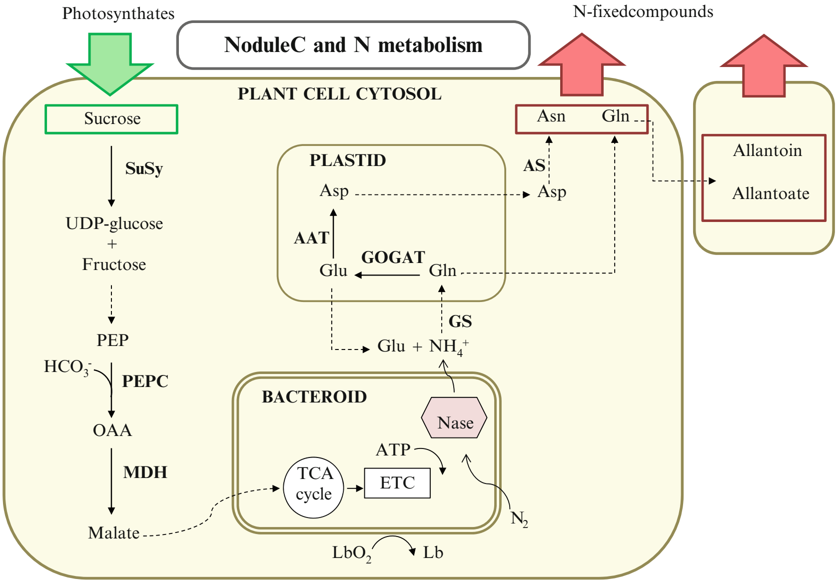


**Fig. 1** Schematic representation of the main nodule metabolic pathways in amide- and ureide (allantoin and allantoate)-exporter legumes. *C* carbon, *N* nitrogen, *Asn* asparagine, *Gln* glutamine, *Asp* aspartic acid, *Glu* glutamic acid, *UDP-glucose* uridine diphosphate glucose, *OAA* oxaloacetate, *AS* asparagine synthetase, *AAT* aspartate aminotransferase, *GOGAT* glutamate synthase, *GS* glutamine synthetase, *MDH* malate dehydrogenase, *Nase* nitrogenase, *ETC* electron transport chain, *Lb* leghemoglobin, *PEP(C)* phosphoenolpyruvate (carboxylase), *SuSy*: sucrose synthase, *TCA* tricarboxylic acid

The availability of C in nodules to fuel bacteroid respiration and nitrogenase activity is one of the mechanisms suggested to regulate SNF (Gordon et al. 1997). SuSy is one of the first enzymes to decline under drought stress in soybean (González et al. 1995), pea (González et al. 1998; Gálvez et al. 2005) and common bean (Ramos et al. 1999), leading to the accumulation of sucrose and the depletion of organic acids, principally malate, in nodules (González et al. 2001). However, SuSy decline seems not to be the main factor controlling SNF in forage legumes such as *M. sativa* (Naya et al. 2007) and *M. truncatula* (Ladrera, González and Arrese-Igor, unpublished). In these studies, significant declines in SuSy activity were found only after the inhibition of SNF and concomitant to malate accumulation, suggesting that C availability was not the limiting factor for the inhibition of SNF in these plants. Moreover, using metabolomics, C respiratory substrates were found not to be limiting in drought-stressed *M. truncatula* nodules (Larrainzar et al. 2009).

Durand et al. (1987) showed that photosynthesis activity under drought stress is maintained longer than SNF. Therefore, nodule sucrose supply is not limiting during primary drought stages. Additionally, it has been reported than starch content decreases in nodules of drought-stressed plants, leading to an extra sucrose supply within the nodule tissue (González et al. 1995; Gordon et al. 1997; Ramos et al. 1999). Both factors may contribute to increase the level of sucrose in nodules, as it has been observed in pea, soybean, alfalfa, and *M. truncatula* (González et al. 1995, 1998; Gordon et al. 1997; Ramos et al. 1999; Naya et al. 2007; Larrainzar et al. 2009). In contrast, the levels of malate vary depending on the legume plant under study (Gálvez et al. 2005; Naya et al. 2007). Malate is a ubiquitous vacuolar anion and the measurement of the malate pool of nodule tissue may mainly represent the vacuolar pool, and this may not have a direct correlation with C availability for bacteroids and/or plant mitochondria. Talbi et al. (2012) used a *Rhizobium etli* strain overexpressing the bacterial cytochrome *cbb3* oxidase to inoculate common bean plants. This led to an increase of the respiratory capacity of the bacteroids and, consequently, a higher C-skeleton demand. This demand was supported by an induction of SuSy expression in nodules, which consequently increased the levels of malate available to bacteroids, leading to plants more tolerant to drought. Recently, Nasr Esfahani et al. (2014) investigated the response of nodule metabolism of the drought-tolerant legume, chickpea, and found that SuSy was not affected by the stress but AI was significantly reduced. As a result, the pool of C was maintained, thus excluding C limitation as a possible cause for inhibition of SNF. Thus, whether C limitation is a factor regulating SNF during drought stress appears to be species-dependent and cannot be generalized.

## *2.3 Regulation Based on N-Related Compounds*

It is generally accepted that bacteroids assimilate a very little amount of the fixed ammonia ($NH_3$) that is mainly exported to the host plant (Brown and Dilworth 1975; Vance et al. 1994). In the plant cytosol initial $NH_3$ assimilation is carried out by the coordinated activities of glutamine synthetase (GS) and NADH-glutamate synthase (GOGAT; Fig. 1). GS catalyzes the ATP-dependent incorporation of $NH_3$ to glutamate to produce glutamine (Cullimore and Bennett 1988), and at least two cytosolic and one plastid isoforms have been described in *M. truncatula* (Carvalho et al. 2000). NADH-GOGAT is responsible for the transfer of the amide group from glutamine to α-ketoglutarate leading to the production of glutamate (Ta et al. 1986). NADH-GOGAT is primarily found in non-photosynthetic plant tissues, such as roots and legume root nodules, where it is located in plastids (Trepp et al. 1999a, b), being essential for N assimilation (Cordoba et al. 2003). Furthermore, in certain legume species such as pea and common bean, an amino-acid cycling system has been shown to be necessary for an effective $N_2$-fixing symbiosis (Lodwig et al. 2003; Prell et al. 2010). Mutations on the bacterial amino-acid transporters amino acid permease (Aap) and the branched-chain amino acid permease (Bra) led to $N_2$-fixing nodules but N-starved plants (Prell et al. 2010). Thus, in the absence of amino-acid transport across the symbiosome, plants were not able to efficiently assimilate the ammonium released. Based on these results, they proposed a model in which glutamate (or one of its biosynthetic precursors) is transported into bacteroids, in addition to dicarboxylates (Lodwig et al. 2003). However, a *Sinorhizobium meliloti aap bra* double mutant showed no phenotype on alfalfa plants (Prell et al. 2010).

Legumes can be classified into amide or ureide exporters according to the compounds used for the mobilization of fixed N (Schubert 1986). Most of the temperate-region legumes, such as pea, alfalfa, or clover, export amides, mainly asparagine, and in relatively lower amount glutamine, which requires the coordinated activity of enzymes aspartate aminotransferase (AAT) and asparagine synthetase (AS; Reynolds et al. 1981; McGrath and Coruzzi 1991). Tropical-origin legumes such as soybean, cowpea, or common bean export ureide compounds (namely, allantoin, allantoic acid, and citrulline) when plants are actively fixing $N_2$ (Schubert 1986). The ureide biosynthetic pathway is a complex compartmentalized process, with some of the enzymes being expressed selectively in neighboring non-infected cells (Fig. 2). Glutamine, together with other amino acids, is incorporated through the purine pathway to finally form ureides. Inosine monophosphate, the product of the purine pathway, is oxidized via xanthine dehydrogenase (XDH), located in the cytosol of the infected cells, to produce urate. The urate produced in the infected cells of nodules is then transferred to uninfected cells and further oxidized by urate oxidase (UO) eventually forming allantoin (Smith and Atkins 2002). XDH and UO are considered key enzymes in the biosynthesis of ureides in legume nodules (Triplett et al. 1980; Tajima et al. 2004). From the point of view of the C economy for N export, ureide-exporting legumes can be considered more efficient as the C:N ratio of ureides is more favorable (4:4) when compared with glutamine (5:2) and asparagine (4:2).

In addition to $O_2$ and C, a N-feedback mechanism involving the N-status of the plant has been proposed to regulate SNF. This hypothesis has received much attention in ureide-exporter tropical legumes, mostly due to studies conducted in soybean. Several N-related compounds have been suggested as inhibitory signal molecules such as ureides (Serraj et al. 1999b; Vadez et al. 2000), glutamine (Neo and Layzell. 1997), asparagine (Bacanamwo and Harper 1997; Vadez et al. 2000), and aspartate (King and Purcell. 2005). The restriction in the export of N-related compounds, with their subsequent accumulation in nodules, under a water deficit situation has also been postulated (Pate et al. 1969; Walsh et al. 1989a, b). Serraj et al. (2001) refined the model by proposing two possible origins for the feedback inhibition: a direct feedback within the nodules and an indirect feedback due to N compound signals coming from the aerial parts. Recent studies showed that before any accumulation occurs at shoot level, ureides were accumulated in nodules suggesting a local regulation of SNF (Ladrera et al. 2007; Gil-Quintana et al. 2013b). On the contrary to ureide exporters for which numerous references have been published (Serraj et al. 1999b; Vadez et al. 2000; Neo and Layzell. 1997; Bacanamwo and Harper 1997; Vadez et al. 2000; King and Purcell. 2005), little was known about how/whether this mechanism operates in temperate and amide-exporting legumes species. Experimental evidence in this regard has been limited to the observation of an accumulation of N-related compounds, mainly asparagine, in salt-stressed *M. sativa* nodules (Fougere et al. 1991). Similarly, studies on the sap composition upon the application of N fertilization in *M. truncatula* supported the hypothesis of asparagine as signaling molecule responsible for N-feedback regulation of SNF (Sulieman et al. 2010). Recently, the overall accumulation of amino acids in nodules of different legume species (Gil-Quintana et al. 2013a, b; Nasr Esfahani et al. 2014) has revealed the existence of a more intricate N signaling regulation mechanism than the one suggested so far with individual candidate amino acids (e.g., asparagine or aspartate).

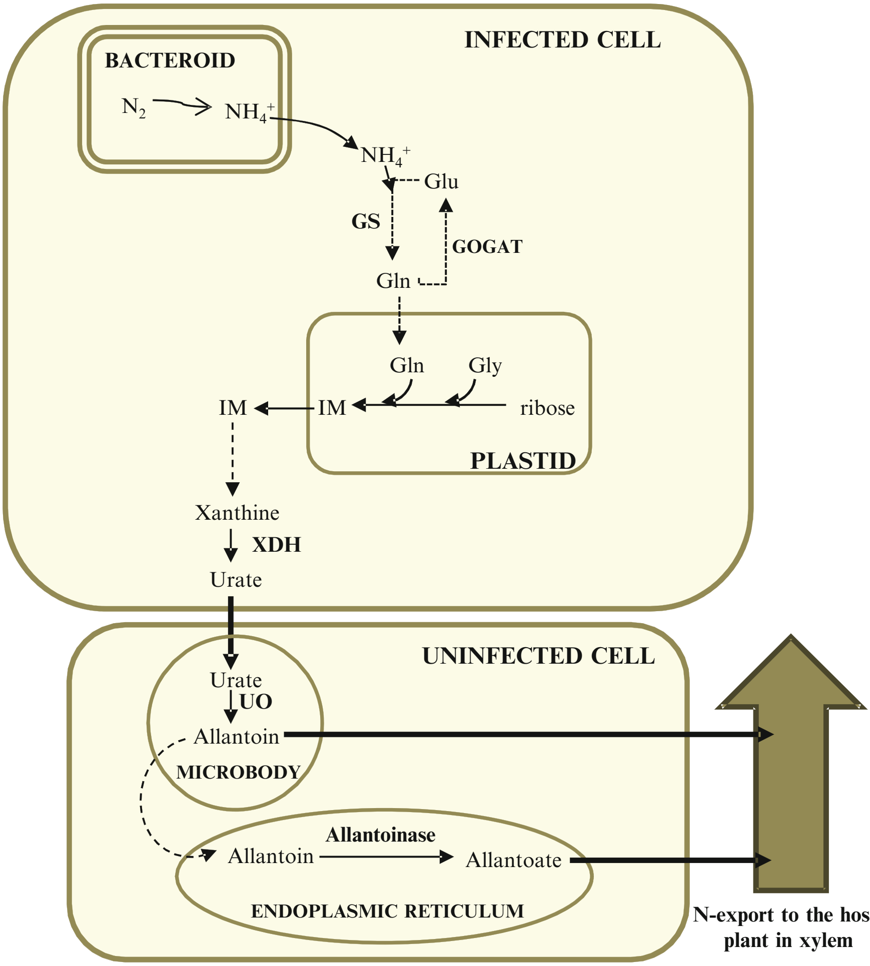


**Fig. 2** Schematic representation of ureide biosynthesis pathway in tropical legume nodules. *Glu* glutamic acid, *Gln* glutamine, *Gly* glicine, *GOGAT* glutamate synthase, *GS* glutamine synthetase, *IM* inosine monophosphate, *UO* urate oxidase, *XDH* xanthine dehydrogenase. Based on Buchanan et al. (2000)

## *2.4 Involvement of Nodule S Metabolism in Response to Drought*

In recent years, proteomic analyses of root nodules in *M. truncatula* have identified a significant number of components related to sulfur metabolism in these organs. These included the enzyme committed to the biosynthesis of methionine (Met), Met synthase, and S-adenosyl methionine (SAM) synthase, whose protein levels were found to decline under water-deficit conditions (Larrainzar et al. 2007, 2009).

Interestingly, the involvement of S metabolism in response to drought stress has been also reported in pea plants in which a decline in the protein level of SAM synthase was observed using 2D gel electrophoresis techniques (Irar et al. 2014).

Both Met synthase and SAM synthase are enzymes committed to consecutive steps in the biosynthesis of S-containing metabolites and amino acids (Fig. 3). SAM is a key metabolite essential for the synthesis of aspartate-related amino acids. It is involved in numerous secondary metabolism pathways and it is required for the biosynthesis of the plant hormone ethylene. To study the connection between S metabolism and ethylene, the full pathway from the synthesis of cystathionine to ethylene

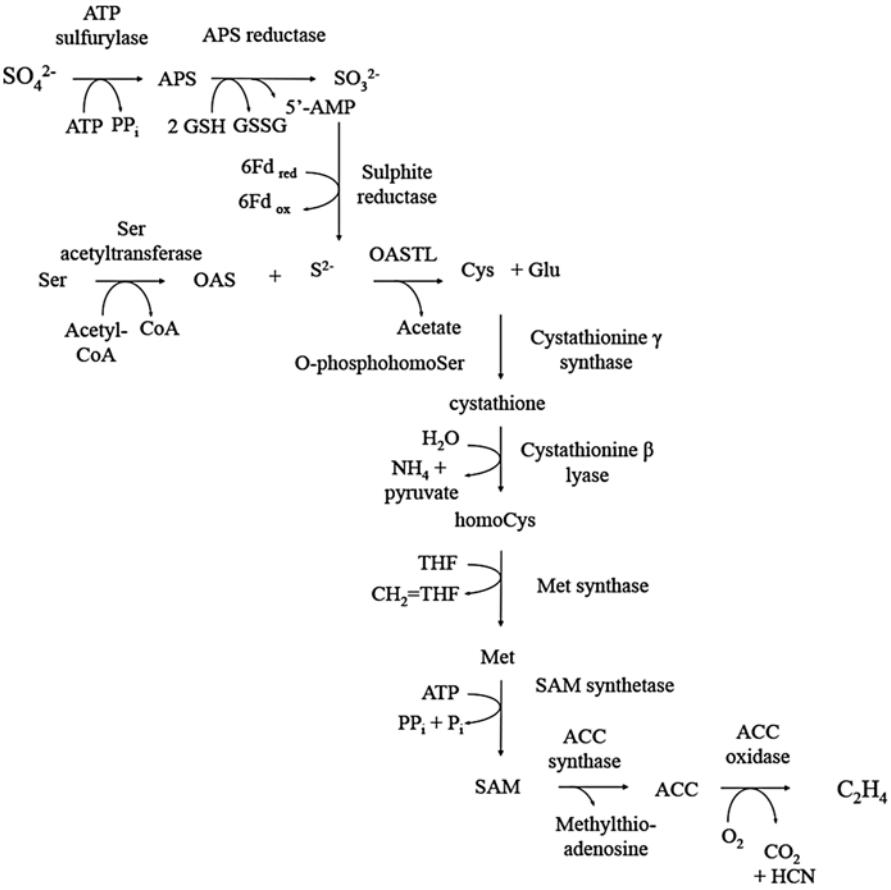


**Fig. 3** Schematic representation of the main reactions from sulfate assimilation to the biosynthesis of ethylene in plants. *GSH* glutathione, *GSSG* reduced glutathione, *Cys* cysteine, *Met* methionine, *Ser* serine, *Glu* glutamic acid, *APS* adenosine 5′-phosphosulfate, *Fd* ferredoxin, *OAS* O-acetylserine, *SAM* S-adenosylmethionine, *THF* tetrahydrofolate, *ACC* 1-aminocyclopropane-1-carboxylic acid, *APS* reductase: adenosine 5′-phosphosulfate reductase, *OASTL* O-acetylserine thiol lyase, *SAM synthetase* S-adenosylmethionine synthetase

was characterized in *M. truncatula* roots and nodules exposed to drought stress (Larrainzar et al. 2014a). To better understand the regulation of the pathway during drought, plants were subjected to a progressive water deficit and, subsequently, rewatered so that control water potential values were reached. Besides the physiological characterization of the plants, the enzymes involved in the pathway were analyzed both at the transcript and protein levels using real-time quantitative PCR (RT-qPCR) and absolute protein quantification techniques, respectively. Interestingly, the gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which is considered the rate-limiting enzyme in the biosynthesis of ethylene (Kende 1993), was found to be one of the genes being most responsive to the imposed drought. This gene showed a decline at the transcript level that was not fully alleviated after rewatering. The observed decline in the content of Met synthase in nodules was also detected in roots, although there was not a direct correlation at the transcript level. This finding suggests that additional posttranslational regulatory mechanisms may regulate the levels of Met synthase in nodules during stress conditions.

The plant hormone ethylene is generally considered a stress-responsive hormone (Ecker 1995; Wang et al. 2002). However, the study of the effects that drought stress provokes on ethylene production has led to contrasting conclusions. Some of the first studies in the topic, in which excised parts of plants were subjected to water stress, suggested that ethylene biosynthesis increases under drought conditions (El-Beltagy and Hall 1974; Apelbaum and Yang 1981). In contrast, later work using whole plant systems actually showed that ethylene production decreases in plants experiencing drought (Morgan et al. 1990; Narayana et al. 1991). Results from our work (Larrainzar et al. 2014a), along with expression analysis in *Arabidopsis thaliana* (Huang et al. 2008), suggest that indeed the enzymes responsible for ethylene biosynthesis are downregulated in roots under water-deficit conditions. Interestingly, this limitation in the production of ethylene in drought-stressed roots has been attributed to the effect of endogenous abscisic acid (ABA) in *A. thaliana*. This ethylene-ABA interaction would be then responsible for maintaining primary root growth at low water potential values, as shown using ABA-deficient maize seedlings (Sharp 2002). Thus, during drought stress the increase in ABA concentration in roots may inhibit ethylene production and, therefore, the associated downstream gene responses. Understanding how this regulation occurs at the molecular level and which the downstream responses are will be important challenges to address in the future.

## 2.5 *Water Stress Induces Redox Alterations That are Involved in SNF Regulation*

Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical are unavoidably generated as a consequence of aerobic life due to incomplete reduction of oxygen to water (Apel and Hirt 2004). The high metabolic rate needed to ensure adequate energy supply to fuel SNF is surely the main source of ROS in symbiotic root nodules. Besides this, ROS can be also generated during the autoxidation of the oxygenated form of Lb, and their production catalyzed due to the abundance of catalytic Fe and the oxidation of proteins with strong reducing potential that are abundant in nodules, i.e., ferredoxin and nitrogenase (Chang et al. 2009; Marino et al. 2009; Becana et al. 2010). ROS have a dual action: on one hand, they are cytotoxic because they can virtually oxidize any biomolecule to generate "oxidative stress" and/or "oxidative damage". In this sense, protein carbonylation and lipid peroxidation are commonly used as oxidative stress markers (Apel and Hirt 2004). On the other hand, they are key actors in many signaling pathways including plant response to environmental changes. In fact, ROS are not only generated as by-products of metabolic processes but also "deliberately" generated by enzymatic complexes (Marino et al. 2011, 2012). The complexes involved include class III peroxidases, oxalate oxidases, amine oxidases, lipoxygenases, quinone reductases, and plant NADPH oxidases (known as respiratory burst oxidase homologs; RBOHs). To maintain an adequate balance between ROS signaling or damage/toxicity, legume nodules possess an important army of enzymatic and non-enzymatic antioxidant systems. The non-enzymatic ones are mainly constituted by ascorbate and (homo)glutathione, but there are also other molecules with antioxidative properties, such as bilirubin, tocopherol or flavonoids. The nodule enzymatic antioxidant machinery is composed by superoxide dismutases, catalases, enzymes participating in the ascorbate–glutathione cycle, glutathione S-transferases, thiol peroxidases, thioredoxins, and glutaredoxins (reviewed in Becana et al. 2010).

Plants are continuously exposed to alterations in their environment such as pathogen attack, high light, low temperature, high temperature or drought stress. They can cope with this changing environment by adjusting their metabolism so that the homeostasis between energy production and consumption is maintained. This metabolic adjustment is generally associated with ROS overproduction, which in turn activates key stress signaling responses but may also overtake nodule antioxidant capacity and, ultimately, generate oxidative damage (Suzuki et al. 2012). In fact, severe water deficit has been shown to generate oxidative damage (i.e., protein carbonylation and/or lipid peroxidation) both in indeterminate and determinate nodules in several legume species including pea (Gogorcena et al. 1995), soybean (Porcel and Ruiz-Lozano 2004), and alfalfa (Naya et al. 2007). When oxidative damage is evident $N_2$ fixation inhibition has been associated to a direct effect of ROS on Lb and nitrogenase (Gogorcena et al. 1995; Naya et al. 2007). However, when the applied stress has generated a general nodule breakdown, it becomes difficult to discern whether ROS are acting as toxic or signaling molecules. In this sense, application of a mild water deficit in pea provokes an alteration of the redox status of the cell, and inhibits SNF prior to any effect observed on Lb or nitrogenase content (Marino et al. 2007), thus, suggesting a potential ROS signaling role in the control of SNF.

Drought, similarly to other environmental stresses, affects plant performance independently of ROS action. To analyze the effect of oxidative stress on nodule performance without the influence of the complex interactions that natural environmental constraints might create, paraquat (PQ; methyl viologen or N,N′-dimethyl-4,4′-bipyridinium dichloride), a compound that exacerbates $O_2^-$ and $H_2O_2$ production

(Farrington et al. 1973), can be applied. This strategy was employed by Marino et al. (2006), who applied PQ to nodulated pea roots at two concentrations: 1 and 10 μM. The application of 1 μM PQ mimicked nodule response exposure to a mild water stress in pea since SNF inhibition was related to SuSy downregulation, and thus to a limitation of the energy supply to bacteroids (Marino et al. 2006). Interestingly the application of a higher PQ dose (10 μM) mimicked the conditions of a severe water deficit and inhibition of SNF was associated to a direct effect on Lb (Marino et al. 2006).

Redox modifications have been shown to not only regulate in vivo SuSy activity but also its gene expression (Marino et al. 2008). Moreover, in vitro experiments have demonstrated that SuSy is regulated by a reversible posttranslational oxidation mechanism. In this sense, a proteomic approach focused on the identification of sulfenylated proteins in *M. truncatula* revealed that SuSy is sulfenylated both in the initial steps of the infection by *S. meliloti* and in mature nodules (Oger et al. 2012). In another attempt to decipher the mechanisms governing the regulation of SNF under oxidative stress conditions, it was shown that cadmium (Cd) treatment inhibited SNF in *M. truncatula* prior to the occurrence of oxidative damage and this inhibition was related to an $O_2$-related effect on Lb and nitrogenase (Marino et al. 2013). Thus, besides the mild and severe oxidative stress regulation of SNF, it seems that in *Medicago* species SNF regulation in relation to redox alteration is not associated to SuSy inhibition but to an $O_2$ related Lb-nitrogenase downregulation (Naya et al. 2007; Marino et al. 2013).

Under water deficit conditions antioxidant systems are induced so as to maintain an adequate redox homeostasis and, as the stress progresses, they are generally downregulated due to a general nodule protein breakdown (Marino et al. 2009; Becana et al. 2010). Thus, it has been hypothesized that an increase in nodule antioxidant capacity might alleviate the effect of abiotic stresses on SNF (Bashor and Dalton 1999; Zabalza et al. 2008; Redondo et al. 2012). For instance, the application of sewage sludge in alfalfa reduced nitrogenase activity probably because the sewage sludge also represented a source of N (Antolín et al. 2010). However, this treatment also provoked an increased nodule tolerance to drought concomitant to the induction of the antioxidant machinery and the prevention of Lb degradation. Indeed, Bashor and Dalton (1999) showed that external ascorbate supply to nonstressed soybean plants led to striking increases in nitrogenase activity. However, exogenous treatment with the ascorbate precursor (galactono-1,4-lactone) to pea plants led to increased ascorbate content in nodules but failed to prevent SNF inhibition (Zabalza et al. 2008). In the same line, genetic approaches have shown that *M. truncatula* transgenic plants partially impaired in glutathione (GSH) biosynthesis presented reduced SNF associated to lower levels of expression of Lb (El Msehli et al. 2011). Furthermore, the overexpression of glutathione biosynthesis correlated with higher SNF, which could be related to higher SuSy and Lb expression levels (El Msehli et al. 2011). Besides these observations, the heterologous overexpression of a flavodoxin from the filamentous cyanobacterium *Anabaena variabilis* in *M. truncatula* plants partially prevented the effects of salt stress on SNF (Coba de la Pena et al. 2010). Flavodoxins are prokaryotic electron carrier proteins and have been suggested to play a positive role in ROS detoxification (Redondo et al. 2009). In a complementary approach, the same research group overexpressed this flavodoxin in *S. meliloti* and *M. truncatula* infected with this strain also presented increased tolerance of SNF to salt stress (Redondo et al. 2012). Thus, it can be concluded that the antioxidant capacity of both partners is crucial to maintain adequate levels of SNF. In this regard, it will be very interesting to check in the future whether the overexpression of such antioxidant systems in the plant and/or the bacterial partners contributes to maintain nodule performance when exposed to water deficit.

Taken together, these results indicate that oxidative stress/signaling is involved in at least two regulation mechanisms regarding SNF: inhibition due to SuSy down-regulation and SNF impairment because of Lb-nitrogenase degradation. It appears that these mechanisms can operate within the same legume species (e.g., pea) depending on the intensity of the stress. However, in other species (e.g., *Medicago* spp.) it is the $O_2$-related Lb-nitrogenase mechanism the one playing a crucial role in the regulation of SNF under oxidative stress.

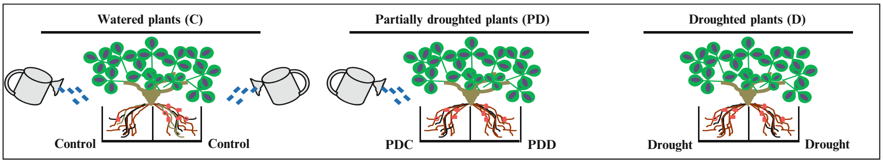
In addition to ROS, reactive nitrogen species (RNS), are also generated in plants upon abiotic stress exposure and also have a dual function: in signaling processes and provoking nitrosative damage (Boscari et al. 2013). In contrast to ROS, RNS function in the regulation of nodule functionality under abiotic stresses has been barely studied, and, so far, their role in relation to drought is unknown. Nitric oxide (NO), the most studied RNS, has been proposed to play a role in maintaining cell energy status in nodules under hypoxic conditions via a cyclic respiration pathway (Horchani et al. 2011; Boscari et al. 2013). Besides this, it has also been reported that control of the NO content is crucial for nodule performance, since a decrease in NO levels leads to a delay in nodule senescence and increased amounts lead to SNF inhibition and acceleration of nodule senescence (Cam et al. 2012). In agreement with these results, Navascues et al. (2011) demonstrated that green Lb is produced by the nitration of the haem group in soybean nodules. Thus, RNS seem to be key players in legume aging. However, their role in stress-induced senescence or in nodule C/energy metabolism remains to be investigated.

## 3 Regulation of SNF: Local or Systemic?

The origin of the inhibition of SNF under drought stress, whether local (i.e., at the root-nodule level) or systemic (i.e., involving the aerial part), has been matter of debate in the symbiosis research community. Most studies have traditionally been approached assuming a local regulation of SNF at the nodule level. However, the hypothesis of a N-feedback regulation, one of the most studied hypotheses, suggested the existence of a systemic regulation mechanism (Serraj et al. 2001). Early studies carried out in tropical ureide-exporter legumes showed an accumulation of N-related compounds in legume nodules when subjected to water deprivation (Schubert et al. 1995; Sinclair and Serraj 1995; deSilva et al. 1996; Serraj and Sinclair 1996, 1998; Serraj et al. 1999a; Purcell et al. 1998; Vadez et al. 2000). One of the proposed explanations for these observations was the impairment in the export of nodule N-related compounds derived from SNF to the aerial part provoked by either a reduction in water supply (Pate et al. 1969; Walsh 1990) or lowered evapotranspiration rates (Devisser and Poorter 1984; Minchin and Pate 1974). However, these hypotheses were not empirically proven under physiological drought conditions. Similarly, studies carried out in legumes subjected to stresses other than drought (i.e., the application of inorganic N to $N_2$-fixing plants) suggested that the N status of the plant and/or some N-related compounds could act as signals regulating SNF through a N-feedback mechanism (Silsbury et al. 1986; Oti-Boateng and Silsbury 1993; Parsons et al. 1993; Hartwig et al. 1994; Bacanamwo and Harper 1997; Neo and Layzell 1997; Serraj et al. 1999b; Vadez et al. 2000; Hartwig and Trommler 2001; King and Purcell 2005). Serraj et al. (2001) refined the model by proposing two possible origins for the feedback inhibition by ureides accumulation under drought stress: a direct feedback within the nodules and an indirect feedback due to N compound signals coming from the aerial part. The local or systemic nature of the regulation of SNF under drought stress remained an open question until the existence of a local inhibition mechanism was demonstrated using a split-root system approach (see experimental setup in the upper panel of Table 1) in pea (Marino et al. 2007), *M. truncatula* (Gil-Quintana et al. 2013a), and soybean (Gil-Quintana et al. 2013b; recently reviewed in Larrainzar et al. 2014b). These studies showed that a general accumulation of N compounds takes place in drought-stressed nodules prior to the drop of evapotranspiration, pointing at a more intricate N signal regulation than previously suggested in the literature. Several studies suggest that the observed accumulation of ureides may be a widespread response to water deficit that does not necessarily relate to the regulation of SNF (Alamillo et al. 2010). Indeed, Díaz-Leal et al. (2012) observed that the content of ureide in common bean varies depending on the plant developmental stage, both under $N_2$-fixing conditions and in nitrate-fed plants. These changes were associated to the activity of the ureide-degrading enzyme allantoinase, which showed a tissue-specific regulation (Díaz-Leal et al. 2012). Collier and Tegeder (2012) have shown that soybean ureide transporters play a key role both in terms of shoot N supply and in nodule development and function, although their role on drought response has not been explored yet. Gil-Quintana et al. (2013b) showed that ureide catabolism in soybean was more affected than the de novo synthesis under drought stress. Furthermore, the observed accumulation of ureides in nodules may be related to a decline in degradation activity rather than to increased biosynthesis. In this regard, Díaz-Leal et al. (2014) identified the allantoate amidohydrolase responsible of allantoate degradation in *Phaseolus vulgaris* and showed that its activity was downregulated in leaves of drought-stressed plants.

Due to the functional interaction between shoots and roots, both local and systemic responses coexist in plants. As discussed above, drought stress provokes a local inhibition of SNF, thus, downplaying the implication of a shoot signal in the regulation of SNF. Similarly, the decline of nodule water potential and the accumulation of N compounds in nodules occur again at the local level (Table 1). Nevertheless, concomitantly, a systemic root to shoot regulation of stomatal conductance and transpiration is observed (Table 1). Abscisic acid, pH and cytokinins, among other factors, are implicated in root to shoot signaling under drought

**Table 1** Upper panel: Schematic representation of the split root system (SRS) experimental setup. Nodulated plants at the late vegetative stage are randomly separated into three groups. Control plants (C) are watered to field capacity and in drought plants (D) water/nutrient solution is withheld. In partial drought plants (*PD* partial drought) a side of the root system is watered to field capacity (*PDC* control partial drought), while in the other side water/nutrient solution is withdrawn (*PDD* drought partial drought). Gradual and progressive drought stress is imposed and physiological determinations and analyses are carried out during the onset of the treatment. Lower table: Summary of locally regulated processes in shoots, roots and nodules of different legumes plants when exposed to partial drought using the above SRS. $\Psi_w$ water potential, *(N)DW* (nodule) dry weight



| **Shoot** | | | |
|---|---|---|---|
| **Leaf $\Psi_w$** (MPa) | ≅ | | pea (Marino et al. 2007); *Medicago truncatula* (Gil-Quintana et al.2013a) and soybean (Gil-Quintana et al. 2013b) |
| **Transpiration** (g $H_2O$ plant$^{-1}$day$^{-1}$) | ⇓ | | |
| **Stomatal conductance** (g $H_2O$ plant$^{-1}$day$^{-1}$) | ⇓ | | *Medicago truncatula* (Gil-Quintana et al. 2013a) |
| **Leaf amino acid content** (mmol g$^{-1}$ DW) | ≅ | | *Medicago truncatula* (Gil-Quintana et al. 2013a) and soybean (Gil-Quintana et al. 2013b) |
| **Shoot ureide content** (mmol g$^{-1}$ DW) | ⇊ | | soybean (Gil-Quintana et al. 2013b) |
| **Root-Nodules** | PDC | PDD | |
| **Nodule $\Psi_w$** (MPa) | ≅ | ⇊ | pea (Marino et al. 2007); *Medicago truncatula* (Gil-Quintana et al. 2013a) and soybean (Gil-Quintana et al. 2013b) |
| **Nitrogen Fixation** (mmol $H_2$ g$^{-1}$ NDW min$^{-1}$) | ≅ | ⇊ | |
| **Nod. sucrose synthase** (mmol NADH mg$^{-1}$ prot min$^{-1}$) | ≅ | ⇊ | pea (Marino et al. 2007) |
| **Nodule malate content** (mmol g$^{-1}$ NDW) | ≅ | ⇊ | |
| **Root amino acid content** (mmol g$^{-1}$ DW) | ≅ | ⇈ | *Medicago truncatula* (Gil-Quintana et al. 2013a) and soybean (Gil-Quintana et al. 2013b) |
| **Nodule amino acid content** (mmol g$^{-1}$ DW) | ≅ | ⇈ | |
| **Nodule ureide content** (mmol g$^{-1}$ DW) | ≅ | ⇈ | soybean (Gil-Quintana et al. 2013b) |
| **Root ureide content** (mmol g$^{-1}$ DW) | ≅ | ⇈ | |
| **Protease activity** (ng Trypsin mg$^{-1}$ protein) | ≅ | ≅ | *Medicago truncatula* (Gil-Quintana et al. 2013a) |

(Schachtman and Goodger 2008). Altogether, these results point at the existence of complex regulation mechanisms governing responses to drought stress in nodulated legumes. Future research will provide us with the answers to further understand the molecular basis of the regulation of SNF during drought stress.

## 4 Molecular Systems Biology for Understanding Legume Drought Stress Response

The response of legumes to drought stresses has been analyzed from several perspectives, including a combination of analytical techniques such as transcriptomics, metabolomics, proteomics, and whole plant physiology (Fig. 4). Despite progress in the field, drought research in legumes is still behind that of the widely studied model

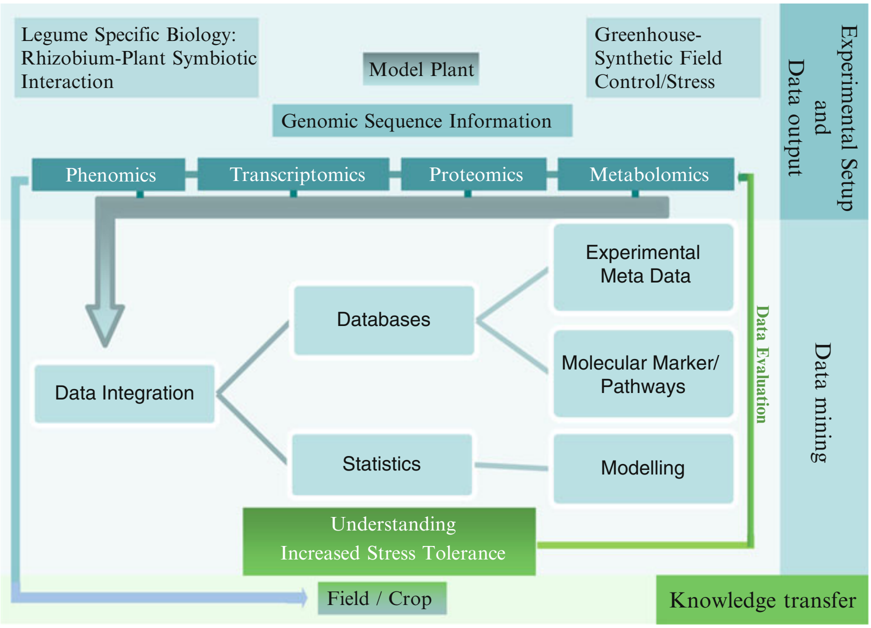


**Fig. 4** Available systems biology-based approaches for the study of drought stress responses. Global data from phenomics, transcriptomics, proteomics, and metabolomics can be integrated and evaluated to predict new regulatory networks and to enable selection of drought-tolerant phenotypes

plant *A. thaliana* (Wienkoop et al. 2010), for which many more resources are available. For instance, in this model plant some attempts to integrate the complex gene regulatory networks governing the plant responses to a number of environmental stresses have already been carried out in the AtGenExpress project (Kilian et al. 2007). In the legume field some first initiatives have been carried out including the analysis of drought-stressed chickpea plants (Molina et al. 2008; Varshney et al. 2009), and at the level of microRNA regulation in *M. truncatula* (Wang et al. 2011; Wang et al. 2013) and cowpea (Barrera-Figueroa et al. 2011). Additionally, new data on the effect of drought stress in the transcriptional profile of different *M. truncatula* organs is now available in the *Medicago truncatula* Gene Expression Atlas website (mtgea.noble.org; Benedito et al. 2008). Despite this progress, our current level of understanding on how legume plants respond to drought stress remains quite limited. A deeper understanding of these interactions will have future implications for the successful breeding of stress-tolerant legumes, which do not depend on external applications of mineral fertilizers and rely on SNF for growth (Charpentier and Oldroyd 2010; Ercolin and Reinhardt 2011).

Gathering complex data sets resulting from systems biology-based approaches and extracting useful biological information, mostly by means of mathematical models, will be a challenging task in the future for plant scientists of diverse biological and technological backgrounds, as well as for communities, such as iPLANT collaborative and the International Plant Proteomics Organization (INPPO) (Goff et al.

**Table 2** Overview of available organizations, and proteomic and transcriptomic databases useful in legume research

| Database | Description | Link | References |
|---|---|---|---|
| ProMEX | Proteomic spectral and meta data library of multiple plant species including legumes | http://promex.pph.univie.ac.at/promex/ | Wienkoop et al. (2012) |
| GelMAP | Universal tool for gel spot visualization | http://www.gelmap.de/ | Rode et al. (2011) |
| P3DB | Plant protein phosphorylation database | http://www.p3db.org/ | Gao et al. (2009) |
| MPPD | Medicago phosphoprotein database | http://phospho.medicago.wisc.edu | Grimsrud et al. (2010) |
| MTDB | *Medicago truncatula* transporter database | http://bioinformatics.cau.edu.cn/MtTransporter/ | Miao et al. (2012) |
| LegumeIP | An integrative platform to study gene function and genome evolution in legumes | http://plantgrn.noble.org/LegumeIP | Li et al. (2012) |
| MtGEA | *Medicago truncatula* gene expression atlas | http://plantgrn.noble.org/LegumeIP | Benedito et al. (2008); He et al. (2009) |
| LjGEA | *Lotus japonicus* gene expression atlas | http://ljgea.noble.org/ | Verdier et al. (2013) |
| LegumeGRN | Gene regulatory network prediction server | http://legumegrn.noble.org | Wang et al. (2013) |
| LIS | Legume information system | http://www.comparative-legumes.org/ | Gonzales et al. (2005) |
| INPPO | Proteomics data aggregation and exchange of plant species from model to crop | http://www.inppo.com/ | Agrawal et al. (2011) |
| iPLANT | Data aggregation and exchange of plant species | http://www.iplantcollaborative.org/ | Goff et al. (2011) |
| iMOP | Proteomics data aggregation of model organisms (not plant specific) | http://www.hupo.org/research/imop/ | Jones et al. (2012) |

2011; Agrawal et al. 2011). In order to succeed, the first goal would be to create platforms for a more efficient data access and exchange, i.e., a database that allows for a systematic search of transcripts, proteins or metabolites previously found to be involved in stress response, accompanied by detailed experimental metadata. In the *Arabidopsis* community, the first steps in this direction have recently been undertaken by initiatives that aggregate databases for open access such as the Multinational Arabidopsis Steering Committee (MASCP)-gator for proteomics data (Joshi et al. 2011). Here, different proteomic databases have been connected to merge all available information of *Arabidopsis* proteins. Amongst these databases, ProMEX contains not only *Arabidopsis* data but also more than 15,000 peptide spectra from different tissues of *M. truncatula* (~3,600 proteins), *Lotus ssp.* (~1,000 proteins), *P. vulgaris* (~2,000 proteins), *G. max* (~500 proteins), together with their rhizobium partners (~600 proteins), which constitutes the largest peptide-spectral library for legumes so far (Hummel et al. 2007; Wienkoop et al. 2012). Furthermore, ProMEX compiles information on tissue of origin and experimental conditions, including drought stress studies. In Table 2, we summarize several databases useful for legume research. Interestingly, all these databases and platforms have only recently emerged, which underlines the increasing interest in gathering and making accessible this type of information in the legume community.

In order to successfully extrapolate laboratory results to applied fields such as agronomy, the experimental design should mimic as far as possible biologically relevant field conditions (Gonzales et al. 2005). Although both controlled-field simulations in the laboratory as well as actual field experiments are necessary, field trials have been often hampered by unpredictable environmental factors and by the difficulty of providing robust and reproducible results (Limpens et al. 2012). Therefore, to overcome some of the above-mentioned limitations, a joint venture "standardized-artificial field" [a similar concept to the project Biosphere2 (www.b2science.org) but at smaller scale] might be a solution. Such standardized-artificial field would greatly simplify the exploration and comparability of the complex interaction networks within live systems, thanks to carefully controlled growth conditions (e.g., temperature, light, diurnal cycle, nutrition solution, growth medium, soil, microbial and other plant communities) in the context of climatic changes. A first attempt towards the creation of this type of controlled large scale artificial-field may be the physiological plant phenotyping platforms that are currently being available at some research centers such as the Jülich Plant Phenotyping Center (http://www.fz-juelich.de/ibg/ibg-2/EN/organisation/JPPC/JPPC_node.html) and the IAP at IPK Gatersleben (http://iap.ipk-gatersleben.de), where automated, high-throughput, and noninvasive techniques are applied for the assessment of developmental states and the degree-of-stress in plants. Figure 4 shows a schematic overview of the post-genomic systems biology approach.

## 5 Future Perspectives

Agriculture is the largest water consumer worldwide, using about 70 % of the total renewable freshwater resources (WWAP 2014). Therefore, changes in the amount of precipitation have a huge impact on agricultural systems. Under the current changing environment, and taking into consideration the consequences of climate change and the expected increase in population, agriculture faces new challenges. During the last three decades, significant progress has made in understanding the regulation of SNF under drought stress. Nodule C and N metabolism have been exhaustively investigated and new factors regulating SNF under moderate stress including oxidative stress and the effects of drought on S metabolism have been analyzed. Additionally, regulation of SNF has been proven to occur at local level rather than systemically. Studies using different legume crops and model plants have highlighted the variability of the metabolic responses in nodules when plants are exposed to drought. Given the advancements in legume genomics (Cannon et al. 2006; Young and Udvardi 2009), it is clear that a better understanding on how SNF is regulated under abiotic constrains at physiological and metabolic level will be instrumental for cultivar selection and the development of varieties with improved performance under stress conditions.

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# Salinity: Physiological Impacts on Legume Nitrogen Fixation

**Carmen Lluch Plá and Libertad Cobos-Porras**

**Abstract** Salinity is of great concern in arid and semiarid regions, where soil salt content is often high and precipitation is insufficient for leaching. High salinity in soil induces a strong decrease in crop yield and productivity of arable land. According to global change prediction models, salinity is expected to expand in the near future. Legumes represent the world's second major crop of agricultural importance. These plants provide an important source of proteins for human and animal diets and they are widely used as green manure, forage, and others. In comparison with other crops, legumes represent a particular plant group thanks to their capacity to fertilize soils through the atmospheric nitrogen fixation. The symbiotic relationship between *Rhizobiaceae* and legumes leads to the development of root nodules, where bacteria fix the atmospheric nitrogen that the host plant incorporates as organic molecules. In general, rhizobia are more salt tolerant than their respective plant host, but the process of nodule formation is particularly sensitive to salt stress (colonization and infection are highly reduced). Under salt stress, plants face at least three major constraints: water deficit, ion toxicity, and oxidative stress. Some plants develop tolerance mechanisms to overcome it, including changes in gene expression patterns and in metabolic responses related with adaptation/tolerance (ion homeostasis, osmoprotectants, and antioxidant system). In this context, the application of new "omics" technologies in combination with traditional agronomic and physiological studies will improve the salt tolerance of symbiotic processes, and it will optimize the biological nitrogen fixation.

**Keywords** Legumes crops • Rhizobia • Biological nitrogen fixation • Salt stress • Metabolic responses • Genomic and biotechnology approaches

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# 1 Introduction to Salinity and $N_2$ Fixation

## *1.1 Salinity Types and Causes*

Salinity problems are critical worldwide; over 20 % of the cultivated areas and half of the irrigated lands are encountering salinity stress of different magnitudes, reducing greatly yield below the genetic potentials (Flowers 2004). Saline soils are defined by Ponnamperuma (1984) as those that contain sufficient salt in the root zone to impose the growth of plants. However, since salt injury depends on species, variety, growth stage, environmental factors, and nature of the salts, it is difficult to precisely define saline soils. The USDA-ARS (2008) defines saline soils as those ones with an electrical conductivity of the saturation extract ($EC_c$) equal or higher to 4 $dSm^{-1}$ (≈40 mM NaCl at 25 °C), and soils strongly saline when ECs exceed 15 $dSm^{-1}$.

Primary salinization occurs naturally when the soil parent material is rich in soluble salts, or in the presence of shallow saline groundwater table. This is caused by two natural processes. The first is the weathering of parent material containing soluble salts of various types, mainly chlorides of sodium, calcium, and magnesium, and, to a lesser extent, sulfate and carbonates. The second is the deposition of microscopic salt particles carried by the wind and rain to oceans (Munns 2009). In arid and semiarid regions, where rainfall is insufficient to wash out soluble salts from the soil, or where drainage is restricted, high concentration of salt is accumulated (USDA-ARS 2008). Secondary salinization results from humane activities that change the hydrologic balance in the soil between precipitation and water used by crops (transpiration). The most common salinity (anthropogenic salinization) occurs when significant amount of poor-quality water is used for irrigation. Therefore salt accumulates in the soil, resulting in saline and thereby unproductive soils (Munns 2009). This process could be avoided with effective irrigation systems that remove salt from the soil profile (Szabolcs and Greenland 1992).

Other causes of salinization are related with the chemical contamination, which often occurs in modern intensive agricultural system as greenhouse and intensive farming systems. This process happens mainly in regions with a poor natural soil cover and high intensive animal husbandry, or by accumulation of airborne/waterborne salts in soils (Shukla et al. 1990; Hastenrath 1991). Szabolcs (1994) has reported that chemicals from industrial emissions may accumulate in the soil, and it can result in salt accumulation in the upper layer of soil.

The long-term consequences of saline irrigation on soil physical fertility are well documented (De Pascale et al. 2003a, b). The saturation level of sodium ion ($Na^+$) is considered the main cause of clay dispersion with the consequent reduction of impermeability, porosity, and hydraulic conductivity (Amezketa 1999). Climatic factors and water management may accelerate salinization. In arid and semiarid lands evapotranspiration plays a main role in the pedogenesis of saline and sodic soils (Yadav et al. 2011). When an excess of $Na^+$ content is involved in the salinization process, it is called "sodicity." Sodicity is defined as the exchange sodium percentage (ESP) threshold that causes degradation of soil structure.

The USDA salinity laboratory defines a soil as sodic if it has the ESP greater than 15 (www.ussl.ars.usda.gob). If the concentration of soluble salts is sufficiently low, hydrolysis of the sodic clay will occur, creating a highly alkaline soil. A soil is considered alkaline (type of sodic soils) when it has a high pH due to carbonate salts (pH of 8.5–10 and EPS of 15 or more). The process of sodicity is complex and occurs over a long period of time; however, saline/sodic soils are widespread in arid and semiarid environments (Munns 2009).

Salinity is the most widespread soil degradation process in the earth and it is regarded as a major cause of desertification. Salinization/sodification is a serious form of soil degradation being considered among the major destruction processes endangering the potential use of world soils (Ladeiro 2012). Deforestation is recognized as a major cause of salinization and alkalinization of soils. It affects salt migration in both the upper and lower layers. Deforestation leads to the reduction in rainfall average and increases surface temperature (Shukla et al. 1990). As a consequence, top thin soil rapidly gets eroded in the absence of green cover. Trees can act as a buffer zone between the soil and the rain, and without them, erosion is practically inevitable. Furthermore, soil erosion leads to a greater amount of runoff and increased sedimentation in the rivers and streams (Hastenrath 1991).

The harmful impacts of salinity include low agricultural production, low economic returns (due to high cost of cultivation), and soil erosion. Crop species show a different range of responses to salt stress. Although all species show some growth under salt conditions, eventually, their yield is reduced. Any increase in the agricultural productivity of saline soils, which are often grouped under marginal or waste soils, can be contributed to the socioeconomic improvement of millions of people who sustain their livelihood from them (Ladeiro 2012). Sustainable agriculture involves ecological managements, which implies considering elements, such as biodiversity, nutrient cycling, and energy flux, in order to avoid the loss of nutrients and soils and to prevent the attack of pest and diseases (Spiertz 2010).

## *1.2 Legumes and Biological Nitrogen Fixation*

Legumes (Fabaceae) are the third largest family of flowering plants with over 650 genera and 20,000 species (Lewis et al. 2005). They are second grass in importance to human diets; and among grain crops, legumes rank world’s third place behind cereal and oilseed production (Graham and Vance 2003). Moreover, legumes are widely used as green manure since the beginning of agriculture. However, this practice has been diminished since industrially produced fertilizers became available. Green manure adds nitrogen to the soil and improves soil quality by increasing the organic matter content of the soil.

Forage legumes have been adapted from wild flora and used in managed grazing lands. These include lucerne (*Medicago sativa*), clovers (*Trifolium* spp.), vetch (*Vicia angustifolia*), and birdsfoot trefoil (*Lotus corniculatus*). These kinds of species provide animal husbandry with high-quality forage (Mikić et al. 2011) and they increase soil organic matter when crops are rotated in farming system (O'Hara et al. 2002). Grain legumes, such as pea (*Pisum sativum*), bitter vetch (*Vicia ervilia*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum*), soybeans (*Glycine max*), peanut (*Arachis hypogaea*), faba bean (*Vicia faba*), and grass pea (*Lathyrus sativus*) are commonly recognized as one of the first domesticated plant species and the most ancient crops. They contributed to the "agricultural revolution" in the Fertile Crescent at the end of the last Ice Age (Bellwood 2005). Subsequently and independently, soybean was domesticated in China, cowpea (*Vigna unguiculata*) in Africa, pigeon pea (*Cajanus cajan*) in India, and common bean (*Phaseolus vulgaris*) in Central and South America. The legumes are major source of food, fodder, timber, phytochemicals, phytomedicines, nutriceuticals, and $N_2$ fertility in agrosystems (Graham and Vance 2003).

Nitrogen is an essential nutrient for plant growth and its availability is a big limiting factor for agricultural systems. For decades, $N_2$ fertilizers have been continuously used to improve crop yield. However, the use of it accelerates the depletion of large amounts of fossil and nonrenewable energy sources. It substantially contributes to environmental pollution through atmospheric emission and leaching of ammonia, nitrates, and nitrous oxide (Velthof et al. 2009).

In this panorama, it is worthy to pay attention to the biological nitrogen fixation (BNF) which has significantly lower economic and environmental costs than the $N_2$ chemical fertilizers coming from industrial process. BNF is crucial from the environmental and agricultural points of view; it is the second more important process to maintain a sustainable biosphere. BNF is the assimilation of atmospheric $N_2$ in the form of organic compounds, and is a sustainable source of $N_2$ in cropping system. Fixed $N_2$ can be used directly by plants and it is less susceptible to volatilization, denitrification, and leaching (Garg and Geetanjali 2007). Thus, this process can reduce the use of chemical fertilizers and consequently mitigate global warming and water contamination (Juárez-Santacruz et al. 2013).

The ability to reduce $N_2$ to ammonia is restricted to some prokaryotes. These species are able to break (with the aid of the key enzyme nitrogenase) the strong triple bond within the $N_2$ molecule. This process requires high cost of energy and the nitrogenase might be rapidly inactivated by high oxygen ($O_2$) concentrations (Bruning and Rozema 2013). Many genera of bacteria and archaea are able to fix and transfer the fixed ammonia to plant through the establishment of mutualistic symbiosis (Sprent and Sprent 1990). The most important $N_2$-fixing agents in agricultural system are the symbiotic associations between legumes and the group of soil bacteria collectively designated as rhizobia. It is estimated that 88 % of legume species examined form $N_2$-fixing nodules with rhizobia, being responsible for up to 80 % of the BNF in agricultural soils (De Faria et al. 1989).

The first symbiosis took place around 58 Mya when the *Papilionoidea* (a subfamily of the *Fabaceae*) underwent genome duplication (WGD) (Young et al. 2011). This early WGD is important for legume research, as it indicates that many genes in the model and agronomic papilionoid legumes exist as paralogous duplicates (although nowadays they have independently evolved for ≈55 Mya) and perhaps have acquired distinct and new functions (Cannon 2013) such as the communications with rhizobia, thereby enabling the legumes to start this almost unique symbiotic relationship. As such, the WGD seems to have had a crucial role in the success of papilionoid legumes, enhancing their utility to humans (Young et al. 2011).

The rhizobia induce the formation of nodules in the legumes that involves an exchange of molecular signals between the symbiotic partners (host plant and microbe) to communicate with each other. Secondary plant metabolites, mainly flavons or isoflavons, are recognized by compatible rhizobia via chemotaxis. The rhizobia approaches to the root while it induces the expression of several genes involved in the synthesis and secretion of lipochito oligosaccharides NOD factors (NFs). These molecules share a "backbone" structure consisting of two to six β1-4-linked *N*-acetyl glucosamine residues, with a fatty acid amide linked to the nonreducing terminal residue (Lerouge et al. 1990; Spaink et al. 1991). NFs from different *Rhizobium* species differ in the number of *N*-acetyl glucosamine residues, the length and saturation of the acyl chain, and the nature of modifications on the basic backbone (sulfate, acetate, fucose, etc.) (Oren 1999). The NF perception leads to physical attachment of rhizobia to the root hairs. It induces a plasma membrane depolarization, intra- and extracellular alkalinization, ion fluxes, and calcium spiking, triggering several early symbiotic responses in root hair growth ("curling") (Brewin 1991). NFs also provoke infection thread that guides the bacteria to the emerging nodular primordium (Oldroyd and Downie 2008). In addition, NFs induce nodulin gene expression on cortical cells, leading to the nodular organogenesis (Radutoiu et al. 2003; Cooper 2007). In the nodule primordium, bacteria are released inside the infected cortical cells by an endocytosis process. Later, it is encapsulated by a membrane of the host plant, forming a symbiosome (Oldroyd et al. 2011). These symbiosomes work as plant organelles and they are responsible for $N_2$ fixation and membrane exchange of metabolites (Udvardi and Poole 2013).

In these symbiosomes, the rhizobia can take up gaseous $N_2$ from the atmosphere and fix it through the nitrogenase to form ammonia or amino acids. Then, it can be transported to the upper parts of the host plant either as amides (mainly asparagine, but also glutamine) or as ureides (Schubert 1986). In return, the plant provides to rhizobia with a carbon (C) source in the form of dicarboxylate (Soussi et al. 2001). The enzyme responsible for the $N_2$ fixation, nitrogenase, is irreversibly damaged when exposed to $O_2$. The plant induces three processes to produce a microaerobic environment around $N_2$-fixing rhizobia in nodules: (1) a barrier to gaseous diffusion in the nodules, outer cell layers limit the rate of $O_2$ influx to the central infected tissue; (2) bacteroids and plant mitochondria, with their high respiration rates, consume $O_2$ as fast as it can enter in the nodules; and (3) plant hemoglobins, which have high affinity to bind $O_2$ in the cytoplasm and rapidly deliver it to mitochondria and bacteroids of nodule cells (L'taief et al. 2007). Leghemoglobins are the most abundant plant proteins in nodules. Steady-state concentrations of free $O_2$ in the infected zones of legume nodules are typically in the tens of nanomolar, approximately four orders of magnitude lower than equilibrium levels in water (Udvardi and Poole 2013).

## 2 Impact of Salt Stress on Rhizobial Biology

### 2.1 *Free-Living Bacteria*

Rhizobia are a group of diazotrophs, most of which belong to the α-proteobacteria. It includes the genera *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, and *Azorhizobium* (Weir 2008). Other non-rhizobial genera showed nodules as *Methylobacterium*, *Devosia*, *Ochrobactrum* (Velázquez et al. 2010; Graham 2008; Rivas et al. 2009), *Phyllobacterium*, and *Shinella* (Trujillo et al. 2005; Valverde et al. 2005). Some nodulating bacteria within the genera *Burkholderia*, *Cupriavidus*, and *Herbaspirillum* (β-proteobacteria) class have also been described (Masson-Boivin et al. 2009).

The sensitivity of different rhizobial species and strain to salt stress varies considerably and some of these bacterial strains can persist and survive in saline soils (Zahran 1999). The growth of many rhizobia was inhibited by 100 mM NaCl, while others (e.g., *S. meliloti*) were able to grow in the range of 300–700 mM NaCl (Talibart et al. 1994). However, the majority of rhizobia are not capable of tolerating the harmful effects of high osmolarity (Talibart et al. 1997). It has been showed that rapidly growing rhizobia (24–48 h) are more tolerant than rhizobia of slow growth (more than 96 h) (Zahran 1999).

Morphological alterations of bacteria under salt stress have been reported. These include the expansion of cell size, distortion of cell envelope, and change of rhizobial cell structure (Vanderlinde et al. 2010). Besides the surface components, several symbiotic bacteria may suffer additional changes in salty environment: alterations such as decrease in the synthesis of exopolysaccharides (Lloret et al. 1998; Vanderlinde et al. 2010), changes in the length of the side chain of lipopolysaccharide (Soussi et al. 2001), and deletion of periplasmic oligosaccharide involved in bacterial osmotic adaptation (Ghittoni and Bueno 1995).

Salty conditions induced different changes in protein profiles on different rhizobia. Laranjo and Oliveira (2011) detected that tolerant strains *M. huakuii*, *M. plurifarium*, and *M. thiogangeticum* could grow under 1.5 and 3 % NaCl concentrations. These bacterial strains overproduce proteins with molecular weights of 40–85 kDa, which may be related to salt stress tolerance. Interestingly, several previously reported salt stress proteins include high-molecular-weight proteins, such as the alanine-tRNA synthetase (Nogales et al. 2002), some chaperones (Domínguez-Ferreras et al. 2006), and the $N_2$ regulation proteins (Nogales et al. 2002). Several high-molecular-weight proteins were overproduced under salt stress, mainly in *Mesorhizobium* spp. For instance, this phenomenon has been described in *M. ciceri* (Soussi et al. 2001) and *Rhizobium* sp. (Hastenrath 1991). The appearance of salt stress proteins (SSPs) of low molecular weight after a specific period of time enabled us to speculate that it is quite possible that at preliminary stages of stress, some other mechanisms of salt tolerance are involved (Saxena et al. 1996). The consistent detection of some proteins only after growth under stress may indicate that they were de novo synthesized, suggesting their importance in the survival and growth of *Mesorhizobium* spp. in stress conditions (Guasch-Vidal et al. 2013).

Plasmids are important genetic components for the divergence and adaptation of microbial populations because they contribute to genomic plasticity (Zhang et al. 2001). The correlation between the selection pressure caused by stress and existence of the same plasmids suggests that plasmid plays a major role in the adaptation to environmental stress (Lakzian et al. 2002). Plasmid-mediated salt resistance may be ecologically important since resistance can be rapidly transferred from resistant bacteria to sensitive bacteria; thus extrachromosomal genes can contribute to survival in saline soils (Pereira et al. 2008). Domínguez-Ferreras et al. (2006) showed that plasmid SymB contains a large number of genes upregulated after an osmotic upshift which may have an active role in the osmoadaptation of *S. meliloti*.

Salt stress induces ionic and osmotic stress in rhizobia, through the change in the concentration of solute around cells, producing water deficit and drying the cell (Zahran 1991). Survival and growth in saline environments are the result of adaptive processes, such as ion transport and compartmentation, and osmotic solute synthesis and accumulation. This leads to osmotic adjustment and protein turnover for cellular repair (Munns and Termaat 1986). Under these conditions, rhizobia should maintain turgor positive across the membrane, allowing accumulation of ions. Past experiments have shown an increase in potassium ($K^+$) levels during early stages of salt stress (Saxena et al. 1996). Under elevated salinity, $K^+$ ions can also be accumulated in some species of rhizobia (Young et al. 2011).

Other mechanisms for osmotic adaptation when rhizobia is exposed to salt stress are through the production, transport, and compartmentation of compatible solutes or synthesis and intercellular accumulation of other organic compounds of low molecular weight (osmolytes) (Botsford and Lewis 1990). The osmolytes lead to osmotic adjustment (Munns and Termaat 1986) including higher intracellular concentration of carbohydrates, such as sucrose, trehalose, and maltose (Wu et al. 2008), and amino acids, such as free glutamate (Le Rudulier and Bernard 1986; Cordovilla et al. 1995a), proline (Soussi et al. 1999), glycine-betaine (Guo et al. 2014), ectoine (Dong et al. 2013), as well as a dipeptide *N*-acetyl-glutaminyl glutamine amide (Shavrukov 2013) and intracellular accumulation of polyamines (PAs) (López-Gómez et al. 2014a).

It was postulated that trehalose and glycine-betaine are accumulated to prevent starvation rather than as osmotic stabilizers (López-Gómez and Lluch 2012; Oren 1999). Osmoregulation is the main strategy employed by rhizobia to cope with salt stress (Ghittoni and Bueno 1995). Trehalose (α-D-glucopyranosyl-(1 → 1)-α-d-glucopyranoside) is a nonreducing disaccharide. Trehalose biosynthetic and degradation pathways are widespread throughout bacteria, archaebacteria, fungi, and plant (Avonce et al. 2006). Bacteria are widely distributed among different genera such as *Streptomyces* (Martín et al. 1986), *Mycobacterium* (Elbein and Mitchell 1973), and *Corynebacterium* (Shimakata and Minatogawa 2000). This disaccharide has a structural role as component of the cell wall. It has also been found in other bacteria including *Rhizobium* spp. (Maruta et al. 1996) where trehalose can constitute the sole C source, be used as a compatible osmolyte, or form part of the cell wall structure. *S. meliloti* accumulates a number of osmolytes which help it to deal with osmotic stress, including trehalose, *N*-acetylglutaminylglutamine amide, and glycine-betaine. The glycine-betaine is one of the most powerful osmoprotectants stimulating *S. meliloti* growth in high-salt media (Le Rudulier and Bernard 1986). Glycine-betaine is typically imposed from the environment or synthesized from choline using a pathway encoded by the chromosomal *betCBA* operon. Yurgel et al. (2013) suggest than truncated betB (glycine-betaine aldehyde dehydrogenase BADH) stimulates glycine-betaine catabolism preventing it from being used as an osmoprotectant. *S. meliloti* can use glycine-betaine either as a source of C and $N_2$ or as a cytoplasmid osmolyte, depending on the osmolarity of its growth medium (Barra et al. 2006).

## 2.2 Signaling Exchange

A complex series of events coordinated by host and bacterial signaling underlie and lead to the development of symbiotic interaction. Rhizobia respond to flavonoids exuded by the leguminous roots by secreting NFs. These are the molecular key signal to trigger the nodulation program in a compatible host (Bruning and Rozema 2013). This signal exchange can be negatively affected by salinity (Miransari and Smith 2009; Oldroyd and Downie 2004).

Moreover, salt stress also increases the biosynthesis of NFs and alters their structure with a great number of different new biologically active NFs being generated (Estévez et al. 2009). A peculiar bacterium is *Rhizobium tropici* CIAT 899, which can survive in the presence of high concentration of $Na^+$, by enhanced nod gene expression and NF biosynthesis. This effect is $Na^+$ specific because high potassium or chloride concentration did not have it. Under salt stress conditions, 14 different new NF structures were identified which were not observed as being produced under neutral or acid conditions (Estévez et al. 2009). Major NF-triggered responses in root hair deformation involved several changes as intra- and extracellular alkalinization, phosphatidic acid and diacylglycerol formation, and accumulation of reactive $O_2$ species (Mulder et al. 2006; Cooper 2007).

Guasch-Vidal et al. (2013) observed that, in the absence of flavonoid inducers, high concentration of NaCl induced nodulation genes (*nod* genes) and the production of NFs. The higher transcriptional activity of the *nod* operon in the presence of NaCl was revealed by the increasing of the β-galactosidase activity of a *nodP*:*lacZ* fusion. It leads to a higher and detectable production of NFs. There have been a number of previous evidences for preincubation of rhizobia with exogenous oxylipin (NF inducers). It significantly alleviates the stress effects of salinity on *nod* gene expression, enhancing thereby nodulation and $N_2$ fixation on fenugreek (*Trigonella foenum-graecum*) (Abd-Alla et al. 2014). In common bean (*P. vulgaris*) inoculated with *R. tropici* or *R. etli* under salt stress, it shows a negative effect on the expression of *nod* genes (Dardanelli et al. 2008). Preincubation of *B. japonicum* with the molecular signal genistein (NF inducers) was described as a method to alleviate the stressful effects of high salinity on soybean-*B. japonicum* symbiosis (Miransari and Smith 2009). In addition, the pretreatment of *B. japonicum* with genistein increased nodulation and $N_2$ fixation of soybean and common bean (Abd-Alla 2011). Preincubation of *R. leguminosarum* with hesperetin and naringenin was found to stimulate nodulation and accumulation of plant dry matter in pea and lentil plants (Begum et al. 2001).

## 3 The Metabolic Modifications of Nodule $N_2$ Fixation Under Salt Stress

Legumes can improve saline soil fertility and help to reintroduce sustainable agriculture to these kinds of lands (Crespi and Gálvez 2000). Saline habitats are N poor (Sprent and Sprent 1990) and one of their sources of N input is $N_2$ fixation by legumes (Zahran 1991). Higher rates of $N_2$ fixation in saline soils compared to nonsaline and agricultural soils were reported (Wollenweber and Zechmeister-Boltenstern 1989). The low oxygen tension in saline soils may favor the process of $N_2$ fixation. However, the diffusion of gasses may be impaired at a higher density and water regime in saline soils, and this might reduce $N_2$ fixation (Rice and Paul 1971).

The effect of salinity on growth and legume nodulation depends strongly on the concentration of NaCl, climatic conditions, soil properties, and growth stage of the plant (Cordovilla et al. 1995a; Flowers 2004). Unfortunately, most crop legumes are on the glycophyte side of the salt tolerance spectrum. Main cereal crops as well as leguminous species are quite sensitive to saline conditions (Cabot et al. 2014). The leguminous plants are classified as sensitive or moderately sensitive (i.e., show 80 % biomass production as compared to nonsaline conditions with 3–6 $dSm^{-1}$ or 30–60 mM NaCl, respectively) (Maas and Hoffman 1977). Some legumes are very sensitive to high salinity, such as *M. truncatula*, *M. sativa*, or *P. sativum*, while other legumes, such as *V. faba*, *G. max*, *P. vulgaris*, or *A. hypogaea*, are more salt tolerant (Läuchli 1984). In general, rhizobia are more salt tolerant than their respective host plant (Manchanda and Garg 2008), but the functional symbiosis is even more sensitive to salinity than either partners is (Mudgal et al. 2010). A review by Zahran (1999) stated that “the best results for symbiotic nitrogen fixation under salt stress are obtained if both symbiotic partners and all the different steps in their interaction (nodule formation, activity, etc.) resist such stress.”

The process of nodule formation is particularly sensitive to salt stress, specially the initial phases (Ikeda 1994) (Fig. 1). Under salt stress, the curly hair’s root and bacterial colonization are inhibited, infection rate is greatly reduced (Fougère and Le Rudulier 1990), and the weight and number of nodule formation are decreased (Manchanda and Garg 2008). Microscopic observations showed that salinity (1) caused the loss of turgor of the nodule peripheral cells, disintegration of the cytoplasm, and loss of rigidity of the cell wall; (2) changed nodule zonation with a decrease in the volume of intercellular spaces; (3) stimulated infection thread enlargement and expansion; (4) caused disturbances in bacterial release from the infection threads; (5) caused the occurrence of variations that lobulated nuclei and chromatin condensation; and (6) induced synthesis of electron-dense material (EDM) and its deposition in vacuoles (Nielson and Griffith 1978). It was also found that cisternae of rough endoplasmic reticulum were involved in the formation of special cytoplasmic compartments responsible for synthesis of EDM (Borucki and Sujkowska 2008). Autofluorescence studies revealed that salinity also increased accumulation of phenolics in pea nodules (Padilla et al. 2013).

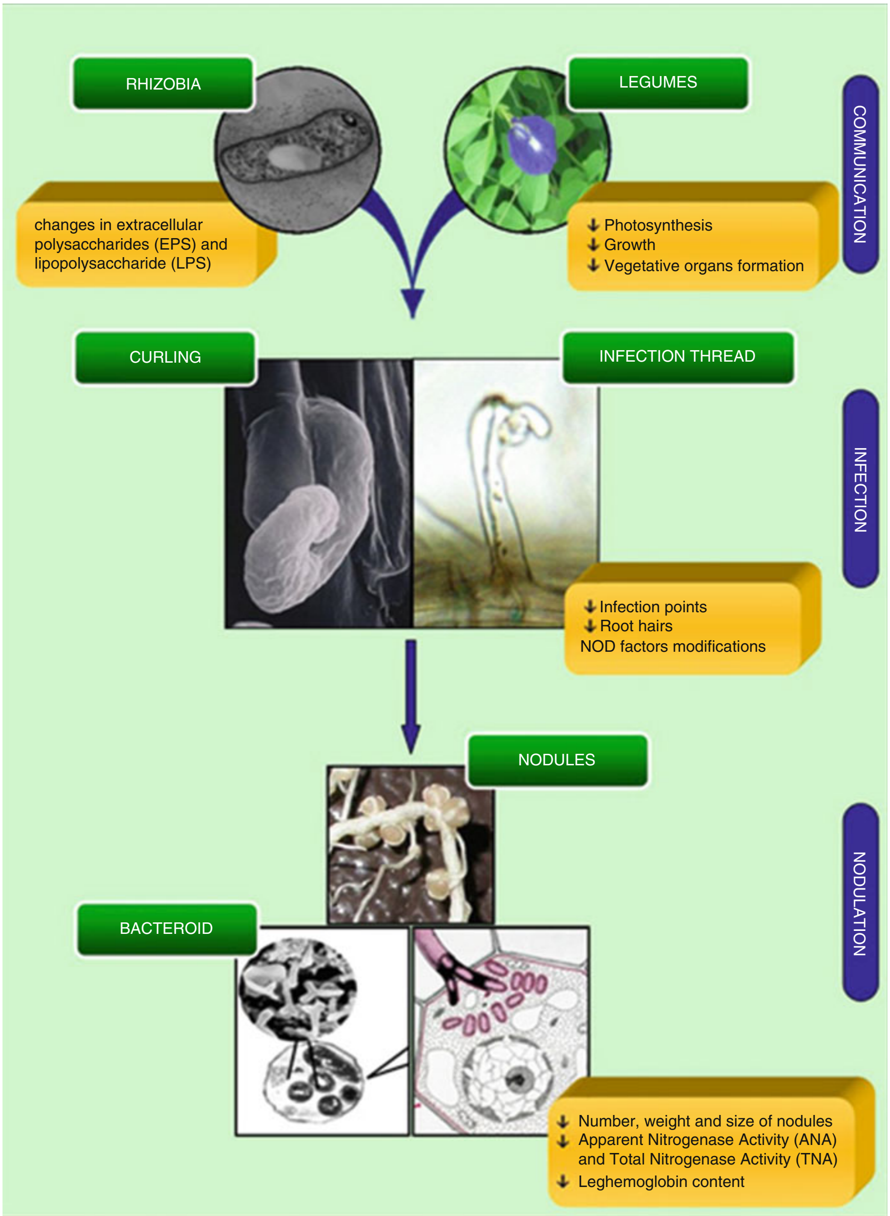

**Fig. 1** Effects of salt stress in different stages of symbiotic process

High salinity affects plant growth, especially shoot growth, and thereby symbiotic relations in legumes (Tejera et al. 2004; López et al. 2008). The negative effect of salinity on *Rhizobium*-legume symbiosis is reflected in inhibition of specific nitrogenase activity. This activity is conditioned by the reduction of the protein leghemoglobin, by oxidative stress and by carbohydrate contents of both the cytosol and the bacteroids (Delgado et al. 1993). Salt stress can also be attributed to reducing C supply to bacteroids, mainly in the form of malate limitation, likely as a result of the salt-induced inhibition of sucrose synthase activity and phosphoenolpyruvate carboxylase. This suggests that under low salinity, host growth is slightly reduced growth of the host (Delgado et al. 1994; Soussi et al. 1999). The supply of $O_2$ to the nodules is a limiting factor. Salt tolerance was associated with higher stability of $O_2$ nodule conductance of the tolerant rhizobia when increasing soil salinity (L'taief et al. 2007).

## *3.1 Mechanisms of Salt Stress Tolerance*

Salinity imposes at least three primary stresses on plants. The first is a hyperosmotic stress caused by the reduction of water potential of the external soil solution. This solution can exceed their osmotic potential of the plant cell, reducing the water availability for the plant. The second consist in a hyperosmotic stress related to the toxic effects of the accumulated ions, specially $Na^+$ and $Cl^-$ that produce a nutritional imbalance (Munns and Tester 2008). The third effect stems from the generation of reactive oxygen species (ROS). The combination of all of these factors causes adverse effects on plant growth and development at physiological, biochemical, and molecular levels (Coba de la Peña et al. 2010).

### 3.1.1 Synthesis of Compatible Solutes

The presence of salt in the growth media often results in the accumulation of low-molecular-mass compounds, termed as compatible solutes or osmoprotectants. These osmoprotectants do not interfere with normal biochemical reactions (Zhifang and Loescher 2003). These compatible solutes are nontoxic and they have low-molecular-weight substances which include mainly simple sugars, disaccharides, sugar alcohols or polyols, amino acids, and sulfonium compounds (Ben Salah et al. 2009). The accumulation of compatible osmolytes works as an osmotic adjustment (raise osmotic pressure) to counteract the high concentration of inorganic salts in the vacuole and the root medium (Zhu 2001). Another function of these accumulated compounds under salinity stress is to act as an energy sink or reducing power, such as C and $N_2$ source, or scavenging ROS (Sairam et al. 2005). Additionally, these compounds also protect some structures against denaturation (Trinchant et al. 2004).

*Sucrose* is supplied to the nodule via phloem, and its metabolism leads to the synthesis of dicarboxylic acids. These are the main products of sucrose degradation supplied to bacteroids to support $N_2$ fixation in legume nodules (Ghittoni and Bueno 1995). However, sucrose metabolism is inhibited under salt stress resulting in a lower supply of dicarboxylic acids (as respiratory substrates) by bacteroids (Soussi et al. 2001). Bacteroids isolated from nodules of pea plants have the respiratory capacity diminished when they are exposed to salt stress. It is associated with a lower content of malate in the nodule cytosol, and it can be reversed by the addition to the incubation medium of exogenous respiratory substrates, such as malate, succinate, proline glutamate, or lactate (Delgado et al. 1994; Ferri et al. 2000). The increase in total soluble sugars has been frequently related to osmoregulation process (Zhu 2002).

*Trehalose* was described in 1980 as the first major carbohydrate in soybean root nodules (Streeter 1985). In later studies with senescent nodules, trehalose becomes the most abundant carbohydrate. The sucrose content (84 %) is reduced during the nodule senescence, while the amount of trehalose remains constant (Müller et al. 2001). The role of trehalose in legume root nodules under salt stress conditions has been studied by the addition of the trehalase inhibitor validamycin A. Validamycin A caused an increase in the amount of trehalase that improved the response to salinity by increasing the biomass production of species as *M. truncatula* (López et al. 2009) and *L. japonicus* (López et al. 2006). In addition, salt stress increased (especially in roots, bacteroids, and nodular trehalose concentration) about 40–100 % in the legumes *L. japonicus* and *M. truncatula*, respectively. These data support a role for trehalose acting as osmoprotectant under stress conditions in the rhizobia-legume symbiosis (López-Gómez et al. 2013; Domínguez-Ferreras et al. 2009). Barraza et al. (2013) propose that breeding for the genetic modification or trehalose degradation in nodules could be a valuable strategy for improving symbiotic $N_2$ fixation.

Transgenic plants that accumulate high levels of *proline* are reported to display increased tolerance to salt and osmotic stress (Kishor et al. 2005). *M. sativa* is one of a few plants, which accumulates simultaneously large amounts of both proline-betaine and proline as osmoprotectant solutes. Proline-betaine within the nodules can significantly alleviate (directly or indirectly) the negative effect of salt on nitrogen fixation (Pocard et al. 1984). It has been accepted that symbiosome behaves as osmometer and the peribacteroid membrane serves to maintain and accommodate changes in osmotic pressure (Ouyang and Day 1992). Bacteroids isolated from *P. vulgaris* root nodules inoculated with *R. tropici* strain CIAT 899 could use proline or lactate for bacteroidal respiration after exposure of plant to salinity (Ferri et al. 2000). Under saline stress, proline levels increase in soybean nodules resulting in high ratios of NADP/NADPH and thereby the activation of the pentose phosphate pathway and the eventual production of purine (Miransari et al. 2013). The purine derivates can act as transporters of fixed $N_2$. Proline can be a transporter of redox reaction product from plant cytoplasm to the bacteroids. It has been verified in the bacteroids of root nodules by the high activity of proline dehydrogenase (ProDH) (Kohl et al. 1990). Within the amino acids, proline shows the largest increase in the nodular tissue of salt-stressed *M. truncatula* and *L. japonicus* plants (López-Gómez et al. 2012).

Salinity induces expression of osmoprotectant-related genes, one of which has been shown to be involved in *pinitol* synthesis (Szabolcs 1994). Pinitol proved to be another carbohydrate that accumulates in nodules of *L. japonicus* plants subjected to salt stress (López-Gómez et al. 2012). Previously, this polyol was described as a compatible solute in plants (Obendorf et al. 2008) and a major carbohydrate (up to 50–60 % of soluble sugar) in soybeans under water stress conditions (Streeter et al. 2001). Palma et al. (2013) suggested that higher increase of pinitol synthesis in nodules of *M. sativa* could be one of the adaptive features used by the plants under salt stress.

### 3.1.2 Ionic Homeostasis

The legumes usually respond to salinity by excluding $Na^+$ and $Cl^-$ ions. In fact, it is thought that salt tolerance in legumes is related to their ability to avoid salt ion accumulation (Läuchli 1984). The relationship between salt tolerance and macronutrient accumulation in legumes was reported in 1995 by Cordovilla et al. (1995b). Later, NaCl tolerance associated with retention of $Na^+$ and maintenance of $K^+$ selectivity seemed to be a strategy used against the salt stress in common symbiotic bean plants (Tejera et al. 2005). Tejera et al. (2006) suggested that maintenance of high tissue $K^+/Na^+$ ratio is a criteria for salt tolerance. The capacity to accumulate $Na^+$ in the root might be related to a protection of the photosynthetic apparatus from $Na^+$ damage (Soussi et al. 1998). The salt effect in nodule functioning can lead to toxic accumulation of ions such as $Cl^-$ and, in particularly, $Na^+$ ions in the cytosol (Fernández-Pascual et al. 1996). Differential distribution of these ions across nodule cell layers (infected and uninfected cells) of two lines of faba bean has been described (Abd-Alla et al. 2001). Unless ions are stored in vacuoles, $Na^+$ and $Cl^-$ are concentrated in compartments such as the cytoplasm. Here ion homeostasis needs to be controlled in order to avoid damage of cellular components, inhibitory effects on cell metabolism, and disturbance of enzymatic activity (Cabot et al. 2014). The disproportionate presence of $Na^+$ (in both cellular and extracellular compartments) negatively impacts on the acquisition and homeostasis of essential nutrients such as $K^+$ and $Ca^{2+}$. Indeed, high levels of $Na^+$ inhibit the $Ca^{2+}$ and $K^+$ absorption which results in an antagonium cation. $Na^+$ and $K^+$ are known to be of the osmotic potential (Asch et al. 1999). $K^+$ is a vital contributor to effective FBN (Høgh-Jensen 2003) and $Ca^{2+}$ signaling is critical for the establishment of a successful plant-microbe symbiosis (Garg and Geetanjali 2007).

### 3.1.3 ROS Scavenging

Salinity, but not osmotic stress, affects apoplastic and intercellular production of ROS, inhibiting root curling and inducing root hair death (Muñoz et al. 2012). Moreover, salt stress affects negatively physiological process in legume-nodulating plants as stomatal conductance, the maximum quantum yield of photosystem II, and total chlorophyll content (Latrach et al. 2014). Some of these effects may involve the oxidative damage of cellular components although they could be prevented by antioxidants. Nodules have a high potential of ROS production due to the elevated rates of bacteroid respiration, the highly reducing conditions required for $N_2$ fixation, as well as the presence of many electron transfer components, including ferredoxin, uricase, and hydrogenase (Dalton et al. 1998). However, ROS production has been shown to play a key role in the rhizobia-legume interaction and maintenance of the symbiosis between the plant and the bacteria (D'Haeze et al. 2003).

Oufdou et al. (2014) suggest that the salinity effect on the ascorbate-glutathione cycle enzymes depended strongly on the rhizobia strain used for inoculation of the faba bean plants. This specific rhizobia strain involved in nodulation appears to play a role in the changes of enzyme activities against the NaCl stress. Ascorbate and glutathione are molecular antioxidants that play important functions on plant growth and stress responses. Both antioxidants can be found in nodules at concentrations ranging from 0.5 to 2.0 mM, which is consistent with its multiple and essential functions (Dalton et al. 1998). They are potent water-soluble antioxidants, acting as direct ROS scavengers, and as ascorbate-glutathione pathway metabolites from hydrogen peroxide detoxification. Although nodule host cells synthesize their own glutathione, some amounts of this critical antioxidant needs to be produced by the bacterial partner to achieve optimal $N_2$ fixation. This is evident because when glutathione (GSH; γ-glutamyl-cysteinyl-glycine) is deficient in rhizobia, it forms nodules with early senescence and diminished symbiotic performance (Muglia et al. 2008).

Under salt stress, $N_2$ fixation and antioxidant enzyme activities in nodules were affected: in *P. vulgaris* catalase (CAT) and ascorbate peroxidase (APX) were inhibited by salt stress (Tejera et al. 2004), whereas superoxide dismutase (SOD) and peroxidase were activated (Jebara et al. 2010). Another study in *P. vulgaris* and *M. sativa* nodules indicated an inhibition of the nodule CAT activity when plants were exposed to NaCl (Tejera García et al. 2007). This inhibition was proportional to the $N_2$ fixation, confirming the correlation between CAT activity and the functioning of symbiosis (Mhadhbi et al. 2004). The protective role of antioxidants in salinity conditions was investigated in *L. japonicum*. This species has several markers of salt stress and it got more tolerant with the expression of antioxidant genes. The result indicates that *L. japonicum* is more tolerant to salt stress than the other legumes which can be attributed to the capacity of the plant to prevent $Na^+$ reacting the shoot and to activate antioxidant defense (Rubio et al. 2009).

### 3.1.4 Other Molecules Involved

The study of the salt stress effects on the growth of nodulated plants and thus, on their ability to fix $N_2$, has been of considerable interest to analyze the interaction and the alterations of signaling molecules. Molecules, such as indole-3-acetic acid (IAA), PAs, salicylic acid (SA), and abscisic acid (ABA), have been researched in symbiotic plant responses. IAA is expressed in both free-living bacteria and bacteroids, and the IAA overproducing rhizobia strain can accumulate a higher level of trehalose (Bianco and Defez 2009). Bianco and Defez (2009) suggest a different system to enhance salt tolerance. They found evidences about overexpression of IAA in *S. meliloti* 1021. This strain can accumulate a higher level of trehalose and play a positive role in the adaptation to osmotic stress.

PAs are polycationic compounds widespread in many organisms and particularly in plants. They have been implicated in the regulation of many physiological processes and stress responses. Spermidine (Spd), spermine (Spm), and putrescine (Put), for example, have been accumulated under abiotic stress conditions (Bachrach 2010). Hernández-Lucero et al. (2008) studied the effect of salt stress on the expression of many genes involved in PA biosynthesis, including *S*-adenosylmethionine decarboxylase (SAMDC) and in common bean cultivars, and suggested that Spm accumulation might be part of the mechanism conferring salt tolerance. Similar results were described by Echeverria et al. (2013). These authors found that Spd and Spm contributed to salt-induced root PA increment in roots of nodulated plants. This suggests that these PAs might mediate an adaptative role to salinity of *M. tianshanense* symbiosis with *L. tenuis* plants. Recently, López-Gómez et al. (2014a) have found homospermidine (Homspd) as the most abundant PA in nodules of *P. vulgaris*-*R. tropici* symbiosis. The presence of 4-aminobutylcadaverine (4-ABcad) was only described before in nodules of *V. angularis*. Both PAs have been detected in bacteroids which indicate the production of these compounds by the bacteria in symbioses. These results indicated that bacterial metabolism alteration towards the production of uncommon PAs (such as 4-ABcad) is one of the mechanisms to tolerate salt stress in the rhizobia-legume symbiosis. However, in *M. sativa*–*S. meliloti* symbiosis, the PAs did not accumulate in nodules during the initial response to salt treatment while proline accumulation has prevalence over PAs at the earliest response to salinity. This is due to $N_2$ limitation under salt stress conditions and the existence of a common nodular precursor for both compounds (López-Gómez et al. 2014b).

Some endogenous low-molecular-weight molecules, called hormones, can regulate protective responses of salt stress in symbiosis. ABA is one of the key hormones regulating plant responses to abiotic stress (Addicott et al. 1968). Under salinity stress, the ABA content increases in *P. vulgaris* and *M. ciliaris* nodules (Ben Salah et al. 2011). Some reports have demonstrated the relation between ABA and proline nodular accumulation (Khadri et al. 2007) and PA metabolism (Ben Hassine et al. 2009) under salt stress. They suggest a role for ABA as a signal involved in the maintenance of the cellular PAs. ABA pretreatment improved the $N_2$ fixation capacity under salt stress conditions by the induction of the nodular antioxidant defenses which may be mediated by the common PAs Spd and Spm that seem to be involved in the antistress responses induced by ABA (Palma et al. 2014).

SA is a water-soluble phenolic compound which can regulate plant growth and has an important role in biotic and abiotic stresses (Palma et al. 2014). SA treatment negatively affected the responses of pea plant to NaCl and this is correlated with an imbalance in antioxidant metabolism (Barba-Espín et al. 2011). Exogenous SA application inhibited the growth of rhizobia and the production of NFs, and reduced the number of total nodules (Mabood and Smith 2007). When the *nahG* gene was expressed in *L. japonicum*, a correlation between reduction of SA level and number of infection was observed. *nahG* gene encodes a SA-hydrolase that degrades SA to catechol (Stacey et al. 2006). It has been reported that SA mitigates the adverse effect of salinity (Nazar et al. 2011). Other reports have demonstrated that the application of SA may promote the ROS formation during salt and osmotic stresses (Nazar et al. 2011). The mechanisms by which SA enhances $H_2O_2$ content are not clear. It could involve inactivation of removing enzymes by this compound as described in bean by Palma et al. (2009) and/or activation of SOD enzyme as reported by Khan et al. (2010). Manipulation of hormone level and/or its signaling could be a useful strategy for selecting/improving legume plant growth and FBN under salt stress condition (Ben Salah et al. 2011) (Fig. 2).

**Fig. 2** Damage and responses induced by salt stress in rhizobia-legume symbiosis

## 4 Application of Biotechnology for Improved $N_2$ Fixation Under Salinity

Identification of mechanisms regulating salt tolerance in plant species has become a priority of research in several countries in order to efficiently develop salt-tolerant crops. It is important to use ecophysiological approaches (Mekhaldi et al. 2008) that can provide an alternative to the attenuation of the soil salinity effect on crop plants. This will enable us to identify salt-tolerant species or varieties to expand our knowledge on salinity adaptation mechanisms. The adaptability of plants may be related to constitutive expression of genes encoding protein synthesis and osmoprotective molecules (Cushman et al. 1990). Changes in the concentration of proteins under salt stress may suggest new lines of research into the molecular mechanisms of salt stress adaptation.

Knowledge of the molecular and physiological basis of plant-microbe interactions and their responses to abiotic stress is of vital importance. It can lead to better and more efficient $N_2$-fixing culture. Recent advances in nodule proteomics, metabolomics, transcriptomics, and fluxomics have provided novel information concerning nodule functioning within a salinity environmental context (Aranjuelo et al. 2014).

### *4.1 Genomics Approach*

Over the last 20 years, many genes that confer salt tolerance were introduced into various crops and test plants (Sun et al. 2013). Traditionally, improved tolerant legumes have been chosen using crosses for higher adaptation to stressed environments and novel methodologies, as omics technology. It has enabled significant progress on C and N exchange understanding between plants and nodules (Aranjuelo et al. 2013). Actually, biochemical tools and cellular and molecular biology are used to modify rhizobia legumes or to make them more resistant to adverse conditions. To make use of genetic engineering, molecular and physiological processes must be studied in stressful situations: circumstances where the plant requires toleration or damage mitigation machinery (Bhatnagar-Mathur et al. 2008). The genome mapping techniques are accelerating the accurate gene position and function identification, genes controlling agronomic traits such as salinity tolerance. The range and accuracy of current breeding programs are enhanced through the use of linked markers with the desirable alleles, such as targeted quantitative trait loci (QTLs) and marker-assisted selection (MAS).

QTLs are stretches of DNA containing or linked to the genes that underlie a quantitative trait. By integrating physiological and genetic strategies, one can get a better understanding of the molecular basis of adaptation of crops to improve abiotic stress tolerance in plants. QTL mapping is revealing genetic components of salt tolerance for genetic improvement of existing varieties (Karan and Subudhi 2012). QTL is associated with salt tolerance at various stages of plant development. It is needed to obtain a more stable plant performance in areas affected by high salinity (Turan et al. 2012). Numerous molecular marker-related techniques have been used in legumes in relation to abiotic stresses. Random amplified polymorphism (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and derivatives have been reported to be used for abiotic stress studies (Lee et al. 2004; Kassem et al. 2004).

MAS is the use of molecular markers linked to useful traits to select individuals with desirable genetic makeup during the variety development process (Karan and Subudhi 2012). The use of genetic and genomic analysis can facilitate breeding strategies for crop improvement. These analyses help to identify regions of DNA tightly linked to agronomic traits of crops, which are called molecular markers (Varshney et al. 2005). The MAS is the result of combining traditional breeding techniques and molecular biology and allows choosing directly the individuals carrying the genes of interest (Collard and Mackill 2008). Combined with traditional selection techniques, MAS is a valuable tool for selection of traits of interest. Besides backcross introgression of characters (controlled by one or a few genes), MAS is used to accumulate QTLs and make genetic improvement in characteristics encoded by several loci (Karan and Subudhi 2012).

As a result, genetic maps for many species with potential stress resistance-related and/or tolerance-related QTLs have been established (Collins et al. 2008). This improved knowledge of the genetic resistance/tolerance control in many vegetables provides information on the number, chromosomal location, and individual or interactive effects of QTLs involved (Dita et al. 2006). Also, Lee et al. (2004) reported that soybean genotypes that are tolerant to salinity have the same QTL allele (*Sat*091). In addition, it has been suggested that the SSR markers Satt237, Satt339, and Satt255 could be used for MAS in soybean breeding program (Hamwieh and Xu 2008). The use of MAS can be useful for crop improvement, such as selecting the common bean drought tolerant (Schneider et al. 1997). However, in legume breeding, its practical application for resistance or stress tolerance has been usually limited by the genetic complexity of traits associated with stress (Dita et al. 2006).

MicroRNAs (miRNAs) are small (20–24 nucleotides) noncoding RNAs which bind to the coding DNA sequences (CDS) or untranslated regions (UTR) of target genes. As a result, they reduce protein expression by accelerating the decomposition of mRNA or repressing the translation of the target mRNAs (Brodersen et al. 2008). Being sessile organisms, plants have to cope with a constantly changing environment to guarantee their survival and reproductive success. Currently, there are several evidences that make us strongly suggest that miRNAs are involved in abiotic stress. The spatial and temporal patterns of miRNA expression during various biological processes to stress responses demonstrate critical regulatory roles for miRNAs (Jones-Rhoades et al. 2006). Recent reports have revealed important regulatory roles of small RNAs in controlling nodulation and BNF in *M. truncatula* and soybean. MTR-miR169 was found to regulate nodule development through regulation of expression of the transcription factor in *M. truncatula* MtHAP2-1 (Combier et al. 2006).

Therefore, miRNAs are involved in different steps during the establishment of symbiosis and BNF. However, the expression dynamics of *miRNA*s are not known, nor it is clear whether expression of them is specifically regulated in $N_2$-fixing nodules, controlling their BNF efficiency.

## *4.2 Transgenic Approach*

Abiotic stresses generally involve the disturbance of diverse cellular functions, activation of complex metabolic pathways, and polygenic traits conferred by Popelka et al. (2004). This complexity, coupled with the lack of good sources of natural tolerance, makes this an area that is not easily amenable to conventional breeding strategies. The successful use of genetic transformation requires better physiological and molecular understanding of these tensions. Recent advances in understanding the physiological and molecular events and pathways involved in abiotic stress tolerance in legumes, along with advances in the development of gene transfer protocols, have helped to generate transgenic legumes with greater abiotic stress tolerance (Coba de la Peña and Pueyo 2012).

The use of biotechnological methods is among the most effective techniques to improve plant and bacterial symbiotic performance under stress (Miransari et al. 2013). Improvement of BNF by biotechnology can be achieved in different ways. In this part of the chapter, we focus on (1) improvement of the plant and (2) rhizobia tolerance to salt stress with emphases on the mechanisms mentioned above.

### 4.2.1 Improvement of Legumes Under Salt Stress

Genes responsible for osmolyte synthesis would be one of the key gene groups important for genetic engineering due to the increasing of BNF under saline conditions. In transgenic *M. truncatula* plants overexpressing the *Δ1-pyrroline-5-carboxylate synthetase* (*P5CS*) gene, which encodes an enzyme involved in proline biosynthesis, more proline is accumulated in leaves, roots, and nodules, resulting in enhanced tolerance to salt and osmotic stresses, improving BNF (Verdoy et al. 2006). The modification of trehalose content in *Rhizobium* is another approach. This modification is due to the overexpression on *Rhizobium* of the *OtsA* gene, which codes for trehalose phosphate synthase (TPS). As a result, expression of several genes involved in N assimilation, C assimilation, $O_2$ transport, and $H_2O_2$ or ROS detoxification was affected in common bean nodules during the symbiotic interaction. It gave rise to improvements in grain yield, $N_2$ fixation, and stress tolerance (Suárez et al. 2008). Suárez et al. (2008) demonstrated that the expression of a bifunctional yeast TPS1-TPS2 in transgenic alfalfa plants increased salinity resistance and the nitrogenase activity having a higher number of nodules and infected cells. DREB is a well-characterized transcription factor known to play an important role in regulating gene expression in plant responses to abiotic stresses. Overexpression of the soybean *DREB1* gene in alfalfa produced significantly higher amount of free proline and total soluble sugars, which might contribute to their higher salt tolerance (Jin et al. 2010).

The expression of a cyanobacterial flavodoxin gene in *S. meliloti* resulted in oxidative stress tolerance in alfalfa pellets, which was attributed to the ROS-facilitating detoxification and protection against oxidative damage. Flavodoxin overproduction in transgenic *M. truncatula* plants also confers salt tolerance (Coba de la Peña et al. 2010).

Another consequence of salinity in plants is the ion damage, caused by the increase of ions, mainly $Na^+$. Genetic manipulation of genes encoding ion channels, contributing to maintenance of $K^+/Na^+$ ratio, has been very successful to increase salt tolerance in plants (Conde et al. 2011). Overexpression of the *Arabidopsis vacuolar $H^+$-pyrophosphatase* (*AVP1*) gene in peanut plants was able to maintain higher photosynthetic rates and transpiration rates under salt stress as compared with control plants (Qin et al. 2011).

### 4.2.2 Genes That Enhance BNF

The development of bacterial strains tolerant to salt stress is one of the targets for genetic engineering to enhance rhizobial symbiosis with leguminous plants to improve yield and BNF (Zahran 2001). Genes involved in stress responses are highly conserved among bacteria and they codify a large set of proteins (Gottesman et al. 1997). The gene of the molecular chaperonin Hsp60 of *E. coli* (*groEL*) is probably one of the best studied genes. This gene is found in a large number of bacteria, and is required for the proper folding of many proteins (Lin and Rye 2006).

BNF is an energy-consuming process that takes place under microaerobic conditions, since nitrogenase activity is inhibited by $O_2$. Overexpression of the *B. japonicum* terminal oxidase *cbb3* (essential for $N_2$-fixing endosymbiosis) in a *R. etli ntr*C mutant increased $N_2$ fixation (Soberón et al. 1999). Castillo et al. (1999) found that increasing the number of *nodD1* regulatory gene copies in *S. meliloti* strains could significantly improve the activity of nitrogenase, leading to enhanced $N_2$ content in plants and plant growth. Insertion of additional copies of *nifA* and *dctABD* genes in a *S. meliloti* recombinant strain also increased $N_2$ fixation, and thus the yield of alfalfa (Bosworth et al. 1994).

As mentioned above, one of the organism mechanisms used for stress adaptation is the enhancement of the synthesis of osmoprotectants. Boscari et al. (2006) observed that an *S. meliloti* strain overexpressing *betaine transporter* (*betS*), achieving an increase of proline and glycine betaine in bacteroids, resulted in increased $N_2$ fixation of nodulated alfalfa plants.

## 5 Future Perspectives

The BNF is one of the possible solutions of N-source for farmers who do not want to use artificial fertilizer, and plays a key role in the sustainable production of legumes (Miransari et al. 2013). The salt stress causes extensive crop losses in many parts of the world due to lack of salt tolerance in major field crops. Improving salinity tolerance in crops is a major goal of plant breeders to ensure the food supply for a growing world population (Flowers 2004). The combination of the current phenotypic selection and the recent addition of new molecular breeding markers could accelerate the improvement of legumes. Therefore, it is important to take into account individually (1) the genetic variability in the resistance/tolerance to abiotic stresses and (2) genetic variability in the interaction between plant and bacteria (Coba de la Peña and Pueyo 2012). Recently, techniques have been developed for use in MAS breeding programs in legumes. The application of "omics" methodologies, either alone or in combination with more traditional agronomic and physiological studies, may contribute to the elucidation of the mechanisms responsible for plant responses to climate changes. The combination of physiology, transcriptomics, metabolomics, and proteomics will provide key information regarding the plant mechanisms that determine the best or worst performance in a wide range of climate conditions (Ahuja et al. 2010). An interesting perspective on the genetic manipulation is the accumulation of osmoprotectants to improve the salt tolerance. Several candidates have been identified in legumes, such as proline, pinitol, glycine betaine, and trehalose (Shavrukov 2013). The manipulation of genes involved in the metabolism of these compounds has received special attention to increase the accumulation of these osmoprotectants in plant.

Conventional breeding technologies, together with molecular genetic analysis including QTL mapping studies, are revealing important genetic components to improve salt tolerance in field crops (Karan and Subudhi 2012). QTL mapping and MAS have been increasingly used in the effort of the systematic dissection and the use of natural variation in the germplasm available to improve crop yield in saline environments (Collins et al. 2008). In particular, wild crop and land races have enormous potential to remove the upper alleles for improving crop adaptation to salinity (Feuillet et al. 2008). It is expected that genomics technologies will contribute significantly towards discovery of candidate genes for various useful trait components. It can be used to improve elite cultivars using transgenic pyramiding (Takeda and Matsuoka 2008). The utility of transgenic technology can be further improved through the discovery and exploitation of stress-inducible promoters. These promoters could improve salt tolerance with minimal undesirable pleiotropic effects on plant growth, getting a productivity as under normal conditions. However, the collaboration of geneticists, molecular breeders, physiologists, and genomicists is required to implement an integrated approach. It would allow discovering, testing, and integrating superior alleles for improving salt tolerance in major food crops.

In conclusion, salinity tolerance is too complex to be easily amenable for improvement just through selection as a trait itself. However, hypothesized traits to contribute salinity tolerance are genetically more tractable and genes underlying these processes can be discovered using molecular genetics tools and genomics (Roy et al. 2014). This new research would improve the BNF.

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# How Does High Temperature Affect Legume Nodule Symbiotic Activity?

**Iker Aranjuelo, Joseba Aldasoro, César Arrese-Igor, Gorka Erice, and Álvaro Sanz-Sáez**

**Abstract** According to global climate model predictions, environmental conditions such as temperature are going to be altered. Plants will be facing high-temperature conditions that affect their development. Within this context, it is crucial to identify the target processes that influence $N_2$ fixation and crop production under elevated temperature conditions. As it is described in this book chapter, while $N_2$ fixation has been well characterized under other adverse environmental conditions (drought, salinity, elevated $CO_2$ concentration, etc.), very little is known about the effect of heat stress on nodule functioning. While there are a few reports about high-temperature effect on nodule carbohydrate and amino acid contents, there is not any study analyzing oxidative stress in those nodules. Regulation of these three factors is essential for optimized $N_2$ fixation; thus, this is a topic that should be studied in more detail. Available information confirms that high temperature strongly affects $N_2$ fixation and plant growth, especially when plants are exposed to temperature higher than 25 °C. High temperature decreased the growth of plants due to its negative effects not only on plants' photosynthetic performance, but also on nodule growth and development which result in decreased nodule biomass and depletion of nodule total soluble protein content. It is also remarkable that $N_2$ fixation has been showed to be more sensitive to high temperature than photosynthesis. In this chapter, we highlight the variability in performance of various bacterial strains and plant species under high-temperature environments, and discuss about the importance of

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the identification of target plants and rhizobium cultivars to form optimal symbiotic combinations that will be better adapted to predicted climate change conditions. This in turn will enable higher $N_2$ fixation efficiency and consequently plant growth under adverse environmental conditions, including high temperature.

**Keywords** Growth • Nitrogen • Nodule • Photosynthesis • Stress and temperature

## 1 $N_2$ Fixation and Soil Fertilization

The availability and management of nitrogen (N) are major determinants of crop productivity and the associated environmental agro-ecosystems. Soil N content, together with water deficit, is the most important environmental factor limiting plant growth and production (Annicchiarico et al. 2011). Due to the N dependency of plants, synthetic N fertilizer input into global agricultural systems increased by approximately 430 % (≈19 to ≈82 Tg N) from 1965 to 1998 (according to data compiled by the Food and Agriculture Organization of the United Nations (FAO)). The environmental costs of the need for increased yields have therefore become a cause for concern. The abuse of these fertilizers in order to increase plant yield may cause pollution of ground and surface water (rivers, lakes, estuaries, and other inland and coastal waters) through excessive nitrate content (FAO 1996; USGS 2013). In addition, human health problems associated with diets high in nitrates have also been outlined (Ward et al. 2005). It should be noted that as described by the Intergovernmental Panel on Climate Change (IPCC) (http://www.ipcc.ch/), N compounds are one of the main causes of climate change. Furthermore, between now and 2050, the human population is expected to increase from 6 to 10 billion people (United Nations 2013). Therefore, the challenge for the coming decades will be to accommodate the needs of the expanding world population by developing a highly productive sustainable agriculture while at the same time preserving the quality of the environment.

$N_2$-fixing plants represent the second major group of crops worldwide (preceded by grasses), and they cover 12–15 % of the world's cultivated area (FAOSTAT 2002). These legume crops represent an important source of protein and calories for humans and animals (Rogers et al. 2006). In addition, and compared with other cultivars, $N_2$-fixing plants represent a special plant group thanks to their capacity of fertilizing soils through the fixation of atmospheric $N_2$. The fact that these plants have a relationship with symbiotic $N_2$-fixing bacteria gives them access to a large reservoir of natural $N_2$ in the atmosphere. This symbiotic relationship serves leguminous plants by supplying natural N fertilizer through release of the fixed N into the soil. This release is due to exudation and decomposition of roots and nodules. $N_2$ fixation has been described to contribute, at least, 16 % of the global N supply (Liu et al. 2010), with grain and forage legumes being the main contributors of biologically fixed N (Herridge et al. 2008). Furthermore, Burity et al. (1989) found that N transfer between $N_2$-fixing alfalfa plants and non-$N_2$-fixing plants can range from 5 to

20 kg N $ha^{-1}$ $year^{-1}$. The symbiotic $N_2$ fixation (SNF) provides an additional N source to the legumes and the surrounding plants that is of great relevance in impoverished soils like those of the Mediterranean region. As observed by Peoples et al. (1995), the symbiotic relationship is the main source of $N_2$ fixation in terrestrial ecosystems (provides 50 % of SNF) and reduces the need to fertilize soils with chemical compounds, with consequent economic and environmental benefits.

## 2 High Temperature and $N_2$ Fixation

Temperature is one of the most important factors affecting plant growth and thus influences worldwide plant distribution. The temperature range of higher plants is considered to lie between 0 and 45 °C (Hopkins and Hüner 2004) but it is known that legume species have a different tolerance to extreme temperature. Legumes such as common bean (*Phaseolus vulgaris* L.) perform best when grown under temperatures ranging from 15 to 30 °C (Van der Maesen and Somaatmadja 1989) but others like cowpea or soybean have been described as more tolerant to high temperature (Piha and Munns 1987). In the current context of climate change, many countries around the world are predicted to experience an increase in their annual average temperature of 4 °C by the end of the century (IPCC 2013) due to the rising atmospheric $CO_2$ concentration. This phenomenon, together with high solar radiation, can easily cause the leaf surface to reach at least 5 °C above air temperature (Hopkins and Hüner 2004). Within this context, it should be considered that, in addition to direct high-temperature effect in plant-bacteria metabolism, high temperature might also alter plant population dynamics (Bélanger et al. 2005) and phenology (Menzel and Fabian 1999; Chmielewski and Rötzer 2001; Chmielewski et al. 2004).

Plant performance within a climate change context is a major concern, especially in semiarid or tropical regions, where soil temperatures often exceed 40 °C (Dennt 1984; Hafeez et al. 1991) during summer, the main cropping season (Hungria and Vargas 2000). Reductions in SNF have been reported at temperatures just above 28 °C (Hungria and Franco 1993), while it is inhibited at 32 °C. This range is dependent on the bacterial strains/species and on plant cultivar/species. For instance, cowpea (*Vigna unguiculata*) has been proven to be tolerant to heat stress showing a reasonable SNF activity at 28 °C (Simões-Araújo et al. 2008), while common bean showed decreased SNF at the same temperature (Hungria and Franco 1993).

The first factor that could alter SNF is the survival of the bacteria in the soil under elevated temperatures. In semiarid tropical regions of Africa and South America, soil temperatures can reach 40 °C (Dennt 1984; Hafeez et al. 1991). The optimal growth temperature for many rhizobium bacteria in growth media is between 28 and 30 °C. The survival at elevated temperatures of different rhizobial strains in the free-living stage has been studied by several authors (Hungria et al. 1993; Michelis et al. 1994). These authors demonstrated that diverse bacterial species isolated from different plants have different survival capacities, even at the strain level. When heat stress occurs during the free-living stage, several processes might reduce the number of bacteria. Death may take place above 37 °C, and Hungria and Stacey (1997) demonstrated that a difference of 6 °C at a 5 cm depth reduced the soybean bradyrhizobial population by more than $10^4$ cells. Michelis et al. (1994) showed that a strain of *Rhizobium tropici* (CIAT 899), isolated from tropical trees, was more resistant to 40 °C, when grown in a culture medium, than a *Rhizobium leguminosarum* (CNPAF512) isolated from common bean. This lower viability could be caused by genetic modification or plasmid loss (Hungria and Vargas 2000) and can be counteracted by the inoculation of legumes at higher rates. Related to this it is important to distinguish between competitiveness and effectiveness. Rhizobia strains present in soil are usually highly competitive but this does not imply that they are effective, so their use as inoculants may not result in efficient SNF (Roughley et al. 1976; Boonkerd et al. 1978). Early studies have demonstrated the dissociation between the competitiveness and the effectiveness of rhizobia strains (Franco and Vincent 1976). Among the features that contribute to this effect could be preferential recognition by the host (Russell and Jones 1975) or the relative numbers of inoculant and indigenous strains (Bohlool and Schmidt 1973).

Legume symbiosis starts with a dialogue between the macro- and microsymbiont (Neves and Hungria 1987; Vance and Lamb 2001). This communication requires that the growing root segregates a different collection of phenolic molecules, mainly flavonoids, that are dispersed in the soil (Hungria and Stacey 1997). Then the bacteria absorb those flavonoids by passive diffusion. The flavonoids activate the expression of *nod* genes allowing recognition between the two partners and the start of root modification and nodule formation (Hungria and Stacey 1997). If heat stress occurs at this stage this signalling or even nodule formation might be disrupted. It has been demonstrated that high temperatures, such as 39 °C or above, decrease the expression of genes related to the production of flavonoids in roots of bean and soybean, with bean being more sensitive to heat stress than soybean (Hungria 1995). At the same time, high temperatures lead to the loss of *nod* gene expression, which reduces the total number of bacteria that start nodulation with the plant (Hungria et al. 1993). Regardless of the possible miscommunications that may happen due to heat stress, high temperatures also inhibit root hair formation. Root hairs are the structures where the bacteria enter the root and have their first contact with the plant tissue, so reductions in root hair formation decrease the number of nodulation sites and therefore the final number of nodules (Barrios et al. 1963; Lie 1974, 1981). Beyond root hair formation, it has been documented that heat stress could affect nodule establishment due to a reduction in bacterial adherence to the root hairs (Frings 1976), lack of root hair penetration (Dart 1974; Frings 1976), and interruption of nodule initiation and bacteroid formation (Roughley et al. 1976; Pankhurst and Gibson 1973; Vincent 1980).

Once the nodule is established, SNF is contingent on the physiological state of the host plant. This continuous provision may be reduced due to environmental stresses affecting photosynthesis such as soil moisture deficit, high temperature, and salt stress (Aranjuelo et al. 2014 for a review). Some authors have described this association between reduced leaf assimilation and the consequent nitrogenase ($N_{ase}$) diminishment (Gordon et al. 1999; Aranjuelo et al. 2013). Nodule metabolism may be limited by carbohydrate availability, accumulation of nitrogenous compounds,

$O_2$ permeability, and accumulation of reactive oxygen species (ROS) (Aranjuelo et al. 2014). Translocated plant carbohydrates support nodule respiration. Carbohydrate supply from host plant is essential to provide energy and reducing power and to synthesize carbon skeletons that act as $NH_2$ acceptors. Thus, nodule metabolism is often limited by sugar availability from leaves (Vance and Heichel 1991), which may represent between 45 and 50 % of total photosynthetic $CO_2$ fixation (Lluch et al. 2002). On the other hand, the decrease in $N_{ase}$ activity has been associated with the accumulation of nitrogenous compounds in the nodule (Serraj et al. 1999). The limited carbohydrate supply to the nodule or the impairment of xylem transport may lead to the accumulation of nitrogenous compounds. This may provoke a negative feedback mechanism resulting in the inhibition of $N_{ase}$ activity (Serraj et al. 1998, 2001; King and Purcell 2005). Another critical problem facing $N_2$-fixing organisms is the sensitivity of $N_{ase}$ to $O_2$ concentration. In legume nodules, a physical barrier to gas diffusion in the inner cortex, known as oxygen diffusion barrier, seems to be the primary site for the regulation of nodule permeability to gas diffusion (Hunt and Layzell 1993). Although oxygenated leghemoglobin, an $O_2$-binding protein, gradients may have the role of fine control of infected cell $O_2$ concentration (Thumfort et al. 1999). This protein is synthesized by the host plant and found in bacteroid-infected cells. It binds $O_2$ and then releases it in a controlled manner within the bacteroid environment (Hopkins and Hüner 2004) providing the bacteria with a high $O_2$ flux at a low $O_2$ concentration (Gálvez et al. 2000). Abiotic stress could consequently also limit nodule metabolism through alteration of the $O_2$ diffusion barrier that conditions $N_2$ fixation performance (Hunt and Layzell 1993). Leghemoglobin may represent as much as 25 % of the nodule protein content, so its decrease may reduce SNF. ROS are one of the factors that reduce leghemoglobin concentration. Oxidative stress is another mechanism conditioning and inhibiting SNF (Porcel et al. 2003; Naya et al. 2007). The $O_2$ content imbalance caused by several abiotic stresses such as elevated temperature is responsible for nodule senescence and negatively impacts on nodule performance (Zahran 1999). ROS are continuously produced as by-products of various metabolic pathways localized in different cellular compartments (Foyer and Harbinson 1994). Under physiological steady-state conditions, these molecules are scavenged by different antioxidant defense components (Alscher et al. 1997). However, the equilibrium between the production and scavenging of ROS may be perturbed by adverse environmental factors and, as a consequence, intracellular levels of ROS may rise rapidly (Polle 2001). It needs to be pointed out that most of the studies concerning abiotic stress on nodule metabolism and ROS production have been focused on drought effects, and knowledge about how elevated temperature affects $O_2$ metabolism, and especially ROS detoxification, is scarce (Aranjuelo et al. 2014). Together with $O_2$ and $H_2O_2$, reactive nitrogen species (RNS), such as NO•, have been described as a major component of the oxidative burst (Hérouart et al. 2002). RNS have been described to be involved in the plant defense against abiotic stresses and as key signal molecules in plants (Dat et al. 2000; Guan et al. 2000). Although its role in symbiosis has not been unraveled so far, several lines of evidence suggest that NO• could participate in the symbiotic process establishment and/or regulation (Hérouart et al. 2002).

Studying SNF is complex because of the molecular signal exchanges between plants and bacteria, which are coordinated by gene expression patterns from both partners and the interdependent plant-bacteria-environment interaction (Van Rhijn and Vanderleyden 1995; Hungria and Stacey 1997; Prell and Poole 2006). Effective symbiosis between the host plant and the bacterium requires the coordinated expression of genes in both partners (Gonnet and Díaz 2000; Vance and Lamb 2001). Studies about tolerance to high temperature have been mainly focused on the microsymbiont, isolating bacterial strains and assessing their environmental stress response (Hungria et al. 1993; Martinez-Romero et al. 1991), whereas the macrosymbiont (host plant) contribution to thermotolerance still remains unclear (Simões-Araújo et al. 2008). This disconnection between heat tolerance and $N_2$ fixation reveals the lack of knowledge of key aspects of high-temperature stress (Simões-Araújo et al. 2008). There is still a gap in the understanding of molecular responses of rhizobia, including the mechanisms for acquired thermotolerance. In addition, the effects of heat on nodulation and symbiotic gene expression and also on proteome profiles, including enzymes involved in nodulation and $N_2$ fixation at high temperatures under field conditions (Qureshi et al. 2010), have been scarcely explored.

It has been proven that the interaction between rhizobial strains and alfalfa genotypes alters the plant response to different ambient variables including freeze tolerance (Bertrand et al. 2007). Prevost et al. (1999) showed that the selection of cold-adapted rhizobia constitutes a valuable tool to counteract the negative effect of low temperature by improving legume productivity. In a similar way the selection of competitive and efficient rhizobial strains coupled to plant genotypes of interest could be invaluable in facing future climate conditions and the challenge to ensure food supply to increasing population.

## 3 High-Temperature Effects in the Plant-Bacteria Relationship

The effect of climate change on plant growth is a matter of major concern because most of the recent global climate models predict an increase in temperature (between 2 and 6 °C) and a decrease in water precipitation in Europe by 2100 (IPCC 2013). The expected warming is going to be greatest in summer in south-western Europe. More specifically, according to IPCC, the current prediction is for an average rise of 4 °C by 2080 and extreme summers like that of 2003 are likely to become four times as common in Southern Europe (IPCC 2013).

In order to summarize the current knowledge of high-temperature effects on $N_2$ fixation and plant growth, we compiled a dataset (Table 1) from the literature on the effects of high temperature on environmental variables including biomass production, plant N and total soluble protein (TSP) content, gas exchange parameters, and $N_{ase}$ activity. Data on the effects of high temperature on plant growth and the N status of $N_2$-fixing plants were compiled by surveying the peer-reviewed literature on the Web of Science (Thompson-ISI, Philadelphia, USA). Because the main goal of

**Table 1** List of articles used for the analysis of high-temperature effects on the growth and N status of $N_2$-fixing plants

| Manuscript | Plant | Bacteria | Strain |
|---|---|---|---|
| Aranjuelo et al. (2005) | *M. sativa* | *R. meliloti* | 102F78 |
| Aranjuelo et al. (2007) | *M. sativa* | *R. meliloti* | 102F78 |
| Aranjuelo et al. (2008) | *M. sativa* | *R. meliloti* | 102F78 |
| Erice et al. (2006) | *M. sativa* | *R. meliloti* | 102F78 |
| Erice et al. (2007) | *M. sativa* | *R. meliloti* | 102F78 |
| Hungria and Franco (1993) | *P. vulgaris* | *R. leguminosarum* | SEMIA 487 |
| | *P. vulgaris* | *R. leguminosarum* | SEMIA 4021 |
| | *P. vulgaris* | *R. leguminosarum* | F413 |
| | *P. vulgaris* | *R. leguminosarum* | F413 Mn3 |
| | *P. vulgaris* | *R. leguminosarum* | C05 |
| | *P. vulgaris* | *R. leguminosarum* | CIAT 57 |
| | *P. vulgaris* | *R. leguminosarum* | CIAT 255 |
| Hungria and Kaschuk (2014) | *P. vulgaris* | *R. tropici* | BR 814 |
| | *P. vulgaris* | *R. tropici* | BR 817 |
| | *P. vulgaris* | *B. elkanii* | BR 6010 |
| | *P. vulgaris* | *R. tropici* | CIAT 899 |
| | *P. vulgaris* | *R. leucaenae* | CFN 299 |
| | *P. vulgaris* | *R. leguminosarum* | CNPAF 126 |
| Montañez et al. (1995) | *G. max* | *B. japonicum* | E1 |
| | *G. max* | *B. japonicum* | E2 |
| | *G. max* | *B. japonicum* | E3 |
| Sanz-Sáez et al. (2012) | *M. sativa* | *R. meliloti* | 102F178 |
| | *M. sativa* | *R. meliloti* | 102F34 |
| | *M. sativa* | *R. meliloti* | 1032GMI |

this chapter is to compile high-temperature effects on $N_2$ fixation, when defining the search we focused the study on exclusively $N_2$-fixing plants. The temperature databases consisted of data from plants grown under high temperatures. Units for plant growth, N content, and $N_{ase}$ activity varied among studies, with unit interconversion being occasionally problematic or even impossible. Therefore, for comparative purposes, all the parameters presented in this chapter are expressed as a percentage of the control values. Control ambient temperature for Aranjuelo et al., Erice et al., and Sanz-Saez et al. values were 17–19 °C, whereas in the study carried out by Hungria et al. it was fixed at 28 °C. For high-temperature stress, data were grouped under slight (25 °C), mild (28–30 °C), moderate (34 °C), and severe (39 °C) heat stress.

The adverse effects of global warming on crop productivity have been documented in different crops such as alfalfa, wheat, and maize (Ciais et al. 2005; Aranjuelo et al. 2008; Oury et al. 2012). As shown in Fig. 1, the lowest biomass values were registered in plants exposed to 39 °C. Although 2–7 °C above control temperature, DM increased in $N_2$-fixing plants, when grown under temperature higher than 28 °C, plant growth decreased 40 %. A 10-year revision conducted in

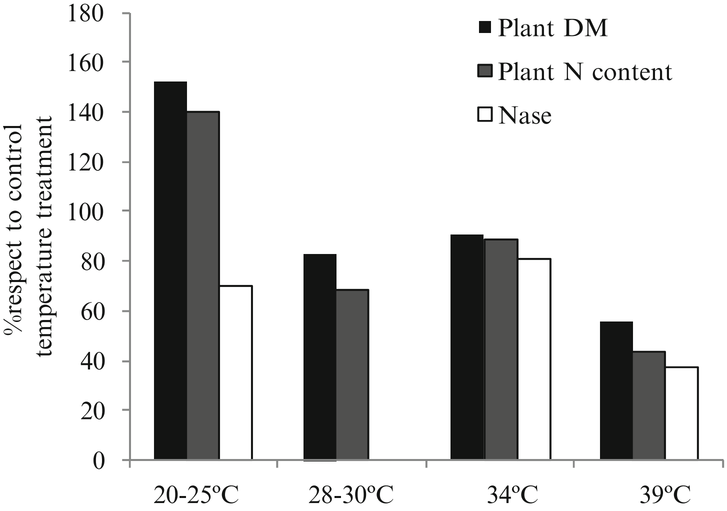

**Fig. 1** Temperature effect on plant dry matter (DM), plant nitrogen (N) content, and nitrogenase ($N_{ase}$) activity of $N_2$-fixing plants grown at 20–25, 28–30, 34, and 39 °C. Values are expressed as percentage of control treatments

different crops revealed a decrease in productivity of 0.42 t $ha^{-1}$ for each °C increase (Oury et al. 2012). A recent review (Galmés et al. 2013) characterizing high-temperature effects on plants indicated that photosynthesis is sensitive to elevated temperature. In agreement with this comment, we have observed that while at 20–25 °C the photosynthetic rates were not affected (compared with control temperature conditions), photosynthesis decreased by 40 % when exposed to 28–30 °C (Fig. 2). In agreement with previous studies (Montañez et al. 1995; Serraj and Adu-Gyamfi 2004), these findings demonstrated that SNF is relatively more sensitive to elevated temperature than photosynthesis. Our study also indicated that no correlation was observed between high-temperature effects on plant growth and photosynthetic rates (Fig. 3). Interestingly, we also observed that depleted photosynthetic activity under high-temperature conditions was due to limitations of lower leaf TSP and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity. This result is in agreement with previous reports identifying Rubisco activase as one of the most heat-sensitive components of the photosynthetic apparatus (Fellers et al. 1998; Salvucci et al. 2003), thus leading to Rubisco deactivation. Among other physiological, biochemical, and biological processes in plants deleteriously affected by high-temperature effects, N status has been described as a factor that determines crop performance. This point is clearly confirmed in Figs. 1 and 4, where a strong correlation is illustrated between plant growth and N content.

The sensitivity of legume-*Rhizobium* symbiosis to ambient stresses like temperature has been observed in the past (Hungria and Vargas 2000; Aranjuelo et al. 2007; Hungria and Kaschuk 2014). Plant N status is tightly conditioned by the plant-bacteria interaction. Within the bacteroid, malate is oxidized by the TCA cycle to

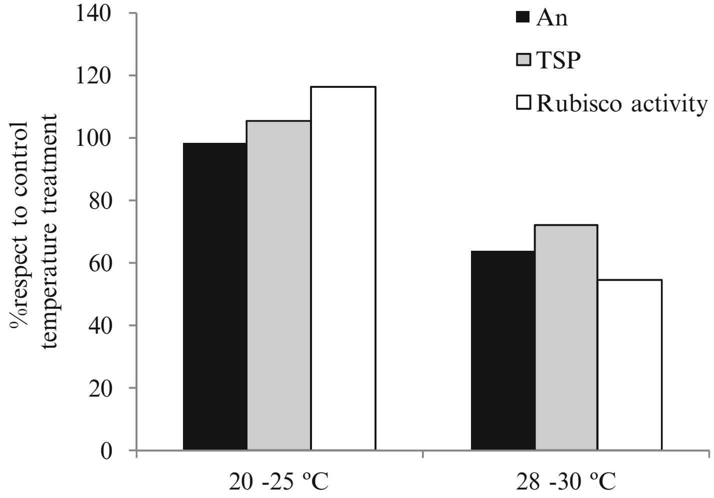

**Fig. 2** Temperature effect on leaf photosynthesis (An), total soluble protein (TSP) content, and Rubisco carboxylation activity of $N_2$-fixing plants grown at 20–25 and 28–30 °C. Values are expressed as percentage of control treatments

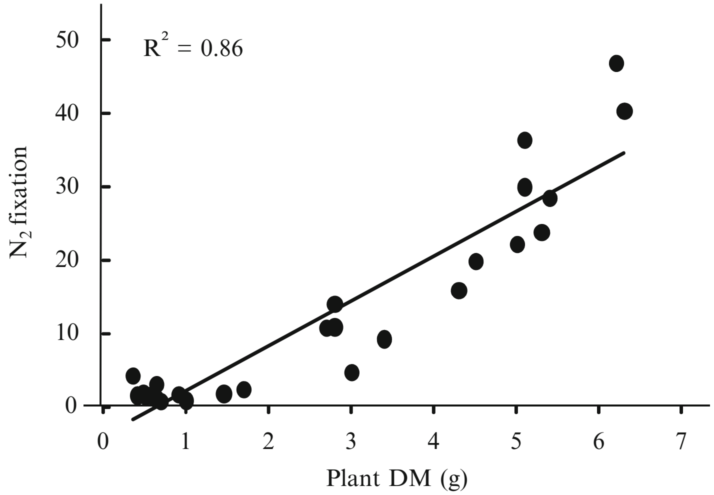

**Fig. 3** Relationship between $N_2$ fixation and plant dry matter (DM). $R^2$ refers to regression coefficient for the corresponding regression line

provide reductant for the $N_{ase}$ complex and for the respiratory chain that fuels $N_{ase}$ with ATP. As a counterpart, the bacteria fix $N_2$ and synthesize amino acids that are partitioned to the plant. Consequently, factors that increase photosynthesis increase

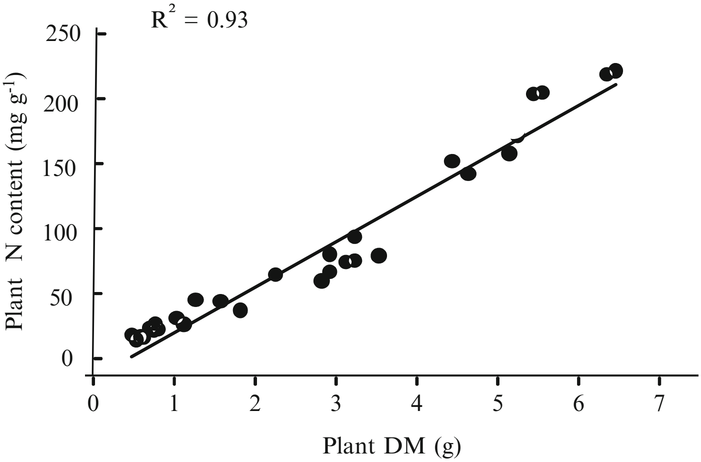

**Fig. 4** Relationship between plant nitrogen (N) content and plant dry matter (DM). $R^2$ refers to regression coefficient for the corresponding regression line

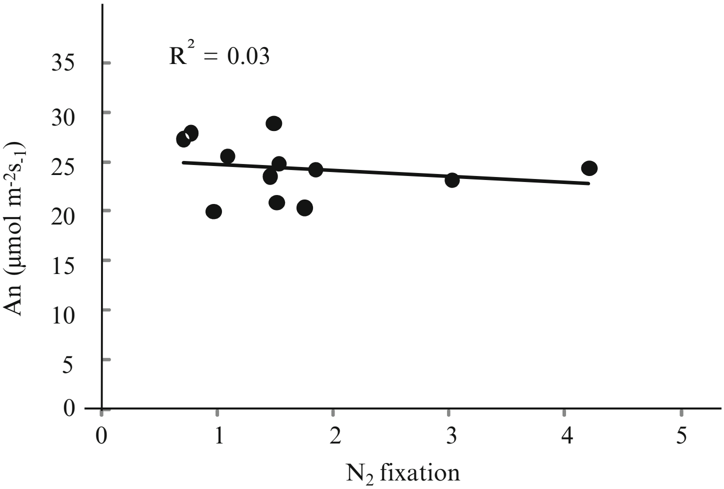

**Fig. 5** Relationship between leaf photosynthetic rate (An) and $N_2$ fixation. $R^2$ refers to regression coefficient for the corresponding regression line

$N_2$ fixation, while factors decreasing photosynthesis tend to decrease $N_2$ fixation (Sanz-Sáez et al. 2012; Aranjuelo et al. 2014). As shown in Fig. 5, no correlation was detected between photosynthetic activity and $N_{ase}$ activity. This could be due to the fact that depleted photosynthetic performance was due to a combination of processes such as stomatal closure (Fig. 6), depleted TSP, and inhibition of Rubisco activity. Such a relationship has been characterized in $N_2$-fixing plants exposed to elevated temperature (Aranjuelo et al. 2007; Hungria and Kaschuk 2014) and water stress (Arrese-Igor et al. 1999; Gálvez et al. 2005; Larrainzar et al. 2009).

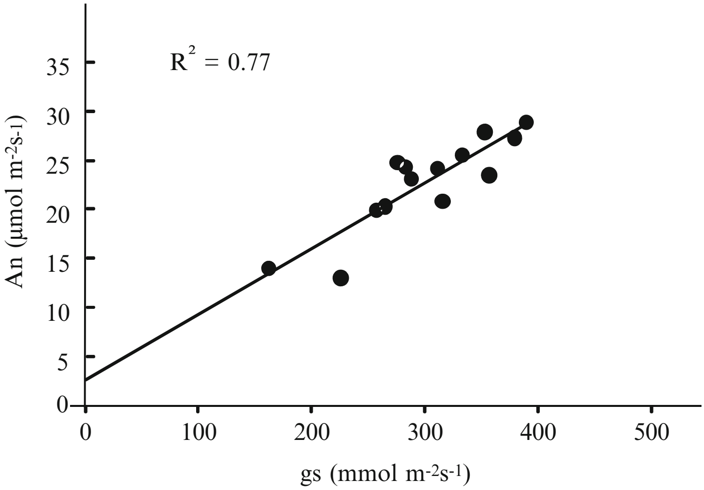

**Fig. 6** Relationship between leaf photosynthetic rate (An) and stomatal conductance (gs). $R^2$ refers to regression coefficient for the corresponding regression line

As observed by these authors, under stressful growth conditions where photosynthetic activity is inhibited, there is a decrease in carbohydrate supply to the nodules with a consequential diminishment in $N_{ase}$ activity. This coupling results in the regulation of $N_{ase}$ activity in plants by photosynthesis (carbon supply), N availability (N source strength), and N demand (N sink strength) (Aranjuelo et al. 2014). Although the current literature review (Aranjuelo et al. 2014) did not reveal a correlation between photosynthesis and $N_{ase}$ activity (Fig. 5), the absence of available data describing nodule sucrose content, and especially organic acid content conditions, confirms the view that under high-temperature conditions, nodules have no carbohydrate restrictions that negatively affect $N_2$ fixation. Moreover, to our knowledge, no study has analyzed the effects of high temperature on nodule oxidative status and the presence of ROS. Because ROS has been extensively observed and characterized in nodules exposed to other abiotic stresses (such as drought and salinity) (Aranjuelo et al. 2011; Larrainzar et al. 2009; Ladrera et al. 2007; Gálvez et al. 2005), the fact that oxidative stress also conditions nodule performance under high-temperature conditions should not be ignored. Despite the absence of a photosynthesis/$N_2$ fixation correlation, as shown in Fig. 3, it is clear that plant growth is tightly linked to plant $N_2$ fixation (Hungria et al. 1993; Hungria and Kaschuk 2014; Bertrand et al. 2007; Sanz-Sáez et al. 2012). Figure 7 shows that nodule dry matter (DM) increased under 28–30 °C conditions, whereas TSP was not affected (compared with control temperature treatment in each experiment). When plants were subjected to 39 °C the nodule DM and TSP content decreased. While available data shows that nodule DM is a key factor conditioning $N_2$ fixation, Fig. 8 reveals the strong correlation between $N_2$ fixation and nodule TSP. Such a correlation indicates that nodule performance under elevated temperature conditions is mediated by protein activity, and this is described in more detail below.

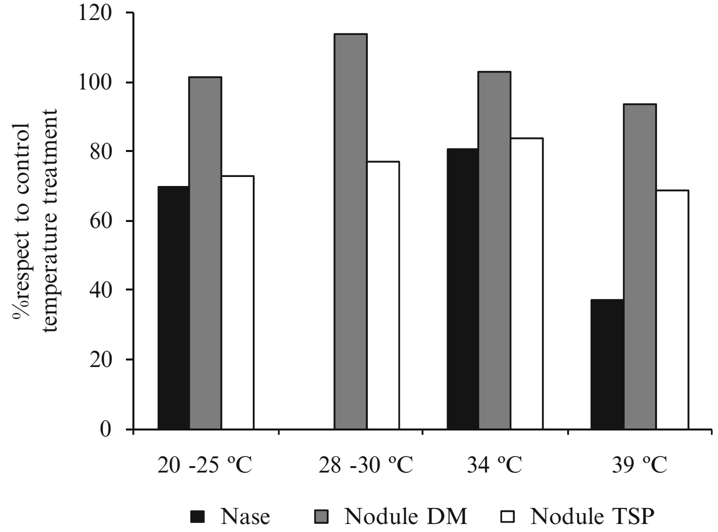

**Fig. 7** Temperature effect on nitrogenase ($N_{ase}$) activity, nodule dry matter (DM), and nodule total soluble protein (TSP) content of $N_2$-fixing plants grown at 20–25, 28–30, 34, and 39 °C. Values are expressed as percentage of control treatments

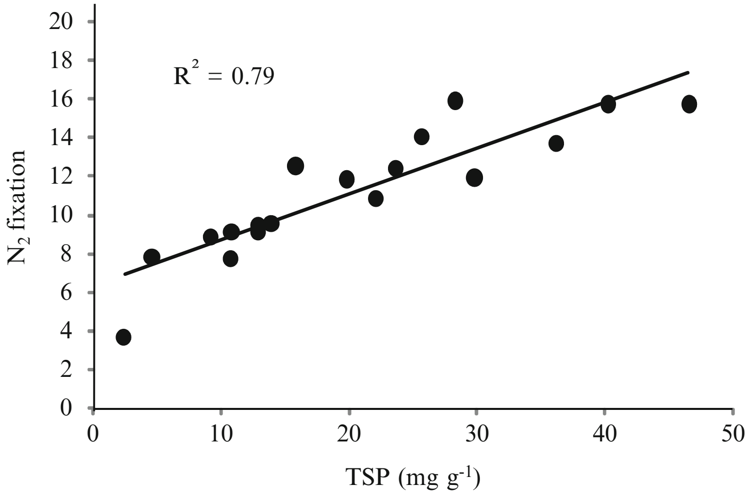

**Fig. 8** Relationship between $N_2$ fixation and nodule total soluble protein (TSP) content. $R^2$ refers to regression coefficient for the corresponding regression line

In summary, our study of the available data showed that plant DM and N content were negatively affected by elevated temperature, especially when exposed to 39 °C (Fig. 1). Moreover, this study did not find correlations between photosynthetic activity and $N_{ase}$ activity. While photosynthetic performance was inhibited above 28 °C (mainly due to limitations in $CO_2$ diffusion and Rubisco activity), the largest depletion in $N_2$ fixation was mostly detected in plants exposed to 39 °C. However, it should be observed that the absence of detailed nodule performance data makes it difficult to conceptualize such an apparent "lack" of correlation between photosynthesis and $N_2$ fixation.

## 4 Nodule Metabolism Under High-Temperature Conditions

Most stress factors influence all of the physiological processes in plants and bacteria as the stress develops. High temperatures can directly and indirectly affect $N_2$ fixation. The indirect effects are usually driven by decreases in bacterial survival in the soil (Michelis et al. 1994) and the disruption of the recognition signals between the micro- and macrosymbiont (Michelis et al. 1994; Hungria and Stacey 1997). These indirect changes under elevated temperature usually affect the total number of nodules formed and therefore the quantity of $N_2$ fixed by the plant, but they have no effect on nodule activity per se. Heat stress also could have a direct effect on the $N_2$ fixation process by altering the function of some proteins in the $N_2$ fixation cycle and/or related cycles. In this section we are going to discuss how the elevated temperature affects different processes that could lead to a diminished $N_2$ fixation (Hungria and Kaschuk 2014).

Under heat stress conditions, bacterial metabolism is altered negatively. This is revealed by the quick decrease in protein synthesis just after the imposition of heat stress (Michelis et al. 1994; Montañez et al. 1995). However, there are some tolerant bacteria such as the previously mentioned CIAT 899 that show maintenance of protein synthesis over time. This resistance to heat stress has been associated with differential and de novo synthesis of heat-shock proteins (Lindquist 1986; Michelis et al. 1994). Heat-shock proteins are involved in important protective cellular functions such as initiation of DNA replication (Pack and Walker 1987; Sakakibara 1988), transcription (Taylor et al. 1984; Wada and Itikawa 1984), protein synthesis (VanBogelen et al. 1983), correct protein folding, assembly of multi-protein complexes (Goloubinoff et al. 1989; Gatanaris et al. 1990), and protein turnover (Gottesman et al. 1981). In the light of the results obtained by different studies it is worth emphasizing that the effect of heat stress on strain survival depends on the rhizobium strain and it is influenced by the synthesis of heat-shock proteins.

Under heat stress, species with both the highest and the lowest levels of $N_2$ fixation show a decrease in the $N_2$ fixation rate, as measured with the acetylene reduction assay and/or with the $H_2$ evolution assay (Fig. 1; Hernandez-Armenta et al. 1989a; Hungria and Franco 1993; Michelis et al. 1994; Montañez et al. 1995; Sanz-Sáez et al. 2012; Hungria and Kaschuk 2014). The downregulation of $N_2$ fixation under other stresses such as drought has been related to a decrease in carbon (C) availability due to the low input of C provided by the plant and/or due to an

N-feedback (Vance and Lamb 2001). The decrease in C availability under heat stress conditions and its link to $N_2$ downregulation have been studied by several authors (Hernandez-Armenta et al. 1989a, b; Hungria and Kaschuk 2014). These authors have shown that high temperature does not decrease the C availability in the nodules. In fact, high temperatures of 38 °C maintained starch and soluble sugars at the same concentrations as the controls in the nodules and stems of stressed plants (Hernandez-Armenta et al. 1989a). In addition, microscopic studies have revealed the accumulation of poly-ß-hydroxybutyrate PHB in bacteroids, which is a carbohydrate storage form, demonstrating that there is enough C supply to the nodule. Although an adequate storage of PHB under non-stressful conditions has been related to high rates of $N_2$ fixation, PHB accumulation under stress has been related to increased reproduction and bacterial survival (Ratcliff et al. 2008; Fig. 1). The carbohydrate accumulation that accompanies the decreased activity of sucrose synthase and phosphoenolpyruvate carboxylase (PEPc) (Silvente et al. 2003; Hungria and Kaschuk 2014) suggests an inhibition of carbohydrate utilization within the nodule, which could lead to the inhibition of $N_2$ fixation. However, this effect will be observed only under stress conditions because under non-stressful conditions $N_2$ fixation is, in fact, not limited by C availability due to sink stimulation of photosynthesis by the nodule symbiotic activity (Kaschuk et al. 2010, 2012).

The hypothesis of N-feedback regulation of $N_2$ fixation is similar to any end-product downregulation, such as photosynthesis downregulation, due to sucrose accumulation (Drake et al. 1997; Moore et al. 1999). In this case, $N_2$ fixation is limited when the products of this reaction, $NH_4^+$ or N-ureides, are accumulated in the nodules (Parsons and Sunley 2001; King and Purcell 2005). In a mild heat stress situation, when the temperature was increased from 28 to 34 °C, the activities of nodule enzymes such as PEPc, glutamine synthetase (GS), glutamate synthase (GOGAT), and $N_{ase}$ were maintained or slightly reduced (Hungria and Kaschuk 2014). In that situation the nodule is still able to export N-ureides to the shoots, and because of this the concentration of $NH_4^+$ and N-ureides does not become concentrated at nodule level. However, under a severe heat stress such as a temperature increase from 28 to 39 °C, all the nodule enzymatic activities drop dramatically, with $N_{ase}$ activity being mostly affected (Hungria and Kaschuk 2014). Under heat stress the increase in nodule $NH_4^+$ and N-ureides concentrations confirmed that the N-feedback hypothesis is applicable under elevated temperature stress (Hungria and Kaschuk 2014). Besides these findings, it has been shown in microscope studies that nodule structure is damaged when temperatures increase from 22 to 38 °C (Hernandez-Armenta et al. 1989a). During this stress, the cytoplasm of the plant cells and the peribacteroid membrane, which is the membrane separating the bacteroids from the plant's cell cytoplasm, showed signs of damage. This structural damage would support the fact that after a heat stress, the plant's $N_2$ fixation activity recovers slowly. Depending on the legume-*Rhizobium* partnership, and the exposure time to heat stress, the time needed to recover the $N_2$ fixation capacity could vary from 7 to 14 days (Hernandez-Armenta et al. 1989a; Hungria and Franco 1993; Michelis et al. 1994; Hungria and Kaschuk 2014).

## 5 Future Perspectives

Global climate models predict that environmental factors, including temperature, water availability, and atmospheric $CO_2$ concentration, will be altered in future (IPCC 2013). Climate change will reinforce the need to develop high-production crops. $N_2$-fixing plants represent the second major crop of agricultural importance worldwide. For this purpose, it is essential to identify target legume cultivars that will be better adapted to predicted climate change conditions such as soybean (Bishop et al. 2014), common bean (Hungria and Kaschuk 2014), and cowpea (Piha and Munns 1987). As it has been highlighted in this chapter, temperature increase has a strong inhibitory impact in $N_2$ fixation and plant growth. However, we also would like to observe that, compared to other environmental factors such as water availability, $CO_2$ concentration, and salinity, little is known about high-temperature effects on nodule and plant performance. Taking into account the fact that ambient temperature is expected to rise 1.5–4 °C by the end of the century, it is crucial to improve our understanding on high-temperature effects on performance of $N_2$-fixing plants.

Traditional N management on commercial farms has been generally based on empirically selected practices that maximize yields (Thompson et al. 2007). This approach has been successful in the past to increase yields but unsuccessful in avoiding environmental risks, frequently associated with high productivity. The application of molecular methodologies (in combination with the more classical agronomic and physiological approaches) might contribute to the elucidation of mechanisms responsible for the plant responsiveness to high-temperature conditions. This novel information might be of great interest to assign priorities in future breeding programs by providing important information for establishing selection criteria. There is a clear requirement to develop new strategies and tools for N management in agriculture to ensure that high levels of production are maintained while minimizing deleterious environmental impacts. To do so, it is essential to identify, understand, and quantify mechanisms associated with crop responses to N management and with environmental impacts.

When analyzing how high temperature affects nodule performance, it must be considered that SNF is a complex and sensitive process that depends on multiple factors (e.g., carbohydrate supply, accumulation of nitrogenous compounds, and oxidative stress). It should be noted that the identification of these three factors is almost entirely based on studies analyzing nodule performance under drought, salinity, high nitrate content, and low phosphorus availability and conditions (Aranjuelo et al. 2014; Sulieman et al. 2014). Little is known about high-temperature effects on nodule carbohydrate availability, N accumulation, and oxidative stress.

When thinking on high-temperature impact on $N_2$ fixation and plant growth, it should also be considered that for breeding purposes, screening of rhizobial strains that could enhance $N_2$ fixation, photosynthesis, and plant production represents a target research field. In fact, several authors (Sanz-Sáez et al. 2012; Hungria and Kaschuk 2014) have found that depending on analyzed rhizobial strain, high-temperature effect on $N_2$ fixation changes considerably. As highlighted by those studies, more research is needed to isolate more rhizobial strains from heat-resistant plants, and inoculate them to agronomical important species such as soybean and common bean to study if they are able to nodulate and maintain a high $N_2$ fixation under heat stress (Martinez-Romero et al. 1991; Hungria et al. 1993, 2000). Although those studies look for strains better adapted to different changing environments, it should not be forgotten that plant performance does not exclusively rely in nodule functioning since nodule activity is also conditioned by its symbiotic relationship with the host plant. Another source of breeding for better adapted legumes to changing environments could be possible by selecting better adapted plant and rhizobial symbionts.

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# Does Elevated $CO_2$ Provide Real Benefits for $N_2$-Fixing Leguminous Symbioses?

**Saad Sulieman, Nguyen Phuong Thao, and Lam-Son Phan Tran**

**Abstract** Feeding the growing world population will be a significant challenge for agricultural development in the twenty-first century. Simultaneously, global climate change will provide an additional challenge by significantly modifying the potential capability of the cultivated plants, particularly for those favoring symbiotic association with soil bacteria. Hence, the whole-plant nutritional metabolism is expected to reprogram basically to meet these climatic variables that collectively become a major concern for future agriculture. In the frame of the current and projected climate scenarios, this chapter attempts to address whether symbiotic legumes would benefit greatly from the current and projected higher levels of carbon dioxide ($CO_2$) in the atmosphere—sometimes called the $CO_2$ fertilization effect. On the basis of the results obtained by several researchers, nodulated legumes are projected to have a stronger response to elevated $CO_2$ whose level is continuously rising. In sharp contrast, another group of researchers has questioned such beneficial responses by symbiotic legumes. Apparently, the experimental findings dealing with the effects of elevated $CO_2$ on legume growth and function have revealed significant discrepancies and variability. In this chapter, we briefly outline the nature of global climate change in terms of rising atmospheric $CO_2$ and then discuss the potential biotechnological targets for improving $N_2$-fixing symbioses in a world of increasing $CO_2$ level. Current interest in understanding legume responses to changing global climate makes this overview timely.

**Keywords** Climate change • C/N metabolism • Elevated $CO_2$ • $N_2$ fixation • Legumes • Symbiosis • Nodules • Productivity

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# 1 Background

The United Nations Population Division estimated a rapid increase of world population by the end of the twenty-first century (UNPD 2010). The population is predicted to exceed 9 billion in 2050, while the records are close to 7 billion nowadays (Charpentier and Oldroyd 2010). Such unprecedented population growth raises the question whether there is a limit to what the world can sustain. Thus, it remains necessary and in top priority to produce more foodstuffs and maximize the overall production on sustainable basis. In this regard, legumes would be of great importance and special concern. Compared with other plant families, this group of plants occupies an important front position with respect to the function of natural and artificial ecosystems through the acquisition of atmospheric dinitrogen gas ($N_2$) (Oldroyd and Dixon 2014). With the aid of the symbiotic association with rhizobial bacteria, the leguminous plants are able to inject sustainable non-limited nitrogen (N) that could have major implications on N dynamics and ecosystem productivity (Udvardi and Poole 2013). The largest contribution to symbiosis is carried out by legumes that have abilities to associate with a wide range of bacteria, collectively referred as rhizobia. Because of these and other huge benefits, leguminous plants represent vital components in maintaining or upgrading soil fertility and exhibit great potential for use in soil stabilization.

The beneficial nodulation event is a multiphase process that comprises several developmental stages, resulting in differentiated bacteria (bacteroids) enclosed in intracellular compartments called *symbiosomes* within nodules on the root system (Udvardi and Poole 2013) (Fig. 1). At the most general level, the induction of symbiotic $N_2$-fixing nodules requires two developmental processes, namely bacterial infection and nodule organogenesis (Terpolilli et al. 2012). For driving the functional mutualistic association, the host plant must power the bacteria with carbon (C) and source of energy (ATP) for growth and functions, while the rhizobia fix atmospheric $N_2$ and supplement the host with organic fixation products (Fig. 1). As such, the legume–rhizobial symbiosis is an energy-intensive process that imposes heavy C burden on its hosting leguminous plant. The C cost (~5–12 g C per gram fixed N) for the symbiotic process represents around 25 and 176 % of C used for shoot and root growth, respectively (Minchin and Witty 2005; Schubert 2007). The C assimilates translocated from the shoots have multiple functions in generating energy and reducing power for atmospheric $N_2$ fixation, C skeleton for fixed-N assimilation in addition to nodule growth and maintenance. Carbohydrates imported in the form of shoot-derived sucrose are converted to organic acids, principally dicarboxylates that are supplied to bacteroids for supporting nitrogenase activity (Fig. 1). Malate is believed to be the sole respiratory C substrate for bacteroids (Schulze et al. 2002; Sulieman et al. 2013a, b). Consequently, the nodules have an intense metabolic respiratory activity resulting in a large sink of photosynthates in the nodulated root system.

The rise in the human population has been proportionally followed by a dramatic increase in the atmospheric concentrations of carbon dioxide (${}_{\text{atm}}CO_2$) (Krausmann et al. 2009). Anthropogenic activities, such as combustion of fossil fuels, rapid

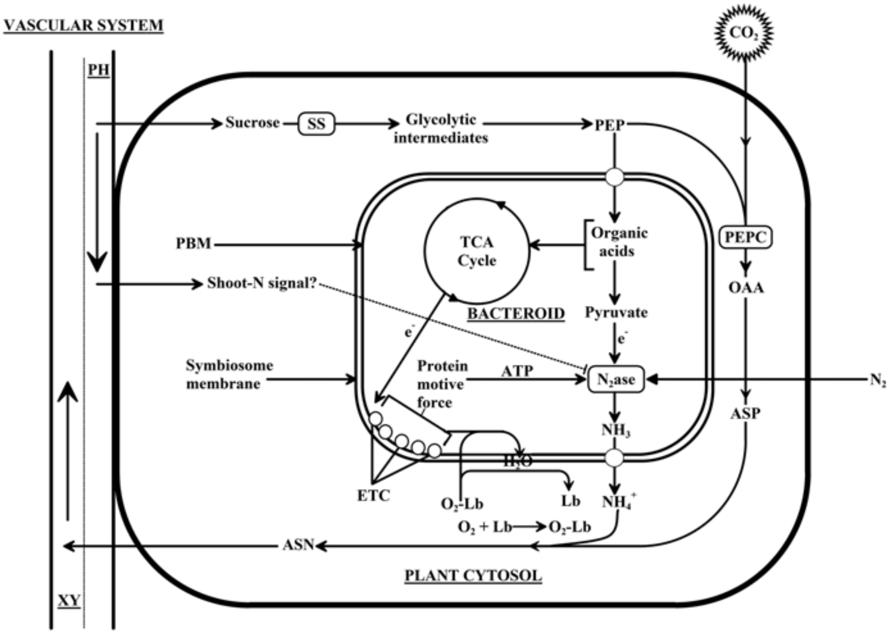

**Fig. 1** Schematic model of symbiotic nitrogen fixation (SNF) in the nodules of amino acid (amide) exporting legumes. *ASN* Asparagine, *ASP* aspartate, *ETC* electron transport chain, *Lb* leghemoglobin, *$N_2$ase* nitrogenase, *OAA* oxaloacetate, *PEP* phosphoenolpyruvate, *PEPC* phosphoenolpyruvate carboxylase, *PBM* peribacteroid membrane, *PH* phloem, *SS* sucrose synthase, *TCA* tricarboxylic acid cycle, *XY* xylem. See main text for further details

deforestation, and changes in land use patterns, are behind such remarkable increase in global $CO_2$ from the end of the eighteenth century to the present day (Irigoyen et al. 2014). The level of $_{atm}CO_2$ has been constantly increasing from approximately 316 ppm in 1959 to a current average of 396 ppm in 2013 (Fig. 2). Based on the predictions of the Intergovernmental Panel on Climate Change, $_{atm}CO_2$ is anticipated to surpass above 800 ppm by the year 2100 (IPCC 2007). At present rates of emissions, these changes in $_{atm}CO_2$ along with other more potent greenhouse gases can entirely change our global climate. Given the fact that $CO_2$ has greenhouse gas properties, such continuous rising of the $_{atm}CO_2$ concentration will concomitantly lead to an apparent increase in atmospheric mean temperature (≥3 °C before the end of this century), i.e., global warming (Rosenthal et al. 2014). Furthermore, rising temperature will concomitantly increase evaporation rates and exacerbate problems associated with water deficit and salinity.

The predicted climatic changes will have important critical implications on legume crop growth and productivity. In such N-limited systems, the response of nodulated legumes to *elevated* $_{atm}CO_2$ (*e*$CO_2$) may be an important determinant of natural and agro-ecosystem responses to global climatic changes. Long-term performance and survival of legumes are therefore highly dependent on the

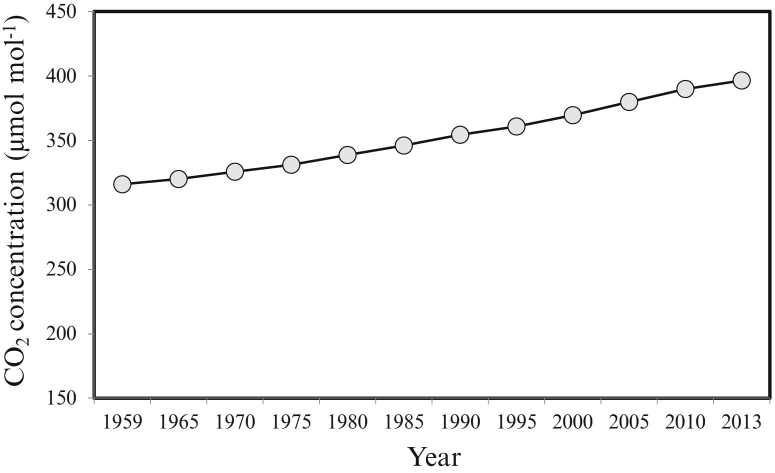

**Fig. 2** The average concentrations of $CO_2$ in the atmosphere. The year 1958 represents the start of precise $CO_2$ measurements in the atmosphere according to the data provided by Mauna Loa Observatory: NOAA-ESRL, United States

eco-physiological adaptations of rhizobia, legumes, and their interactions to the current and future climate variability. Since $_{atm}CO_2$ concentration remains among the main drivers affecting future food production, this chapter focuses on current research challenges that are relevant for climate change scenarios, with view at the most likely predictions, i.e., an increase of $_{atm}CO_2$ concentration associated with other climatic factors and a likely increase in climate variability. Current interests in understanding responses of plants, in particular legumes, to changing global climate make this review timely.

## 2 Impact of *e*$CO_2$ on Rhizobial Biology

The legume growth and productivity largely depend on the ecosystems' functional processes. Toward sustainable agro-ecosystems, it is necessary to understand the impact of *e*$CO_2$ on the functional diversity, composition, structure, metabolic potential, and dynamics of soil rhizobial communities. Little information is available today about the response of soil rhizobia to increases in $_{atm}CO_2$ concentration (Libault 2014). Understanding of how *e*$CO_2$ affects soil rhizobial community will be essential for robust prediction of $_{atm}CO_2$ concentration in the future. It has been suggested that the soil rhizobial bacteria might be more responsive to *e*$CO_2$ when compared with equivalent symbiotic state (Reed et al. 2011). Principally, the patterns of rhizobia response to *e*$CO_2$ are influenced by several factors including soil properties, nutrient status, vegetation type, analysis method, and diversity of microorganisms in the ecosystem (He et al. 2014).

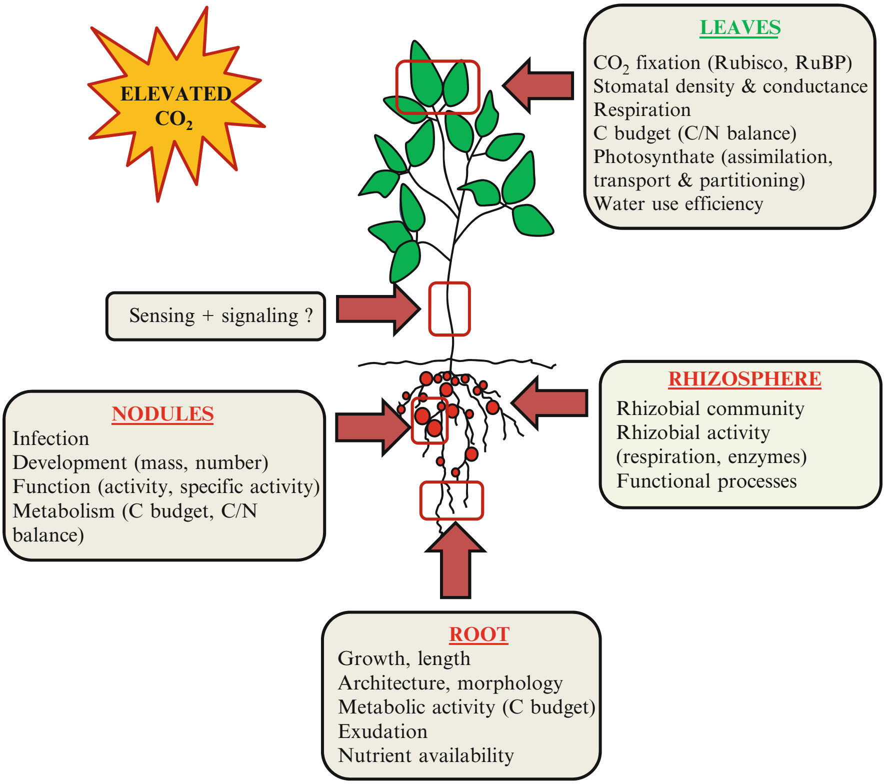

**Fig. 3** Summary of the main morphological, physiological, and biochemical characteristics by which nodulated legumes can be affected by rising atmospheric $CO_2$ concentrations

Several researchers have noticed dramatic changes in the population structure of soil microbial communities under *e*$CO_2$; with rhizobial colonizing the rhizosphere (and rhizoplane) being mostly affected (Schortemeyer et al. 1996; Haase et al. 2007; Sugawara and Sadowsky 2013) (Fig. 3). For example, Marilley and coworkers reported that *Rhizobium leguminosarum* populations increased in white clover (*Trifolium repens* L.) rhizosphere when plants were exposed to *e*$CO_2$ (Marilley et al. 1999). Moreover, the authors showed that the proportion of rhizobia on the total rhizospheric microbial community was remarkably enhanced by *e*$CO_2$ treatment. The subsequent genetic and phenotypic analyses further confirmed this finding and revealed that the genetic background for *R. leguminosarum* strains, which were isolated from nodules of plants exposed to *e*$CO_2$, differed greatly from those isolated from control plants grown under ambient $CO_2$ (*a*$CO_2$) (Montealegre et al. 2000). Based on transcriptomic analysis of rhizoplane-attached *Bradyrhizobium japonicum* cells, *e*$CO_2$ resulted in obvious and remarkable changes in the expression of genes involved in C/N metabolism, microaerobic respiration, and nodulation (Sugawara and Sadowsky 2013). Nevertheless, some other reports have contrarily indicated that *e*$CO_2$ had no effect on soil rhizobial population level (e.g., Prévost et al. 2010).

Since the $CO_2$ concentration in the soil (i.e., 2,000–6,000 μL $L^{-1}$) is much greater than that found in the atmosphere (i.e., 380 μL $L^{-1}$), it was hypothesized that the responses of soil microorganisms to *e*$CO_2$ are expected to be indirect owing to enhanced plant's carbohydrate status and related stimulatory responses (Deiglmayr et al. 2004). Consequently, *e*$CO_2$ could remarkably influence the physiology and metabolism of the soil rhizobia through increased root growth and nutrient availability, C allocation to the roots, rhizodeposition and root exudates, and enhanced water use efficiency by the exposed plants (Freeman et al. 2004; Haase et al. 2007) (Fig. 3). A remarkable variation in rhizobial population structure was found strongly related to the quality and quantity of root exudates, including flavonoids, which are well known to control the expression levels of rhizobial nodulation genes (Montealegre et al. 2000).

# 3 Impact of *e*$CO_2$ on Legume–*Rhizobium* Symbiosis

## *3.1 General Legume Response*

Although there has been a considerable debate about the response of legumes to *e*$CO_2$, most researchers believed that leguminous plants have a substantial competitive advantage over non-leguminous ones when grown under *e*$CO_2$ conditions (Rogers et al. 2009; Sanz-Sáez et al. 2013). The symbiotic $N_2$ fixation (SNF) trait is likely to be the key secret factor behind the predicted strong responses of legume growth to *e*$CO_2$. Under such climatic conditions, the leguminous plants will sustain the ability to shunt excessive carbohydrate levels to root nodules where they can serve as C and energy sources for nitrogenase activity. Accordingly, the leguminous plants are expected to have a critical vital role in adjusting/controlling future $CO_2$-enhanced environment (Irigoyen et al. 2014). Although evidence of growth stimulation of legumes by *e*$CO_2$ is well documented (Table 1), the influences of such increases in $_{atm}CO_2$ concentration on nodule physiology have revealed significant discrepancies and variability (Rogers et al. 2006; Bertrand et al. 2007a). Thus, it remains not fully understandable why some reports have shown positive responses of nodulation to *e*$CO_2$ while others have not. This discrepancy could be partially explained by the fact that legume responsiveness to the predicted $CO_2$ enhancement is dependent on certain principle factors including plant genotype, bacterial symbiont, plant-nutritional status, and surrounding other environmental conditions (Rogers et al. 2006; Haase et al. 2007; Bertrand et al. 2011). All these together have made the relationship between nodulation and *e*$CO_2$ quite complex, requiring studies on this relationship be conducted in a comprehensive manner.

Many studies of $CO_2$ enrichment have shown positive responses of root systems to *e*$CO_2$ conditions (Deiglmayr et al. 2004; Fischinger et al. 2010; Cabeza et al. 2014) (Fig. 3). Exposure of plants to elevated levels of $CO_2$ could significantly enhance the photoassimilate partitioning to roots, and therefore increasing the capacity and/or activity of belowground C sinks. Based on this observation, it was

**Table 1** Response of different nodulated legumes to elevated $CO_2$ concentrations

| Test plant species | Cultivar | Exposure system | Response | | | | $[CO_2]$ | References |
|---|---|---|---|---|---|---|---|---|
| | | | | Nodulation | | | | |
| | | | Plant growth | Number | Mass | Specific activity | | |
| *Glycine max* (L.) Merr. | Lotus | Controlled environmental chamber | Positive | Positive | Positive | nd | 400/800 | Prévost et al. (2010) |
| *Pisum sativum* L. | Frilene | Controlled growth cabinet | Positive | No | Positive | nd | 360/1,000 | Aranjuelo et al. (2013) |
| *Pisum sativum* L. | Frilene | Controlled growth cabinet | Positive | No | Positive | nd | 360/1,000 | Aranjuelo et al. (2014) |
| *M. truncatula* Gaertn. | Jemalong | Open-top field chambers (OTCs) | Positive | Positive | nd | nd | 390/750 | Guo et al. (2013a) |
| *M. truncatula* Gaertn. | Jemalong | Open-top field chambers (OTCs) | Positive | Positive | nd | nd | 390/750 | Guo et al. (2013b) |
| *M. truncatula* Gaertn. | Jemalong | Plexiglass containers | Positive | No | nd | Negative | 400/1,200 | Cabeza et al. (2014) |
| *Phaseolus vulgaris* L. | Hilds Maxi GS | Controlled environmental chamber | No | Positive | Positive | nd | 400/800 | Haase et al. (2007) |
| *Lens culinaris* Medic | ILL6995 (Idlib) | Tightly sealed ventilated chambers | Positive | Positive | nd | nd | 400/700 | Nasser et al. (2008) |
| *Medicago sativa* L. | nm | Glasshouse chambers | nd | Positive | nd | nd | 400/640 | Ryalls et al. (2013) |
| *Trifolium repens* L. | nm | Free air carbon dioxide enrichment (FACE) | Negative | No | No | nd | 360/475 | Watanabe et al. (2013) |
| *Medicago sativa* L. | Aragón | Temperature gradient tunnels (TGTs) | nd | nd | Positive | Negative | 405/730 | Aranjuelo et al. (2005) |
| *Medicago sativa* L. | Aragón | Greenhouses | Positive | nd | Positive | nd | 392/700 | Baslam et al. (2014) |
| *Medicago sativa* L. | Aragón | Temperature gradient tunnels (TGTs) | No | nd | nd | nd | 400/700 | Aranjuelo et al. (2006) |
| *Medicago sativa* L. | Hunter River, Trifecta, Aurora, Sequel | Glasshouse chambers | Positive | Positive | nd | nd | 400/600 | Johnson et al. (2014) |

Negative values indicate a decrease under elevated $CO_2$. Plant growth and nodule mass data were reported on dry matter basis. $[CO_2]$, $CO_2$ concentration in air in μmol $mol^{-1}$ ambient/elevated; *nd* not determined, *No* no response, *nm* not mentioned hypothesized that shoot/root ratio is going to reduce under conditions of *e*$CO_2$ (Erice et al. 2007b). The shoot/root ratio is an agronomical parameter that is known to be greatly affected by modifications in C supply, as well as environmental factors such as nutrient availability (Sulieman et al. 2010, Sulieman et al. 2013a, b). The predicted stimulation of root growth may enhance the ability of plants to explore the soil and alleviate nutrient limitations to legume growth. Moreover, $CO_2$ fertilization could trigger strong plant responses by modifying the architecture, morphology, biomass, length, and size of root system as well as nutrients uptake kinetics (Prasad et al. 2005; Tu et al. 2009; Pritchard 2011) (Fig. 3). Of particular interest, associations with $N_2$-fixing bacteria or mycorrhizal fungi are widely believed to be strongly affected by such modifications in root growth and architecture. Nevertheless, some scattered reports showed that *e*$CO_2$ had no considerable impact on root growth and biomass (e.g., Zhang et al. 2011).

## 3.2 Effects of Increased $CO_2$ on Host Plants

The direct effects of rising $_{atm}CO_2$ on legumes include both quantitative and qualitative changes in the shoot aerial parts. For example, remarkable alterations in plant photosynthetic rates besides other changes connecting with the physiological and biochemical reactions, such as C/N ratio, concentrations of starch, sugars, and total nonstructural carbohydrates, have been reported (Erice et al. 2011; Guo et al. 2014) (Fig. 3).

While $CO_2$ has been classified among the greenhouse gases that drive the global climatic changes, this gas remains an important stimulant for plant C uptake (photosynthesis) and hence vegetative growth and plant productivity. Since the photosynthetic pathway in $C_3$ plant species is suboptimal under current *a*$CO_2$ levels, it has been anticipated that the photosynthetic rates and consequently biomass production for most plant species are more likely to be stimulated by predicted rising in $_{atm}CO_2$ concentrations (Sanz-Sáez et al. 2013; Tausz et al. 2013). In this updated survey, some reports have shown strong and persistent enhancements of $C_3$ photosynthetic rates upon exposure to *e*$CO_2$ (Prasad et al. 2005; Nösberger et al. 2006; Zhang et al. 2011), while others have contrarily noted remarkable reductions in photosynthetic responses of $C_3$ plants to *e*$CO_2$ (Ainsworth and Rogers 2007; Aranjuelo et al. 2013; Irigoyen et al. 2014).

The photosynthetic upgrading in nodulated legumes is usually observed in short-term (i.e., hours to days) *e*$CO_2$ exposures, while this potentiality is normally lost after quite often prolonged exposure (i.e., weeks or months) to *e*$CO_2$ concentrations (Baslam et al. 2014). The lack of $CO_2$ favorable effect on photosynthetic machinery might be a consequence of widely recognized phenomena called *photosynthetic acclimation* or *photosynthetic downregulation* (Leakey et al. 2009; Aranjuelo et al. 2014; Goicoechea et al. 2014). This phenomena could be resulted from either of two processes: (a) diffusional limitations resulted from lower stomatal/mesophyll conductance at *e*$CO_2$ levels, (b) certain metabolic limitations commonly related to the reduced carboxylation activity and protein content and downregulation of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and/or ribulose-1,5-bisphosphate (RuBP) regeneration at higher levels of $CO_2$ (Kanemoto et al. 2009; Leakey et al. 2009; Rosenthal et al. 2014). Rubisco is known to be the key chloroplast enzyme (comprising 25–50 % of leaf total cellular N) in the photosynthetic process with potential activity sensitively influenced by ${}_{atm}CO_2$ and N availability (Goicoechea et al. 2014; Irigoyen et al. 2014). Obviously, the photosynthetic downregulation is a complex phenomenon that has been intensively focused, particularly in the recent years.

Photosynthetic downregulation to $CO_2$ enrichment is closely related to carbohydrate status in plant aerial parts. Exposure of plants to *e*$CO_2$ could strongly accelerate the C assimilation rates, thereby leading to cellular carbohydrate accumulation particularly in the leaves (Prasad et al. 2005; Rogers et al. 2006). A massive production and buildup of carbohydrates in the leaves could exert a negative feedback control on photosynthetic capacity by reducing the synthesis of photosynthetic enzymes. Rubisco is known to be among the most sensitive enzymes which are frequently affected by photosynthetic acclimation in conditions of *e*$CO_2$ (Kanemoto et al. 2009; Goicoechea et al. 2014). Thus, exportation of surplus carbohydrates out of the leaves seems to be decisive in determining plant ability to avoid the inhibition of photosynthetic activity (Tausz et al. 2013). Sucrose is the major form of translocated C in most plants and the main substrate for sink development (Nasr Esfahani et al. 2014a; Sulieman et al. 2014). However, the degree of feedback repression of the photosynthetic apparatus and the form in which excessive carbohydrate could be stored might differ considerably among plant species. Starch remains the major form of leaf cellular carbohydrate reservoir (Almeida et al. 2000; Haase et al. 2007; Sanz-Sáez et al. 2013).

Based on the published literature, two postulated hypotheses have been emphasized to explain the photosynthetic downregulation phenomena: (1) the *C-sink limitation* hypothesis and (2) the *N limitation* hypothesis (Rogers et al. 2009). Firstly, C source–sink relationship has been proposed as a major determinant of plant ability to invest the fertilization effect of *e*$CO_2$ and maintain higher assimilation rate, and therefore avoiding photosynthetic acclimation (Ainsworth and Rogers 2007). Consequently, the plant's ability to induce new C sinks and/or expand the capacity of already existing ones was noted particularly influential (Erice et al. 2011). *Sink* has been defined as the plant parts which, at a given stage of development, are consuming the plant photoassimilates either for construction, respiration, or translocation into storage tissues or organs (Ainsworth et al. 2004). For symbiotic leguminous plants, sink induction or stimulation could represent a good strategy that allows legumes to invest such unlimited supply of photosynthates coming from rising ${}_{atm}CO_2$ levels. Hence, nodulated plants may be able to enhance SNF rate, perhaps as a consequence of greater nodule numbers, nodule mass, or greater nitrogenase activity in the bacteroid fraction of nodules (Rogers et al. 2009) (Table 1). Several results have shown that when grown at *e*$CO_2$, strictly nodulating roots are able to provide sufficient N to the host's shoots in support of the increased N demand in the absence of any limiting factors. Indeed, a growing body of evidence highlights the relevance of root nodules, and more specifically the bacteroids, as strong sinks for photosynthetically derived C (Bertrand et al. 2007b; Imtiaz et al. 2011).

Besides root nodules, inductions of other sink types were reported to provide tremendous benefits for legume growth. For example, mycorrhizal symbiosis was found to be highly beneficial to alleviate the photosynthetic acclimation due to increased C sink strength as a result of the fungi C demand (Kaschuk et al. 2009; Goicoechea et al. 2014). While the C sink strength of rhizobial symbioses is basically related to the respiration associated with SNF, the C sink strength of mycorrhizal symbioses is mostly associated with the growth respiration of mycelium. Fortunately, the majority of leguminous plants have the ability to establish double symbiotic associations with $N_2$ fixing bacteria and mycorrhizal fungi in order to directly modulate the C source–sink balance (Baslam et al. 2014; Irigoyen et al. 2014). Once mycorrhizal symbiosis is functional, the rate of SNF in nodules is expected to be markedly enhanced. Additionally, vegetative storage proteins (VSP) in the taproot were found to represent an additional potential C sink, particularly in forage legumes such as alfalfa (Erice et al. 2007a, b).

A large body of information has emphasized that the capacity of plants to ameliorate the negative photosynthetic acclimation and ultimately maximize the productivity in response to $e\mathrm{CO}_2$ is critically depending on other variables, of which N availability is the most commonly restrictive resource (Luo et al. 2004; Thomas et al. 2006; Guo et al. 2013b). It has been noticed that N concentrations in plant tissues were generally reduced when exposed to $e\mathrm{CO}_2$, which, for instance in leaves, was obviously connected with concomitant reductions in Rubisco content and activity, and therefore, linked to photosynthetic acclimation (Leakey et al. 2009; Sanz-Sáez et al. 2013). Accordingly, and as an alternative to *C sink limitation* mentioned above, the *N limitation* hypothesis was put forward to explain the photosynthetic downregulation at $e\mathrm{CO}_2$ levels. Hence, photosynthetic stimulation and plant growth under $e\mathrm{CO}_2$ conditions are usually accompanied by a corresponding higher internal N demand (Ellsworth et al. 2004; Kanemoto et al. 2009). Under such conditions, extra required N input could be acquired either through intensification of $N_2$ fixation in nodulated legumes and/or with the aid of external mineral-N fertilization (Soussana and Lüscher 2007). In this context, leguminous plants are exceptionally unique referring to their symbiotic association with $N_2$-fixing microorganisms that could support the plant with a vital unlimited N source. Thus, any estimated enhancement of SNF would compensate for cellular reductions in N concentration and maintain optimum C/N ratios and, therefore, would help to ameliorate any possible photosynthesis downward under $e\mathrm{CO}_2$ (Gray et al. 2013). Moreover, it was found that surplus application of 15 mM ammonium nitrate to nodulated alfalfa incubated under $\mathrm{CO}_2$ enrichment was found to be extremely effective in maintaining the photosynthetic stimulations compared with those without combined-N treatment (Sanz-Sáez et al. 2010). One interesting remark that deserves to be mentioned is that external-N supplementation is known to markedly inhibit $N_2$ fixation capacity (e.g., Sulieman et al. 2010, 2014), while its stimulatory response was observed only under conditions of $\mathrm{CO}_2$ enrichment.

Given the fact that N content is usually well correlated with forage quality, it was suggested that N dilution effects (higher C/N ratios) derived from carbohydrates accumulation would alter the feeding values of forage legumes, such as alfalfa (Soussana and Lüscher 2007; Baslam et al. 2014). Exposing plants to *e*$CO_2$ decreased forage quality and in vitro digestibility through a drop in crude protein and an enhancement of fiber content, which collectively would have negative impacts on animal production (Milchunas et al. 2005; Sanz-Sáez et al. 2012). On the other hand, such N dilution in plant tissues would affect the crop resistance to many insect pests (e.g., leaf-chewing insects) (Zavala et al. 2013). Since rising $_{atm}CO_2$ concentrations are expected to alter plant resistance and nutritional quality, i.e., quantity and quality of amino acids, it was hypothesized that nodulated legumes might suffer greater damage from herbivorous insects such as aphids in future climate change (Guo et al. 2014; Johnson et al. 2014).

## 3.3 *Effects of Rising $_{atm}CO_2$ on Nodules*

Although the impact of $CO_2$ on plant growth and photosynthesis has been studied extensively, comparatively less attention has been directed to the role of root nodulation in legume performance under *e*$CO_2$ conditions. Generally, several mechanisms have been highlighted to explain the expected increase in nodule performance under *e*$CO_2$. Nonetheless, only limited information is available today in connection with the factors critically determining the symbiotic efficiency under such climatic changes. Since the nodulation process involves multiple steps and thus offers a number of potential opportunities for modification, this part of the chapter aims to review the major themes of plant nodulation which are thought to be mostly related to mechanisms that regulate the nodule symbiotic efficiency under elevated levels of $CO_2$.

### 3.3.1 Infection and Nodule Initiation

The mutualistic legume–rhizobia symbiosis starts with rhizobial infection and followed by nodule formation, which occur after the bacteria invade the host plant, most usually through root hairs (Oldroyd 2013). Since there are many critical steps in the formation of symbiosis, it seems to be difficult to determine precisely which stage is mostly affected by environmental changes, including *e*$CO_2$. Generally, nodule initiation, development, and function are believed to be sensitively affected by various components of climate change. A significant body of evidence supports the idea that infection of the host plant by root nodule rhizobia increases under $CO_2$ enrichment (Haase et al. 2007). For example and as mentioned above, elevated concentrations of $CO_2$ are known to stimulate the secretion rate of root exudates. Based on the published reports, remarkable increments in root exudation of signals and chemo-attractant substances, such as malate, phenolic compounds, and flavonoids (e.g., genistein, daidzein and coumestrol) that induce Nod factor encoding genes, were frequently noticed at *e*$CO_2$ exposures (Marilley et al. 1999; Montealegre et al. 2000; Prévost et al. 2010). Of particular importance, malate was emphasized as a moderately potent chemo-attractant for the soil bacteria (Yurgel and Kahn 2004). Malonate was additionally revealed as another secreted organic acid that might contribute to symbiosis establishment at *e*$CO_2$ (Haase et al. 2007). Further studies are required to clarify the stimulating effect of increased malonate exudation in the infection and nodule initiation.

### 3.3.2 Nodule Development and Function

The effects of global environmental change on the nodulation process are largely uncertain. $CO_2$ enrichment is expected to influence the legume–rhizobia symbiosis by altering the nodule development (i.e., nodule biomass and nodule number per plant) and nodule function (i.e., nitrogenase activity, $N_2$ fixation, and whole-plant N status) (Almeida et al. 2000; Thomas et al. 2006; Rogers et al. 2009). In a general sense, most researchers believe that long-term exposures to elevated levels of $CO_2$ could enhance SNF capacity mainly via upgrading the nodule development, while short-term exposures are expected to have remarkable impacts on nodule function. Numerous findings documented that the possible enhancement in the rate of SNF as a result of exposure to *e*$CO_2$ is primarily driven by higher increments in nodule number and biomass (Tu et al. 2009; Cernusak et al. 2011; Lam et al. 2012; Guo et al. 2013b). As shown in Table 1, nodule number and biomass of several leguminous plant species, such as soybean, common bean, *Medicago truncatula*, pea, alfalfa, and lentil, remarkably increased at *e*$CO_2$ exposures. However, it remains unclear the extent to which various species achieve greater nodulation performance under elevated levels of $CO_2$. The higher nodule biomass and the possible increase in the nodule number per plant might suggest that there is remarkable enhancement in the photosynthesis and level of photoassimilate supply to the nodulated roots, presumably reflecting a greater sink for C substrates to enhance nitrogenase activity (Aranjuelo et al. 2013). Hence, nodule number and biomass represent important indices for SNF under *e*$CO_2$ treatments.

In addition to the nodule development, many authors have suggested that *e*$CO_2$ could enhance SNF by stimulating the activity of the key enzyme nitrogenase (Rogers et al. 2006; Tu et al. 2009; Lam et al. 2012). Since nitrogenase activity positively correlates with host plant photoassimilate supply, increased photosynthetic C fixation under *e*$CO_2$ is suggested to provide more energy for bacteroids to facilitate SNF within the symbiotic tissues (Prévost et al. 2010; Nasr Esfahani et al. 2014a). Although it is generally believed that SNF is not directly regulated by the availability of C assimilates in the whole plant (Weisbach et al. 1996; Zanetti et al. 1996), some published reports have showed that *e*$CO_2$ caused apparent increase in the rate of nitrogenase activity (Dakora and Drake 2000; Feng et al. 2004; Bertrand et al. 2007b; Guo et al. 2013b). In contrast, other investigations revealed that *e*$CO_2$ concentrations promote SNF mainly by increasing root nodule development rather than by enhancing nitrogenase or its specific activity, i.e., the rate of fixed N per unit nodule and time (Cabrerizo et al. 2001; Rogers et al. 2009; Zhang et al. 2011). Lack of *e*$CO_2$ response on SNF has been attributed to the limitation of nitrogenase to upgrade the specific capacity of fixation (Vance and Heichel 1991; Cen and Layzell 2004; Cabeza et al. 2014). In conformity with this finding, electron allocation coefficient (EAC) of nitrogenase was unchanged by the presence of threefold increase in $CO_2$ concentration around the shoots of *M. truncatula* for duration of 3 weeks exposure time (Cabeza et al. 2014). EAC is known to provide reliable estimates for the relative efficiency of SNF, basically by quantifying the proportion of total electron flow through nitrogenase that is allocated to SNF rather than proton ($H^+$) reduction to $H_2$ (Hunt and Layzell 1993). According to Watanabe and coworkers, *e*$CO_2$ caused significant reductions in the number of *nifH* genes and gene transcripts per nodule of field-grown white clover which could imply a simultaneous reduction in the rate of SNF efficiency, i.e., a lower fraction of plant N derived from atmospheric $N_2$ (%Ndfa) (Watanabe et al. 2013). Thus, the effect of *e*$CO_2$ on nodule function was found to be sometimes positive, neutral, or even negative, based on the test plant examined and experimental conditions assayed (Table 1).

## 4 Regulation of SNF Under *e*$CO_2$ Conditions

SNF is a complex process which is tightly regulated by the hosting plant (reviewed in Minchin and Witty 2005; Sulieman 2011). On the side of the host plants, several complex regulatory mechanisms have been proposed to control SNF and fine-tune the fixation rate according to their actual demand (Nasr Esfahani et al. 2014b). The suggested regulatory mechanisms include, but not limited to, changes in C supply, N-feedback regulation, oxygen ($O_2$) supply, and oxidative stress (Schulze 2004; Schubert 2007; Sulieman and Tran 2013; Sulieman et al. 2013a, b, 2014). Hence, these regulatory checkpoints seem to have great adaptive significance for the symbiotic partners, particularly under nonoptimal conditions (e.g., stresses). In this regard, there has been a considerable debate about the regulation of SNF and its relation to *e*$CO_2$ in the frame of current and projected climatic conditions. Carbohydrate supply and plant N-feedback mechanism are likely to be the main modulators of SNF under *e*$CO_2$. In this part of the chapter, we discuss our current understandings on the regulatory mechanisms of SNF under *e*$CO_2$ on the basis of the recent advances in this research field.

### *4.1 The Role of C/N Balance*

A strong relation between C and N metabolism is widely accepted as a crucial interplay in the regulation of the whole-plant symbiotic performance, particularly under suboptimal conditions (Vadez et al. 2012; Libault 2014). As an example, strong interrelations have been indicated between the levels of cellular amino acids and sugars in different environmental circumstances. Despite the considerable importance of C/N relationship for regulation of SNF at whole-plant level, relatively little information is affordable today about the response of the symbiotic efficiency to C/N balance when exposed to *e*$CO_2$ conditions. Increasing the concentration of $_{atm}CO_2$ is known to directly modify the rates of photosynthesis, thereby affecting the C/N balance as well as the nodulation efficiency (Fig. 3). Hence, maintenance of the C/N balance remains of particular importance for the various biochemical reactions occurred inside the symbiotic tissue and plant regulatory network. For cellular metabolic adjustments, raising plant N concentration with the increase of SNF efficiency will be necessary as an integral part of maintaining an optimal C/N balance at $CO_2$-enriched conditions (Zanetti et al. 1996; Libault 2014).

## *4.2 C Supply*

An adequate supply of carbohydrate is an essential requirement of nodule functioning as SNF is expensive in terms of energy and C for synthesis of fixed-N products (reviewed in Minchin and Witty 2005; White et al. 2007; Valentine et al. 2011). An increase in $CO_2$ concentration was supposed to stimulate photosynthesis and C allocation to belowground processes, thereby increasing C supply to support the function of nodules. Based on the results of some studies, photoassimilates were able to reach nodules within the range of few minutes (Cabeza et al. 2014). Sucrose is known to be the predominant sugar quantified in nodules mainly to support the nitrogenase activity (Sulieman and Schulze 2010a, b; Udvardi and Poole 2013) (Fig. 1). In most cases, higher photosynthetic rates of plants exposed to *e*$CO_2$ concentrations were translated into larger nodule carbohydrate levels (Arrese-Igor et al. 1999; Cabrerizo et al. 2001; Thomas et al. 2006; Haase et al. 2007). The metabolism of sucrose in nodules represents a vital regulatory process which is likely to be central to co-ordinate C and N metabolism in $N_2$-fixing legumes (Schulze 2004; Sulieman and Tran 2013).

The key enzyme of sucrose degradation appears to be sucrose synthase (SS, EC 2.4.1.13), although an alkaline invertase (AI, EC 3.2.1.26) is also present in small amount (Arrese-Igor et al. 1999; Nasr Esfahani et al. 2014a). Further metabolism of the products of sucrose degradation is most likely to occur through the glycolytic pathway and involve another potential regulatory enzyme: phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) (Fig. 1). This enzyme links carbohydrate metabolism to both organic acid and amino acid metabolism since the product of the reaction, oxaloacetic acid, may be reduced to malate or transaminated to aspartic acid. Malate is believed to be the major substrate to enter into the $N_2$-fixing bacteroid respiration to produce the energy and reducing power required for the reduction of $N_2$ to ammonia (Schulze et al. 2002; Sulieman et al. 2013a, b). Enhanced malate accumulations in nodules of plants exposed to *e*$CO_2$ might reflect an improved supply of nodules with organic C (Arrese-Igor et al. 1999). Higher levels of malonate were additionally observed in nodule of plants exposed to *e*$CO_2$ concentrations (Haase et al. 2007). According to the findings of some published reports, this $C_3$-dicarboxylic acid represents an important C source during SNF (Schramm 1992). Nevertheless, the role of malonate in nodulated roots during symbiosis deserves further investigations (Karunakaran et al. 2013). Overall, a significant body of evidence highlights the relevance of $CO_2$ concentration to the organic acid status and nodule symbiotic activity (Arrese-Igor et al. 1999). Other contrary results have showed that elevated levels of $CO_2$ did not either affect or enhance alfalfa nodule activity besides the status of some related parameters such as plant and bacteroid soluble proteins (Aranjuelo et al. 2008). Interestingly, the activities of some key metabolic components (i.e., PEPC, aspartate aminotransferase, malate dehydrogenase, isocitrate dehydrogenase) were found to be significantly reduced in response to *e*$CO_2$ treatment. Since stored carbohydrates act transiently as a C source particularly in conditions of surplus C supply, it was assumed that the availability of stored carbohydrates would be largely affected by $CO_2$ fertilization. Elevated $CO_2$ significantly increased sugar concentrations in nodules as well as root starch concentrations (Cabrerizo et al. 2001). Starch is known to be one of the principle storage carbohydrates in nodules (Garg 2007). Instead of C consumption for plant growth stimulation, remarkable massive starch accumulation has been hypothesized as one of the possible causes for the absence of growth responses to *e*$CO_2$ in some plant species (Haase et al. 2007).

In addition to the major role played by host plant carbohydrate supply for supporting nodule activity, some other findings have emphasized nodule direct $CO_2$ fixation and its vital role in C provision under conditions of *e*$CO_2$ levels (Yamakawa et al. 1997, 2004; Fischinger and Schulze 2010; Fischinger et al. 2010) (Fig. 1). Nodules were recognized to have good potential capabilities of fixing substantial amounts of $CO_2$ through the combined action of PEPC and carbonic anhydrase (CA, EC 4.2.1.1), both of which represent vital components of nodule C metabolism, thereby resulting in a more efficient SNF system (Yamakawa et al. 2004; Fischinger et al. 2010; Fotelli et al. 2011; Tsikou et al. 2011). Hence, substantial amounts of organic acids (i.e., malate) would be available for supporting the bacteroid activity as well as provision of required C skeletons for N assimilation in the symbiotic tissues (Tsikou et al. 2011). Indeed, nitrogenase activity was noted to be well correlated with nodule $CO_2$ fixation. In this way, nodule $CO_2$ fixation may represent a *C-saving* mechanism particularly in occasions of limited C availability, i.e., when the fixed C is used for N assimilation and N transport to shoots (Fischinger and Schulze 2010; Fischinger et al. 2010). According to the authors' opinion, nodule $CO_2$ fixation is expected to be highly beneficial mainly in conditions which impair the photosynthetic activity and assimilate supply to functioning nodules as observed under phosphorus (P) deprivation conditions (Fischinger and Schulze 2010).

### 4.3 N-Feedback Regulation

It has been generally believed that external environmental conditions strongly affect legume growth and development, thereby altering the potential demand for essential nutrients including N (Vadez et al. 2000; Sulieman et al. 2010). Host-N demand is known to determine the response of nodules to external stimuli, including $CO_2$ enrichment. Pending on the surrounding environmental triggers, SNF might be enhanced, preserved, or sometimes entirely suppressed according to the potential N requirement of the host plant. The higher C cost of nitrogenase activity is the major reason behind such physiological modulations. As a result, the plant's demand for symbiotically fixed N is believed to play a central role in adjusting both nodulation and SNF activity with whole-plant N-feedback being the master regulatory mechanism (Schulze 2004; Schubert 2007; Sulieman and Tran 2013). The N-feedback concept suggests that nodule development and function are continuously regulated in accordance with the N-sink strength.

As put forward by Parsons and others, the N-feedback mechanism could be the main trigger for regulating both nodulation and SNF activity in mature nodules of various leguminous plants (Parsons et al. 1993). Such hypothesis is so far supported by many physiological data in connection with the internal soluble-N pool of the host plant (in shoot, phloem sap, or nodules); thus it looks very plausible. Several N substances (satiety-N signals) have been proposed to take part in the modulation of the symbiotic activity (Fig. 1). Part of the suggested substances include asparagine (Almeida et al. 2000; Sulieman and Tran 2013), glutamine (Neo and Layzell 1997), glutamate (Fischinger et al. 2006), ureides (Ladrera et al. 2007; Serraj et al. 2001), γ-aminobutyric acid (Sulieman and Schulze 2010b; Sulieman 2011), proline (Larrainzar et al. 2009), polyamines (Whitehead et al. 2001), or combinations of these compounds (Lima and Sodek 2003; Vadez et al. 2000).

The N-feedback hypothesis was initially proposed as the mechanism by which nodulation responds to combined-N supply (Parsons et al. 1993). Currently, this concept has been formulated to represent the master control by which SNF plants respond to several unfavorable conditions including P deficiency (e.g., Almeida et al. 2000; Sulieman et al. 2013a, b), water stress (e.g., Nasr Esfahani et al. 2014b), defoliation (e.g., Hartwig and Trommler 2001), partial leaf darkening (e.g., Fischinger et al. 2006), and partial nodule excision (e.g., Sulieman and Schulze 2010b; Sulieman 2011). However, the nature of the whole-plant N-feedback mechanism remains to be elucidated. In this context, some possible features of the N-feedback regulation of nodule activity particularly under drought stress are discussed by González and colleagues in this volume (Chap. 2).

On the other hand, regulation of SNF at elevated levels of $CO_2$ appears to be under tight control of the host-N demand. This response has been arising from numerous experiments aiming to investigate the impact of *e*$CO_2$ on legume symbiotic performance (Lüscher et al. 2000; Serraj and Sinclair 2003). The higher plant growth and biomass production under future $CO_2$-enriched environments are expected to bring additional N requirement to support the plant growth and development, consequently leading to higher stimulations in nodule symbiotic activity (Daepp et al. 2000; Haase et al. 2007; Nasser et al. 2008; Prévost et al. 2010). Some documented findings have revealed that there was an enhancement in SNF in response to the $CO_2$-induced increment in the N demand of the examined plant species (Rogers et al. 2006; Tu et al. 2009; Lam et al. 2012). Given the fact that accumulation of various soluble N substances in the vascular tissues of the symbiotic plant has been closely linked to the downregulation or suppression of nitrogenase activity (Fischinger et al. 2006; Sulieman et al. 2010; Sulieman and Tran 2013) (Fig. 1), it has been suggested that $e\mathrm{CO}_2$ is going to ameliorate the accumulation of N compounds in plant tissue, thereby reducing their negative impacts on nodule function (Serraj 2003; Serraj and Sinclair 2003). If this scenario is correct, one would expect that SNF plants would be highly tolerant to adverse conditions, such as water deficit (Serraj 2003) or P deficiency (Almeida et al. 2000), in the frame of future global environmental changes associated with increased $_{\mathrm{atm}}\mathrm{CO}_2$. Hence, the anticipated advantage of leguminous plants under global climate change is likely to be greater than originally presumed.

## 5 Interaction of $e\mathrm{CO}_2$ with Other Climate Change Elements

Although $e\mathrm{CO}_2$ may stimulate nodule symbiotic activity, numerous reports have indicated that the overall response of leguminous plants varies greatly pending on the interaction with other environmental factors (Leakey et al. 2012; Goicoechea et al. 2014; Rosenthal et al. 2014). In this context, somewhat inconsistent or contradicted findings are perhaps not surprising because SNF is a complex process and highly related to surrounding environment as well as genetic background of the tested plant (Bertrand et al. 2007a; Zhang et al. 2011). In most cases, environmental factors override the genetic factors in terms of response to $e\mathrm{CO}_2$ (Tausz et al. 2013). Thus, for drawing any reliable prediction or valid conclusion regarding the impact of $e\mathrm{CO}_2$ on future performance of legumes, it is quite essential to simulate the combining effect of $e\mathrm{CO}_2$ with other climatic elements (e.g., temperature, nutrient availability, radiation, wind speed, relative humidity, water availability) as realistically as possible (Thomas et al. 2006; Erice et al. 2007a; Irigoyen et al. 2014).

Many comparative studies have estimated that the response of plant growth to $e\mathrm{CO}_2$ is much stronger in field conditions compared with those performed in control environments (Almeida et al. 2000; Suter et al. 2002; Gray et al. 2013). Hence, the performance of plants grown in the field substantially differs from those produced in controlled environmental conditions, i.e., growth chambers, greenhouses etc. (Sanz-Sáez et al. 2013; Watanabe et al. 2013). The lack of trials that integrate measures of physiology and structure to understand whole-plant function is particularly acute in the field. Fortunately and during the recent decades, several new approaches which simulate the natural field conditions have been developed and favored by many research bodies. Some adopted environmental techniques include, but not limited to, free-air $CO_2$ enrichment (FACE) systems, free air temperature increase (FATI), open top chambers (OTCs), and temperature gradient tunnels (TGTs) (Table 1). These approaches have been widely used to evaluate the impact of several important environmental factors in plant growth responses to different climate change scenarios (Leakey et al. 2012; Irigoyen et al. 2014; Rosenthal et al. 2014).

## 6 Application of Biotechnology for Improved SNF Under *e*$CO_2$

Predicted increases in $_{atm}CO_2$ concentrations critically bring many challenges for plant breeders to cope with current and future climatic changes (Ainsworth and Ort 2010; Tester and Langridge 2010). Climate change scenarios suggest that suitable improved cultivars for successful future cultivation must be able to withstand variable conditions including *e*$CO_2$. Understanding the nature of such climatic constraints will facilitate breeders to design appropriate adaptation strategies which could have profound significance for improving agricultural sustainable production (Prasad et al. 2005). On the other hand, omics and biotechnology provide numerous advances and sophisticated techniques for better understanding and possible manipulations of plant performance at various biological and environmental levels, thereby boosting conventional plant breeding approaches (Pritchard 2011; Tausz et al. 2013). Recently, the whole genome sequencing for several model plants, especially *M. truncatula* and *Lotus japonicus*, has enabled researchers to discover novel genes at high-throughput manner and understand gene functions which contribute to the improved production of plants under such variable climatic conditions.

While the impact of *e*$CO_2$ on legume growth and nodulation has been intensively focused, not much effort has been directed for identifying the molecular mechanisms controlling such response (Rogers et al. 2009; Libault 2014). Accordingly, intensive breeding and plant molecular genetics research programs have to be considered mainly to facilitate the development of climate-resilient cultivars for predicted environmental changes. Cultivar creation with increased utilization of *e*$CO_2$ for photosynthesis is an urgent requirement that seems to be one of the most promising strategies in this platform (Kant et al. 2012; Libault 2014). Some progress has already been made in this regard. Another promising genetic engineering strategy for improving SNF efficiency might be related to better improvement of nodule $CO_2$ fixation particularly under conditions of global environmental changes associated with *e*$CO_2$ noted above (Yamakawa et al. 2004; Fischinger and Schulze 2010; Fischinger et al. 2010) (Fig. 1). We believe that genetic manipulation of legumes for positive acclimation is an extremely useful strategy to obtain optimal crop yields under predicted changing global climate. Future genetic breeding programs could focus on developing elite *e*$CO_2$-adapted cultivars based on these and other related directions.

## 7 Future Perspectives

The exact consequences of alarming rise in $_{atm}CO_2$ concentration are difficult to predict due to the existence of its interactive relationships with several other climatic elements. Despite the fact that there is much current effort dedicated for identifying the responses of legumes to *e*$CO_2$, there are still key knowledge gaps (Rogers et al. 2009). Addressing such gaps should be of high priority as it is vital for future legume breeding programs that aim to maximize plant production in a changing environment. Improved mechanistic understanding of leguminous plant responses to *e*$CO_2$ will require a collaborative international effort among plant physiologists, agronomists, molecular biologists, modelers, and breeders, because many disciplines are likely to be involved. Therefore, a thoughtful framework will be necessary to develop ambitious robust strategies for creating elite adaptive cultivars if we are to meet global food and feed demands (Prasad et al. 2005; Erice et al. 2011). This approach will also enable development of new avenues of research for improving the symbiotic efficiency of the leguminous plants under future climatic changes. The potential of exploiting genetic diversity, along with using adaptation strategies, could help mitigate the expected negative impacts of climate change and potentially improve legume production in the future. The potential of exploiting sophisticated techniques such as high-throughput omics, bioinformatics, and molecular genetics tools will be highly beneficial in this regard (Araújo et al. 2015). Further studies are needed to extend understanding of legume responses to climate changes, as well as to identify *e*$CO_2$-adapted leguminous cultivars that will achieve greater and more efficient production values under hostile climatic conditions.

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# Physiological Implications of Legume Nodules Associated with Soil Acidity

**Brett J. Ferguson and Peter M. Gresshoff**

**Abstract** The mutualistic legume-rhizobia symbiosis that leads to nodule formation and ensuing nitrogen fixation is of great importance to soil health and agriculture sustainability. The processes of nodulation and nitrogen fixation can improve the nitrogenous content and overall structure of the rhizosphere; however, they are highly sensitive to environmental parameters, such as soil acidity. Indeed, low pH conditions can appreciably diminish the benefits associated with legumes and can decrease their biomass and yields. This is of considerable economic and agricultural importance as legume crop and pasture species are commonly grown on acidic soils. Acidic growing conditions can reduce the overall fitness of both the legume host and the rhizobia partner, in addition to directly hindering the establishment of nodule structures. This includes impairing the signal exchange between the two symbiotic partners and inhibiting their ability to express critical nodulation genes. The effect of soil acidity on legume and rhizobia growth is addressed here, with a focus on nodule development and function. Practices to help overcome limitations associated with growing legumes in low pH environments are addressed, with an emphasis on how current biotechnology and molecular genetic approaches could aid in overcoming the negative effects associated with low pH.

**Keywords** Autoregulation of nodulation • Legumes • Nitrogen fixation • Plant signalling and development • Rhizobia • Symbiosis

## 1 Introduction

Nodulation is a powerful mechanism that improves the competitiveness of legume plants by enhancing their ability to acquire nitrogen. It involves a symbiotic relationship with nitrogen-fixing rhizobia bacteria, which provide the host plant with an available form of nitrogen (e.g. ammonia) in exchange for an energy-providing carbon source (e.g. malate). This exchange takes place in highly specialised legume

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organs, called nodules (Ferguson et al. 2010; Desbrosses and Stougaard 2011; Oldroyd 2013). The formation of these nodules by the host plant requires substantial resources and is balanced with other developmental processes such a lateral root formation; thus, legumes have developed various mechanisms to tightly regulate the number of nodules they form.

In favourable conditions, legume plants regulate their nodule numbers via a systemic process called the Autoregulation of Nodulation (AON). The process was first described by Gresshoff and Delves (1986) and Delves et al. (1986), based on physiological and genetic evidence and has continued to be validated by molecular breakthroughs that support the earlier findings (e.g. Wopereis et al. 2000; Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003, Schnabel et al. 2005; Okamoto et al. 2009, 2013; Mortier et al. 2010, 2012; Reid et al. 2011b, 2013; reviewed in Reid et al. 2011a; Hayashi et al. 2013). By controlling the number of nodules that form, the AON process essentially provides the host plant with a means of balancing nitrogen acquisition (i.e. nitrogen fixation by its rhizobia partner) with resource expenditure (i.e. developing and maintaining nodule structures, and sustaining the rhizobia).

The nitrogenous content of the surrounding rhizosphere can also regulate the extent of legume nodulation by acting as a strong inhibitor of nodule formation (e.g. Carroll et al. 1985a, b; Reid et al. 2011b). This mechanism likely evolved so that the host plant can avoid forming and maintaining nodules when ample nitrogen is already available. In addition to nitrogen, the plant hormone ethylene, which is often associated with stress, can be a strong inhibitor of nodulation (e.g. Penmetsa and Cook 1997; Oldroyd et al. 2001; reviewed in Guinel and Geil 2002; Gresshoff et al. 2009; Ferguson and Mathesius 2003, 2014). By shutting down nodule development, ethylene could help the plant to save resources for coping with the immediate stress. Other environmental factors that can regulate nodulation include water stress (González et al. 2015, Chap. 2), salinity (Lluch and Cobos-Porras 2015, Chap. 3), temperature (Aranjuelo et al. 2015, Chap. 4), and soil pH, the latter factor being the focus of this chapter.

Acidic soils are considered to be those having a pH of 5.5 or less. Over 30 % of the world's soils are deemed to be acidic, including many located in key agricultural regions, with an estimated 40 % of arable land being acidic (Von Uexküll and Mutert 1995; Ferguson et al. 2013). Numerous factors can lead to the acidification of soil, including leaching, harvesting of crops, low nutrient cycling and excess fertilisation. Thus, intensive agricultural practices can reduce soil pH, and this is expected to worsen as factors associated with climate change intensify and agricultural activities increase to help feed the world's growing population. Acidic conditions have a considerable influence over the makeup of the rhizosphere, typically reducing the availability and retention of essential nutrients and increasing the content of toxic $Al^{3+}$ and $Mn^{2+}$ ions (Kinraide 1991; Marschner 1991; Ryan et al. 1993; Eswaran et al. 1997). Indeed, $Al^{3+}$ can be highly toxic and is widely associated with reduced crop productivity in acid soil regions (Kopittke et al. 2015).

In agriculture, acidic growing conditions are frequently associated with significant reductions in crop biomass and yield. Yield losses of over 50 % have been attributed to low pH for a wide range of crop species, including both legume and

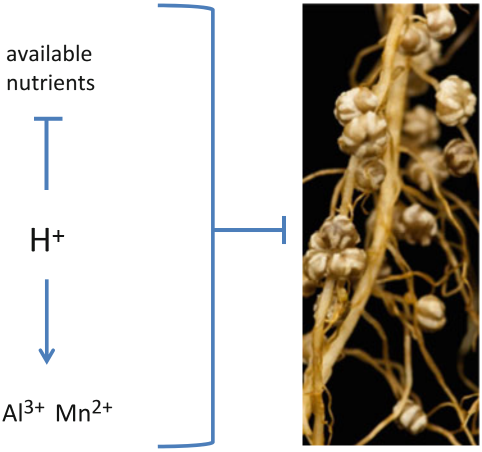

**Fig. 1** Effect of low pH on legume nodulation. Acidic growing conditions increase the amount of $Al^{3+}$ and $Mn^{2+}$ in the soil and reduce the amount of available nutrients. All of these factors have a negative effect on legume nodulation

non-legume varieties (Ferguson et al. 2013). In regards to plant physiology, acidic growing conditions can restrict nutrient uptake, which can considerably impair plant growth and development. Moreover, the presence of $Al^{3+}$ can inhibit cation uptake and negatively regulate the division and elongation of root cells (Ryan et al. 1993; Kopittke et al. 2015).

Legumes represent some of the most important food, feed, pasture and biofuel crops (Jensen et al. 2012; Gresshoff et al. 2015). They are frequently used in crop rotation and in intercropping due to their ability to nodulate and enhance the nitrogen status of the soil. Indeed, nodulation and nitrogen fixation can extensively benefit the soil content and structure, thus helping to improve the surrounding environment and increase crop biomass and yields. It also helps to alleviate the excessive use of nitrogen-based chemical fertilisers in agriculture, which are expensive and often pollute (Peoples et al. 2009; Jensen et al. 2012; Ferguson 2013; van Hameren et al. 2013). However, these benefits of legumes can be derailed by soil acidity, which can substantially impair nodulation and nitrogen fixation, and considerably diminish their advantages (Fig. 1; Ferguson et al. 2013). This is of considerable importance for many legume crops, such as soybean, which is one of the world's most economically significant crops (Ferguson 2013; van Hameren et al. 2013) and is predominately grown on acidic soils (Lin et al. 2012). The effect of soil acidity on legume nodulation and nitrogen fixation is reviewed here, with future strategies put forth that could help to overcome the negative effects low pH growing conditions exert on these critical processes.

## 2 Impact of Soil Acidity on Rhizobial Biology

Low pH markedly influences rhizobia by negatively affecting their growth and overall ability to function. This can dramatically reduce rhizobia populations and can impair their facility to enter into a symbiotic relationship with legume plants (e.g. Munns 1968; Wood et al. 1984; Whelan and Alexander 1986; Vargas and Graham 1988, 1989; Brockwell et al. 1991; Glenn and Dilworth 1994). These negative effects are often directly attributed to the elevated presence of $H^+$ ions in acidic soils, and also to the resultant increase in toxic metal ions, such as $Al^{3+}$ and $Mn^{2+}$ (Fig. 1). Collectively, these ions can disrupt the general function of the rhizobia and can noticeably weaken their overall competitiveness (e.g. Keyser and Munns 1979; Wood et al. 1984). Acidic growing conditions can also reduce the expression of key nodulation genes of rhizobia, including *nodA* (Richardson et al. 1988). This can lead to a decrease in the biosynthesis of the rhizobia-derived Nod factor signal (McKay and Djordjevic 1993), which is a component involved in the signal exchange and recognition of the two symbiotic partners and is essential for the Nod factor-induced nodulation pathway.

The severity of the effects that low pH exerts on rhizobia is highly dependent on the strain. Although acidic growing conditions are inhibitory to most rhizobia, acid-tolerant strains have been isolated for many species. Slow growing *Bradyrhizobium* species are typically more tolerant than faster growing *Rhizobium* species; however, acid-tolerant strains of both *Bradyrhizobium* and *Rhizobium* have been isolated (e.g. Graham et al. 1982, 1994; Cunningham and Munns 1984; Wood et al. 1988; Correa and Barneix 1997; Vargas and Graham 1988, 1989; Appunu and Dhar 2006). Tolerance can be achieved via a number of mechanisms, including variations in membrane properties and structure, extracellular polysaccharide production, cytoplasmic buffering and the active exclusion of protons (Cunningham and Munns 1984; Aarons and Graham 1991; Glenn and Dilworth 1994; Correa and Barneix 1997; Rojas-Jiménez et al. 2005). However, despite the successful isolation of acid-tolerant rhizobia strains, it is important to note that they often fail to provide acid-tolerant symbiosis in agriculture.

A number of candidate rhizobia genes have also been identified that act in acid stress response and/or tolerance. Two genes of *R. meliloti*, called *actS* and *actR*, are thought to function as a two-component protein pair in pH sensing and/or response (Tiwari et al. 1996). An additional gene of *S. meliloti*, called *Rpoh1*, acts in low pH stress response by regulating the expression of other genes (de Lucena et al. 2010). Moreover, in *R. tropici* strain CIAT899, the genes *sacA* (encoding a ClC chloride channel homolog) and *olsC* (encoding a putative β-hydroxylase that modifies ornithine-containing membrane lipids) were found to contribute to the symbiotic performance and acid tolerance of the bacteria (Rojas-Jiménez et al. 2005).

## 3 The Metabolic Modifications of Nodule $N_2$ Fixation Under Soil Acidity

Acidic growing conditions can reduce the extent of nodulation in an extensive range of legumes, including both determinate- and indeterminate-nodule forming species (reviewed in Ferguson et al. 2013; Miransari et al. 2013). Nodulation is typically more sensitive to low pH than other aspects of plant development (Evans et al. 1990) and can be completely abolished, particularly when acid-sensitive species are grown in low pH conditions. The processes of legume-rhizobia communication, nodule initiation and development, nodulation control and nitrogen fixation are all tightly regulated by signalling mechanisms that can be interfered with by low pH. This can be due to acidic conditions directly regulating the production of key plant and bacterial factors and/or influencing the overall fitness of the symbiotic partners to indirectly impair nodule development and associated nitrogen fixation.

Low pH conditions profoundly interfere with host-microbe communication. Legume exudates are reduced by low pH and the addition of compatible flavonoid molecules can help to alleviate decreases in nodulation attributed to acidic conditions (Hungria and Stacey 1997; Miransari and Smith 2007). The production of critical rhizobia-produced Nod factor signal molecules is also hampered by low pH (McKay and Djordjevic 1993). Acidic growing conditions reduce the extent of Nod factor-induced events occurring during the initial stages of nodulation, including root hair deformation and curling (Munns 1968; Miransari et al. 2006) and early rhizobia infection events, such as the attachment to root hairs (Caetano-Anollés et al. 1989; Howieson et al. 1993). As with flavonoids, the application of compatible Nod factor molecules helps to overcome the inhibitory effects that low pH exerts on nodulation (Miransari et al. 2006).

The expression of a number of key early nodulation genes is also reduced in plants grown in acidic conditions (e.g. Lin et al. 2012). Treatment with various pH solutions to split-root grown soybean plants demonstrated that this decrease in early nodulation gene expression is systemic (Lin et al. 2012). Later stages of nodulation, including cell division events, nodule primordia establishment and nodule growth are also reported to be impaired by acidic growing conditions (e.g. Lie 1969; Vassileva et al. 1997). These reductions are further compounded by the inhibitory effects that low pH has on the growth and development of essential roots and root hairs that are needed for nodulation. In addition to issues relating to the initiation and formation of nodule structures, nitrogen fixation can also be considerably diminished by growth in acidic conditions (e.g. Döbereiner 1966; Graham 1981; Vassileva et al. 1997; Taylor et al. 1991). This reduction in nitrogenase activity and subsequent loss of nitrogen acquisition is typically offset by the application of chemical nitrogen-based fertilisers, thus removing one of the central benefits to using legumes as crop species.

In addition to interfering with the initiation and development of nodules (see Ferguson et al. 2010 for a developmental description of such stages), acidic growing conditions can reduce nodulation by inducing regulatory mechanisms of the host. This is achieved indirectly by reducing plant fitness and stimulating stress responses (e.g. the production of ethylene, a strong inhibitor of nodulation; reviewed in Guinel and Geil 2002; Gresshoff et al. 2009; Ferguson and Mathesius 2003, 2014). It can also be achieved directly by influencing molecular mechanisms, such as the AON pathway that acts in nodulation control (reviewed by Ferguson et al. 2010; Reid et al. 2011a). The AON pathway commences within hours of rhizobia inoculation (e.g. Bhuvaneswari et al. 1981; Turgeon and Bauer 1982; Reid et al. 2011b) and is signified by the production of novel, plant-derived peptide hormones (reviewed in Hastwell et al. 2015). In soybean, these peptides are called GmRIC1 and GmRIC2 (Reid et al. 2011b, 2013), with orthologues also discovered in *Lotus japonicus*

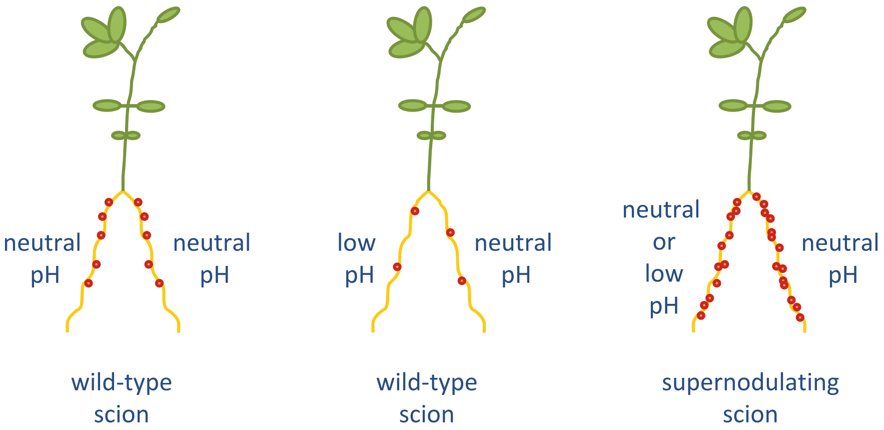

**Fig. 2** Systemic effect of acidic growing conditions on legume nodulation. Low pH conditions reduce the extent of legume nodulation. However, supernodulating mutants of soybean form significantly more nodules than wild-type plants, even when grown in acidic conditions. Split root and grafting studies showed that treatment with low pH can inhibit nodulation systemically in plants having a wild-type scion, but a systemic reduction was not observed in plants having a supernodulating mutant scion

(Okamoto et al. 2009, 2013), *Medicago truncatula* (Mortier et al. 2010, 2012) and *Phaseolus vulgaris* (common bean; Ferguson et al. 2014). These rhizobia-induced peptides are transported in the xylem from their site of synthesis in the root up to the shoot, where they are perceived by an LRR receptor kinase (Okamoto et al. 2013). This receptor is called GmNARK in soybean, and its orthologues are LjHAR1, MtSUNN, PsSYM29, GsSYM29, PvNARK in other species (Krusell et al. 2002; Nishimura et al. 2002; Searle et al. 2003; Schnabel et al. 2005; Ferguson et al. 2014).

Perception of the CLE peptides by the LRR receptor kinase (possibly in association with other receptor and/or kinase components) leads to the production of a novel shoot-derived inhibitor (SDI) signal that is transported back down to the root where it acts to prevent further nodulation events from occurring (Lin et al. 2010, 2011). The nodulation phenotype of supernodulating *Gmnark* mutant plants is far less sensitive to low pH than that of wild-type plants (Fig. 2; Alva et al. 1988; Lin et al. 2012). Recent work using split-root plants of soybean demonstrated that acidic growing conditions can influence a systemic mechanism to reduce nodule formation in wild-type plants, but a similar systemic reduction was not observed in grafted split-root plants having a supernodulating *Gmnark* mutant scion (Fig. 2; Lin et al. 2012). These findings suggest that soil acidity may influence the expression and/or activity of a nodulation-suppressing factor, such as a nodulation-inhibiting CLE peptide. Such a mechanism would enable the plant to tightly restrict its nodule numbers when grown in unfavourable pH conditions, and thus conserve its resources for coping with the low pH stress.

A similar molecular mechanism exists in the nitrate inhibition of nodulation pathway, where the nitrogenous content of the rhizosphere triggers the production of a soybean peptide, called GmNIC1, which acts locally in the root via GmNARK to suppress nodule formation (Reid et al. 2011b; reviewed in Hastwell et al. 2015).

A number of legume varieties have been isolated that exhibit acid tolerance. This includes various lines found in the highly acidic Caatinga and Cerrado regions of Brazil (dos Reis et al. 2010). Adaptive tactics of the plant include slowing their overall rate of growth and employing mechanistic strategies, such as increasing the soil pH with root exudates, releasing chelators for Al ions, restricting acid influx and compartmentalising toxic anions (e.g. Marschner 1991; Andersson 1988). Recent evidence has indicated that malate exudation from the root may also be critical for adaptation to acid soil; this process is synchronised by pH, Al, and P via the regulation of the root cell plasma membrane transporter, ALMT1 (Sasaki et al. 2004; Liang et al. 2013).

## 4 Application of Biotechnology for Improved $N_2$ Fixation Under Soil Acidity

Current agricultural efforts to mitigate low soil pH include liming, which refers to the practice of applying calcium carbonate directly to the soil to bind $H^+$ ions and raise the pH (e.g. Calder et al. 1965). Other approaches, such as treating plants with flavonoids, Nod factors or phytohormones, can help to overcome the negative effects of low pH in glasshouse trials but are not always feasible or practical in the field. Additional strategies geared towards improving nodulation, nitrogen fixation and overall crop yields in low pH environments focus on modern-day breeding and engineering strategies to generate acid-tolerant legume cultivars and rhizobia strains. These latter strategies show the most promise for bolstering crop yields and improving agricultural sustainability in the face of a rising global population and the resultant increased demand for food (e.g. Olivares et al. 2013; Ray et al. 2013).

Increasing the molecular understanding of the mechanisms driving acid response and tolerance will provide genetic targets that can be used for the selection of improved legume cultivars and rhizobia strains. This includes identifying key genes and molecular markers associated with phenotypic traits of interest. Traits to enhance could include those associated with legume-rhizobia recognition and interaction, optimising nodule development and control, and increasing nitrogen-fixation rates and nitrogen-use efficiency in acid soils. Achieving these molecular advances will require an interdisciplinary approach involving expertise in areas of biotechnology, genomics, bioinformatics, molecular physiology and applied functional genetics.

The current “omics” era (genomics, transcriptomics, proteomics, metabolomics) is considerably advancing the identification of molecular factors and polymorphic markers associated with the key processes of interest. Indeed, the availability and continued improvement of complete-genome sequences, in tandem with other modern molecular techniques, is enabling rapid progress to be made. Soybean is one species that is benefiting from these technological advances, with genomes from multiple cultivars having been sequenced (Schmutz et al. 2010; Lam et al. 2010) and transcriptomes from an array of tissues having been generated (e.g. Libault et al. 2010; Hayashi et al. 2012; Reid et al. 2012). Collectively, these recent scientific advances, in addition to continued progress in areas of molecular genetics and functional genomics, represent powerful tools for the discovery of genes and polymorphic markers associated with QTLs and phenotypic traits of interest. Breakthroughs resulting from these advances could lead to the optimisation of developmental processes such as nodulation and nitrogen fixation that help to overcome the negative effects of acidic environments on crop biomass and yield, thus benefiting agricultural sustainability and food security.

## 5 Future Perspectives

Optimising the use of legumes and rhizobia and the extent/efficiency of nodulation and nitrogen fixation is critical to sustainable agriculture and food security. Biotechnology and molecular genetics will almost certainly be at the forefront of future breakthroughs in this area, including advances that help to overcome the negative effects imposed on the symbiosis by acidic growing conditions. Indeed, decisions made now will directly impact on future cultivars/strains and agricultural practices, with modern technologies enabling progress to be achieved faster than ever. Current advances in understanding the molecular mechanisms that drive the development and control of nodulation, and the extent of nitrogen fixation, will enable a more targeted approach to enhance breeding programmes. This will be of considerable benefit to the isolation of elite crop varieties and inoculant strains, and could aid in developing policies and practices that improve agricultural sustainability and food security.

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# About the Editors

**Dr. Saad Sulieman** was graduated at the Faculty of Agriculture, University of Khartoum, in 1998 with distinction in Agronomy and was awarded many prizes for being the best graduate. He joined the Department of Agronomy, Faculty of Agriculture, University of Khartoum, as a Teaching Assistant in 1999. Subsequently, he was trained for M.Sc. in Crop Sciences at the University of Khartoum (2002) and then for Ph.D. in Agricultural Chemistry (2009) at Georg-August University of Göttingen, Germany, with a Ph.D. scholarship from German Academic Exchange Service (DAAD). During his Ph.D. pursuit, he focused on investigating the physiological characterization of symbioses contrasting tolerance in nitrogen fixation to major soil limitations (low soil bio-availability and excess of nitrate). After completing his Ph.D., he continued to work as a Research Assistant at the same institute for one and half year. He also worked as a Postdoctoral Fellow at RIKEN Center for Sustainable Resource Science (CSRS), Yokohama, Japan, for one year (2013) with a Postdoctoral Fellowship from the Japan Society for the Promotion of Science (JSPS). Sulieman was promoted to Lecturer in 2005, Assistant Professor in 2009, and Associate Professor in 2013. He is actively involved in the teaching of

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undergraduate and postgraduate students, and throughout his career he trained many students to the level of B.Sc. and M.Sc. Since 2014, he has been holding the position of Head of Department of Agronomy, University of Khartoum. Throughout the years of his research career, he has attended many domestic and international meetings, conferences, seminars, and workshops. He has authored or coauthored more than 30 publications. The main thrust of his research is in the field of molecular plant physiology of abiotic stress.

**Dr. Lam-Son Phan Tran** is Head of the Signaling Pathway Research Unit at RIKEN Center for Sustainable Resource Science, Japan. He obtained his M.Sc. in Biotechnology in 1994 and Ph.D. in Biological Sciences in 1997 from Szent Istvan University, Hungary. After doing his postdoctoral research at National Food Research Institute (1999–2000) and Nara Institute of Science and Technology of Japan (2001), in October 2001 he joined Japan International Research Center for Agricultural Sciences, to work on functional analysis of transcription factors and osmosensors in stress responses in *Arabidopsis*. In August 2007 he moved to the University of Missouri—Columbia, USA, as a Senior Research Scientist to coordinate a research team working on discovery of soybean genes for genetic engineering of drought-tolerant soybean plants. His current research interests are elucidation of the roles of phytohormones and their interactions in abiotic stress responses, as well as translational genomics of legume crops with the aim to enhance crop productivity under adverse environmental conditions. He has published over 80 peer-reviewed papers with more than 60 research and 20 review articles, contributed 7 book chapters to various book editions published by Springer, Wiley-Blackwell, and American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America. He has also edited four book volumes for Springer, including this one.

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