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Chapter 3

LEGUME NODULE OXIDATIVE STRESS AND N_2 FIXATION EFFICIENCY

***Joaquim Albenisio Gomes Silveira*[*1], *Márcia do Vale Barreto Figueiredo*[2], *Fábio Rossi Cavalcanti*[3] and *Sérgio Luiz Ferreira-Silva*[1]**

[1]Research Fellow of the National Research and Technological Development, Brazil. Department of Biochemistry and Molecular Biology, Federal University of Ceara – UFC, Fortaleza, Ceara, Brazil
[2]Research Fellow of the National Research and Technological Development Department, Brazil
Agronomical Institute of Pernambuco IPA/CARHP, Recife, PE, Brazil
[3]Department of Agronomy, Federal University of Ceara/Campus of Cariri, Brazil

ABSTRACT

N_2 symbiotic fixation efficiency by legume nodules involves several metabolic processes and requires favorable environmental conditions. The nitrogenase enzymatic complex demands high energy and depends on oxidative reactions to generate reducing power and ATP. These oxidative reactions are accomplished via O_2 consumption in the electron transporter chain localized in the bacteroid inner membrane (bacteroid oxidative phosphorylation). Moreover, the legume nodule metabolism is also energetically expensive, requiring high O_2 concentrations for mitochondrial activity. This situation generates a very reducing environment that favors the formation of reactive oxygen species (ROS), especially the superoxide radical ($O_2^{\bullet-}$). Similarly, other nodule cell organelles and compartments such as peroxisomes, plastids, cytosol and apoplast are also important sites of ROS production. The most important ROS produced in the infected nodule cells are: superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^{\bullet-}$). The redox balance plays an important role in the regulation of nodule metabolism and N_2 fixation efficiency. To maintain an adequate equilibrium

* Corresponding author: silveira@ufc.br

between the production and scavenging of ROS, root nodules have an efficient biochemical apparatus with enzymatic and non-enzymatic components. ROS are essential to several key physiological processes, such as cellular signaling, defense against abiotic and biotic stresses, senescence and programmed cellular death. Modification of the redox homeostasis and decline in the antioxidative defense, both triggered by ROS accumulation, greatly influence the metabolism of the nodule and N_2 fixation. This situation is especially frequent under abiotic stress conditions such as drought, salinity and high temperature, which are common factors in tropical semi-arid regions. In this chapter, we focus on some aspects of the legume nodule oxidative metabolism associated with N_2 fixation efficiency and highlight the importance of certain abiotic stress conditions.

Keywords: diazotrophic; effectiveness; nodulation; senescence; reactive oxygen species; plant oxidative metabolism.

1. INTRODUCTION

Legume nodule N_2 fixation account for 20% of the estimated biological nitrogen fixed each year on Earth. This percentage is far beyond any other biological systems, which have figures similar to the total N fixed chemically by the industry. Leguminous plants represent the only known crop that can be self sufficient in nitrogen nutrition and that leave fixed N in soils. The contribution of legume N_2 fixation is also important in natural uncultivated systems (Franco, 1977). Legumes are widely distributed in tropical regions of Africa, America and Asia, and they are well adapted to cope with adverse environmental conditions like those of tropical semi-arid regions. In these regions, legume plants might be the most important protein source widely used as food. However, the stress conditions of the semi-arid tropics, such as drought and high temperature, can cause marked restrictions in the N_2 fixation efficiency (Naya et al., 2007).

The N_2 fixation reaction is carried out by the nitrogenase enzyme complex, which is localized inside the bacteroids that are disseminated in the plant cytosol of the infected legume nodules. The nitrogenase reaction is complex and energetically expensive, because it consumes a high amount of ATP and requires significant reducing power; it needs 15 ATP molecules and six electrons per N_2 molecule reduced. The ATP is synthesized by the bacteroid oxidative phosphorylation, which operates via an electron transport chain that contains terminal oxidase systems. These systems can perform with a low or a high O_2 concentration (Robertson and Farnden, 1980). Paradoxically, despite the N_2 fixation process requirement of high O_2 quantities, the nitrogenase is an O_2-sensitive enzyme, (Yates, 1980). Indeed, active legume nodules present high respiration rates compared to other plant tissues. This high O_2 reduction rate creates a strong reducing environment that is potentially capable of producing a high amount of reactive oxygen species.

Legume nodule N_2 fixation efficiency depends on several physiological and biochemical processes, which, in turn, are strongly affected by adverse abiotic stress factors (Silveira et al., 2001; Silveira et al., 2003). Each environmental stressor alone or in combination with others can induce an imbalance in the oxidative metabolism of all the plant tissues, especially in those that exhibit intense aerobic metabolism, such as the legume nodules. The oxidative

metabolism plays a central role in plant growth and productivity, because it represents a common route for signaling, for plant defense and for several other important physiological processes like senescence and programmed cellular death (Cavalcanti et al., 2007a). To avoid oxidative stress caused by an excess of reactive oxygen species (ROS), plants display several defense mechanisms that combine non-enzymatic and enzymatic systems. The effectiveness of this machinery is essential to allow a coordinate balance between all physiological processes (Cavalcanti et al., 2006).

Several abiotic stress factors that are common in tropical regions, such as drought and high temperature, strongly affect the N_2 fixation efficiency of nodules by mechanisms that are not yet completely understood. Surprisingly, the study of oxidative stress in nodules of legumes cultivated under adverse conditions has received less attention compared to other biochemical processes (Naya et al., 2007). However, several lines of experimental evidence have shown that the nodule oxidative stress induced by abiotic factors is important for the N_2 fixation efficiency; however, the precise mechanism responsible for this phenomenon is far from clear. In general, the alterations in the nodule oxidative metabolism are associated with accelerated nodule senescence and an inhibition of sucrose synthetase and nitrogenase activities (Marino et al., 2007).

The infected legume nodule cells present high respiratory activity compared to roots and other plant tissues, and they are constituted by various cellular compartments (mitochondrion, peroxisome, plastid, cytosol and bacteroid), which present active oxidative metabolism (Figure 1). Hence, they should generate great amounts of ROS during nodule growth and development, and they must exhibit an efficient system to maintain their oxidative homeostasis, especially under adverse environmental conditions. The current knowledge regarding oxidative metabolism in legume nodules associated with N_2 fixation efficiency is summarized and discussed herein, with a special emphasis on tropical conditions.

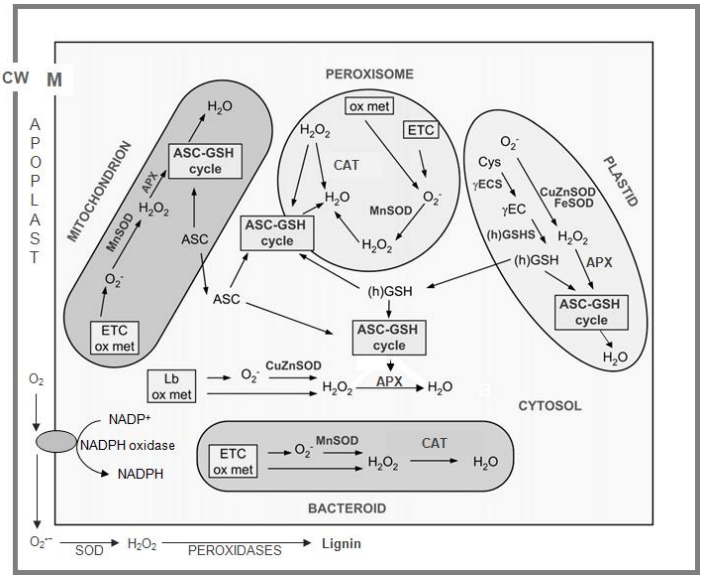

Figure 1. A simplified model of oxidative metabolism in an infected legume nodule cell. The most relevant organelles and compartments are shown. Abbreviations: CW = cell wall; APO = apoplast; M = plasma membrane; ETC = electron transport chain; ox met = oxidative metabolism; ASC-GSH cycle = ascorbate-glutathione cycle; GSHS = glutathione synthase; Lb =leghemoglobin. Adapted from Naya et

al. (2007). The figure shows the most important sites of ROS production as well as the most important enzymatic and non-enzymatic defense systems in an infected legume root nodule cell. It is important to highlight the central role played by the non-enzymatic antioxidants ascorbate (ASC) and glutathione (GSH), which actively participate in the oxidative protection. Actually, oxidative homeostasis depends on the coordinated action of several enzymatic and non-enzymatic agents, which permits the maintenance of adequate levels of ROS in the root nodule cells. This homeostasis, in turn, ensures the proper physiological functioning and cellular signaling and efficiency in the N_2 fixation process.

2. UPDATE ON PLANT OXIDATIVE METABOLISM

Reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet-}$) are unavoidable byproducts of plant metabolism (Halliewll, 2006). In leaves, chloroplasts and peroxisomes are the main ROS producers in the presence of light (Foyer and Noctor, 2003). Chloroplasts produce $^1O_2$ at PSII and $O_2^{\bullet-}$ at PSI (Asada, 2006) and PSII (Pospisil et al., 2004). The peroxisomes produce $O_2^{\bullet-}$ and H_2O_2 in several key metabolic reactions (del Rio et al., 2006). Conversely, in non-green plant tissues or in the darkness, the mitochondria appear to be the main ROS producers (Maxwell et al., 1999; Møller, 2001). The mitochondria produce $O_2^{\bullet-}$ at complexes I and III. An estimated 1–5% of the oxygen consumed by isolated mitochondria results in ROS production (Møller, 2001).

In accordance with Schrader and Fahimi (2006), ROS include oxygen radical species (containing unpaired electrons), such as the superoxide anion ($O_2^{\bullet-}$), which is formed through a one-electron reduction of O_2 ($O_2 + e- \rightarrow O_2^{\bullet-}$). Hydrogen peroxide ($H_2O_2$) is also considered as a ROS, although it has no unpaired electrons, and thus it cannot be classified as a free radical. It can be formed, for example, by the dismutation reaction of $O_2^{\bullet-}$ (catalyzed by superoxide dismutases) via the hydroperoxyl radical ($O_2^{\bullet-} + H + \rightarrow HO_2^{\bullet-}$; $2HO_2^{\bullet-} \rightarrow H_2O_2 + O_2$). Probably the most reactive and toxic form of oxygen, the hydroxyl radical ($HO^{\bullet-}$), can be formed by a metal ion (e. g., iron or copper) - catalyzed decomposition of H_2O_2 ($H_2O_2 + O_2^{\bullet-} \rightarrow O_2 + OH^- + HO^{\bullet-}$). $O_2^{\bullet-}$ can be converted into H_2O_2 by superoxide dismutase (SOD), and H_2O_2 can give rise to $^{\bullet}OH$ through the Fenton reaction, which is catalyzed mainly by free transition metal ions.

Different ROS have different properties. H_2O_2 is relatively stable, and its concentration in plant tissues is in the micromolar to low millimolar range, probably depending on the compartment (Cheeseman, 2006, Puntarulo et al., 1998). The other ROS have very short half-lives and are probably present at very low concentrations. They also have different reactivity. The $HO^{\bullet-}$ reacts rapidly with all types of cellular components, while the $O_2^{\bullet-}$ reacts primarily with protein Fe-S centers, and $^1O_2$ is particularly reactive with conjugated double bonds, as found in polyunsaturated fatty acids (Møller et al., 2007).

In some cases, ROS are produced in plants as byproducts of mainstream enzymatic reactions (e.g., glycolate oxidase in the peroxisomes during photorespiration). However, in other cases, ROS production appears to be an unavoidable accident (e.g., the $O_2^{\bullet-}$ produced by the mitochondrial electron transport chain). Besides the ROS produced in chloroplasts, peroxisomes and mitochondria, the NADPH oxidase enzyme localized in the plasma membrane can also produce $O_2^{\bullet-}$. ROS production by NADPH oxidase in the plasma membrane occurs during several physiological processes, and it is stimulated by pathogen attack and a variety of other processes, such as abiotic stress (Torres and Dangl, 2005). In

general, ROS production and ROS-induced damage increase during abiotic and biotic stress, and at the same time, ROS are important signaling molecules in plants (Mittler et al., 2004).

H_2O_2 is an established messenger in bacteria, yeast and mammals, where transcription factors are sensors (Lee and Helmann, 2006), and it may also be a messenger in plants (Miller and Mittler, 2006). Its relative stability and ability to cross membranes, possibly through aquaporins (Bienert et al., 2006; Bienert et al., 2007), makes H_2O_2 a good messenger molecule. However, all of the ROS forms can, in principle, act as messengers either directly or by using an oxidized product as a secondary messenger. Using a secondary messenger, such as transcription factors, depends on the distance the ROS molecule has to travel before reacting with other cellular components. Although ROS have this signaling role at low concentration, when they are in excess they can be extremely cytotoxic and can cause serious disruptions of the normal metabolism through oxidative damage to lipids, nucleic acids and proteins (Møller et al., 2007).

As a consequence of this noxious effect, a series of cellular degenerative processes are triggered, including peroxidation of membrane lipids and programmed cell death (Gechev et al., 2006). To avoid theses damages, plants have evolved defense systems with both enzymatic and non-enzymatic components (Alscher et al., 2002). The enzymatic antioxidant system includes enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). The non-enzymatic system includes low molecular antioxidants, such as reduced ascorbate (ASC) and reduced glutathione (GSH) (Bowler et al. 1992; Noctor and Foyer 1998, Foyer and Noctor, 2000).

These systems protect membranes against the deleterious effects of ROS, such as $^1O_2$, $O_2^{\bullet-}$, H_2O_2, $HO^{\bullet-}$, which are produced at elevated rates when plants are exposed to environmental stress conditions (Bowler et al., 1992; Baisak et al., 1994; Rao et al., 1996; Noctor and Foyer, 1998). SOD is believed to play a crucial role in the antioxidant systems because it catalyses the dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2 (Bowler et al., 1992). It is present in three isoforms: copper/zinc (CuZn-SOD), iron (Fe-SOD), and manganese (Mn-SOD). CuZn-SOD and Fe-SOD exist in chloroplasts, and Mn-SOD is present in mitochondria. CuZn-SOD also exists in the cytosol (Alscher et al., 2002). The conversion of H_2O_2 into water in the peroxisomes is carried out by CAT, while that same process is carried out in the cytosol and chloroplasts by the ascorbate–glutathione cycle, which also involves APX, ASC, GSH and GR (Noctor and Foyer, 1998). APX catalyzes the conversion of H_2O_2 into water and uses ASC as a substrate. GSH and GR are involved in the regeneration of ASC.

The occurrence of APX in plants illustrates that ASC is an important reducing substrate for H_2O_2 detoxification in photosynthetic organisms (Shigeoka et al., 2002). APX utilizes ASC as a specific electron donor for reducing H_2O_2 into water, with the concomitant generation of monodehydroascorbate (MDHA), a univalent oxidant of ASA. In this reaction, APX uses two molecules of ascorbate to reduce H_2O_2 to water, with the concomitant generation of two molecules of MDHA (Figure 2).

The MDHA is a radical with a short lifetime that can be converted either to ascorbate and dehydroascorbate or directly reduced to ASC by the action of the NAD(P)H-dependent MDHAR enzyme. The dehydroascorbate reductase (DHAR) utilizes glutathione (GSH) to reduce DHA and thereby regenerate ASC. The oxidized GSH is then regenerated by GSH reductase and uses reducing equivalents from NAD(P)H. Thus, APX together with the

effective ASA-GSH cycle functions to prevent the accumulation of toxic levels of H_2O_2 in plant cells (Foyer and Noctor, 2000).

3. NODULE OXIDATIVE METABOLISM AND SITES OF ROS PRODUCTION

After nodulation, the legume root nodules are composed of infected and non-infected cells that differ largely in morphology, structure and metabolism (Robertson and Farnden, 1980). The infected root nodule cells contain the majority of the plant cell organelles and cell components (cell wall, plasma membrane, cytosol, mitochondria, plastids, etc.), together with several bacteroids dispersed in the plant cytosol (Figure 1). The bacteroids and plant cell components exchange metabolites and energy for an efficient N_2 fixation that is carried out by the nitrogenase enzyme complex localized inside the bacteroid. The legume nodules present high metabolic activity (nitrogenase activity, O_2 transport by leghemoglobin, carbohydrate catabolism, NH_3 assimilation, cell division etc.), especially during the period of intense growth and high N_2 fixation rates (Silveira et al., 1998a; Silveira et al., 1999). In those phases, the active nodules exhibit a strong demand for reduced carbon (sugars) and high respiration rates.

The majority of nodule cell components present oxidative metabolism, especially the bacteroid inner membrane, mitochondrion, peroxisome and plastid, as shown in Figure 1. The bacteroids are the most important sites for O_2 consumption and the generation of reducing power for nitrogenase activity (Robertson and Farnden, 1980). However, the plant nodule organelles like mitochondria are also important sites for oxidative metabolism via the mitochondrial electron transport chain. Thus, root nodules present strong reducing conditions represented by intense electron flux and O_2 consumption. Under these conditions, the production of ROS is strongly favored (especially the superoxide radical). Indeed, several reports, reviewed by Chang et al. (2009), suggest that the reducing environment for nitrogen fixation in the nodule may initiate ROS formation. Thus, some electron donors such as ferredoxin, uricase, and hydrogenase are susceptible to autoxidation, resulting in superoxide formation (Dalton et al., 1991). The high concentration of oxyleghemoglobin in the nodules can also produce superoxide (Puppo et al., 1991).

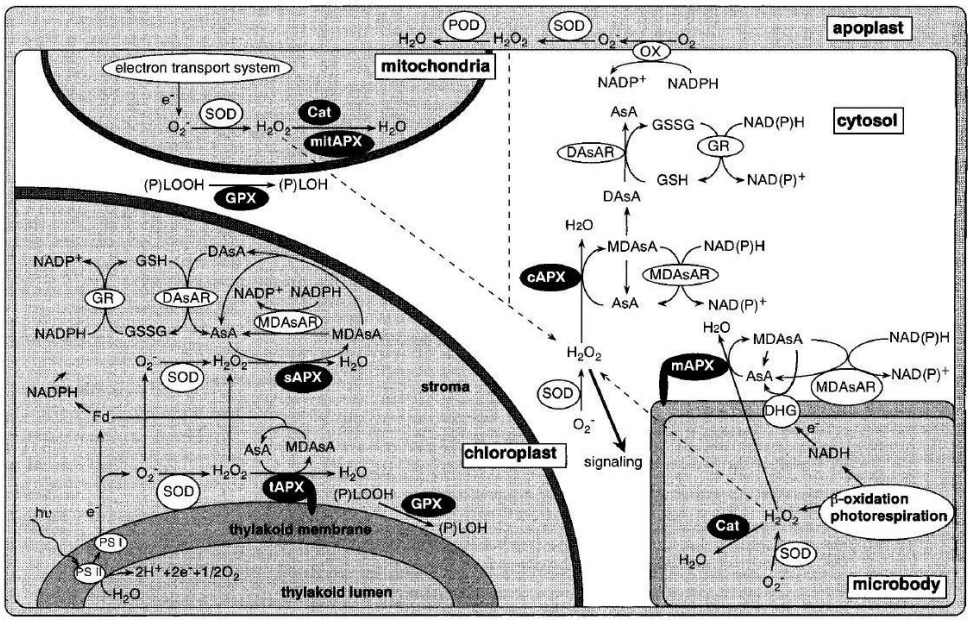

Figure 2. A simplified model of oxidative metabolism showing the major components involved with production and scavenging of ROS in plant cells. (Extracted from Shigeoka et al., 2002).

In bacteroids, oxygen metabolism produces superoxide radicals, which can interact with proteins (especially nitrogenase) and affect the enzymes' activity (Yates, 1980). The excess of superoxide can be detoxified by a combination of superoxide dismutase and catalase. Some experimental evidence with aerobic bacteria have shown that superoxide radicals and hydrogen peroxide inhibit nitrogenase activity and that exogenous SOD but not CAT or POX prevents this inhibition (Buchanan, 1977, cited by Yates, 1980). These authors suggested that $O_2^{\bullet-}$ or its decomposition products, singlet oxygen or the hydroxyl radical, were toxic agents. In nodule mitochondria, superoxide radical and H_2O_2 are the most important ROS produced. These oxidative species are scavenged via SOD and APX activities. APX utilizes using ascorbate as electron donor in the glutathione-ascorbate cycle. The H_2O_2 can be converted into other ROS, especially in the oxygen singlet and the $HO^{\bullet-}$ radical via the Fenton reaction.

The nodule cell peroxisomes are also an important site of ROS production throughout legume nodule development. This organelle mostly produces H_2O_2, and $O_2^{\bullet-}$ to a minor extent. The hydrogen peroxide is a product of the fatty acid catabolism, and it is also produced from $O_2^{\bullet-}$ by the action of the superoxide dismutase enzyme (MnSOD). The peroxisome's H_2O_2 is removed mainly by catalase activity and to a lesser degree by APX activity and the glutathione/ascorbate (ASC-GSH) cycle (Figure 1). The nodule plastids are another important site of ROS production; they produce superoxide radicals that can be converted into H_2O_2 by the action of superoxide dismutases (CuZnSOD and FeSOD). The excess of H_2O_2 can be removed by APX activity and the ASC-GSH cycle, which supplies new reduced ascorbate molecules for APX activity.

The plant nodule cytosol is an important site of production and removal of ROS generated under conditions of intense oxidative metabolism in several nodule cell compartments. Indeed, in plant cells, the antioxidative activity of the cytosol is essential to maintain the oxidative homeostasis in the cells and tissues as a whole. For instance, the

excess of H_2O_2 produced in organelles might migrate to the cytosol, and there it can be removed by the action of peroxidase enzymes, especially via cytosolic-APX. The ascorbate peroxidase operates in the cytosol in conjunction with the ASC-GSH cycle. The leghemoglobin might also generate $O_2^{\bullet-}$ in the nodule cytosol, and this radical can be converted into H_2O_2 by superoxide dismutase activity (CuZnSOD). The H_2O_2 found in the nodule cytosol can generate other ROS, such as the hydroxyl radical via the Fenton reaction. This radical is the most toxic ROS, capable of causing robust damage to crucial cell biomolecules, such as membrane lipid peroxidation, protein oxidation and nucleic acids denaturation.

4. OXIDATIVE STRESS DURING NODULE GROWTH AND SENESCENCE

The Reactive Oxygen Species (ROS) includes singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\bullet-}$) species. These molecules are commonly involved in a large range of physiological processes and are generated by plant cells as a consequence of aerobic metabolism during plant development or in response to abiotic and biotic stresses (Møller et al., 2007). They are highly toxic and are able to react with lipids, carbohydrates, proteins, nucleic acids and other primary constituents of the cell. Their toxicity leads to senescence and cell death (Rivero et al., 2007). Besides, ROS play a role in the regulation of plant metabolism as secondary messengers in biochemical pathways associated with plant development and environmental stress responses (Cavalcanti et al., 2004; Cavalcanti et al., 2007b; Gechev et al., 2006). Such ROS suffer delicate metabolic regulations that enable plants to better acclimate to biological pressures.

Some reports, reviewed by Chang et al. (2009), illustrate that the very reducing environment for nitrogen fixation in the root nodules may trigger ROS formation. In that condition, electron donors, such as ferredoxin, uricase, and hydrogenase, are susceptible to autoxidation, resulting in superoxide formation (Dalton et al., 1991). The high concentration oxyleghemoglobin in the nodules can also produce superoxide (Puppo et al., 1991). In *Medicago sativa,* an increase of ROS has been detected during the interaction with the rhizobia (Santos et al., 2001). The ROS accumulation, detected 12 h after exposure to Nod Factors, is related to the NF signaling transduction pathway (Ramu et al., 2002).

The production and distribution of intracellular ROS and the specific response to NFs were analyzed in growing *Phaseolus vulgaris* root hair cells. ROS levels were dramatically and transiently increased within a few seconds after treatment with NFs. The response was specific for NFs and was clearly different compared to that observed after the addition of H_2O_2, ATP or chitosan, or after exposure to UV radiation (Cardenas et al., 2008). In a previous work, Peleg-Grossman et al. (2007) observed that the elimination of those ROS disrupts the curling of root hairs and the formation of infection threads during the infection process. Therefore, the production of ROS may not be a plant defense response against parasitism but rather a process that is needed for the development of the proper interaction between rhizobia and legumes.

The redox balance is important in the regulation of nodule metabolism. The decrease of N_2 symbiotic fixation under abiotic stress is correlated with a modification of the redox balance and a decline in antioxidant defense (Gogorcena et al., 1997; Jebara et al., 2005). For instance, the enzyme sucrose synthase that acts in the carbon metabolism regulation and in

the nitrogen fixation (Baier et al., 2007; Silveira et al., 1998b) has been shown to be regulated by a redox state in nodules (Marino et al., 2008). Nodules can display all types of antioxidant models already described in plant cells, such as enzymatic systems and non-enzymatic antioxidant molecules (Chang et al., 2009). The Halliwell-Asada pathway performs the disulfide reduction of thioredoxin and glutaredoxin. Whereas this pathway enables the reduction of the two antioxidant molecules by NAD(P)H, the enzymatic systems (e.g., superoxide dismutases, catalases, peroxidases) remove ROS with the assistance of antioxidant molecules in different organelles, such as α-tocopherol, ascorbate and xanthophylls.

The importance of ROS production has been confirmed in an *Ensifer meliloti* strain that over-expresses a catalase, hence, acting as an H_2O_2 sink. This modified strain induces a delayed nodulation and enlargement of infection threads (Jamet et al., 2007). In addition, it is hypothesized that H_2O_2 may be involved in cell wall formation and in the regulation of the rigidity of the infection thread necessary for its progression. The induction of genes encoding *Rhizobium*-induced peroxidase such as *Rip1* (Cook et al., 1995) or *Srprx1* (Den Herder et al., 2007) support this conjecture.

The production of H_2O_2 during symbiosis was detected in infection thread walls of both infection and fixing zones of *Medicago sativa* and *Pisum sativum* nodules (Rubio et al., 2004). It was also detected in the cell walls and intercellular spaces of the cortex. Such H_2O_2 certainly play a role in the oxidative cross-linking needed for restoration of plant cell walls and the infection threads. In contrast to the evidence of high concentrations of ROS during nodule development, NFs have been shown to reduce the ROS amounts in *Medicago truncatula* during the very early responses after inoculation (Shaw and Long, 2003). Hydrogen peroxide was not detected either in the nitrogen-fixing zone of the nodules (Santos et al., 2001; Rubio et al., 2004). Therefore, this lack of detection might be attributed to the antioxidant alleviation system, or to its fast reaction with the substrate in the nodule.

In addition, it has been proposed that the antioxidant defense participates in the regulation of the influx of O_2 into the nodule (Dalton et al., 1998). Coincidentally, the expression of ascorbate peroxidase (APX) is highly induced in soybean nodules (Dalton et al., 1987). The preferential localization of the APX is in the endodermis of indeterminate nodules in alfalfa, pea, and clover and in the exterior edge of the parenchyma of determinate nodules in bean and soybean. These data suggest that this enzyme may participate in the nodule oxygen barrier via the detoxification of ROS produced by the high rates of respiration detected in the endodermis of indeterminate nodules and in boundary layers of determinate ones (Dalton et al., 1998).

Conversely, Chang et al. (2009) suggest that leghemoglobin is a candidate for oxidative damage, because it is present at a very high concentration in nodules and it facilitates oxygen transport to the bacteroids. They also mentioned that the exposure of young or mature root nodules to oxidative stress results in the formation of leghemoglobin radicals that are similar to those observed during natural senescence (Mathieu et al., 1998). Leghemoglobin undergoes autoxidation and forms ROS, such as superoxide radicals or H_2O_2, when linked to O_2 (Puppo et al., 1981). As reviewed, a modification of nodule metabolism under senescence could be partially reproduced by a direct oxidative stress on mature nodules (Mathieu et al., 1998; Marino et al., 2006).

Puppo et al. (2005) concluded that nodule senescence is an active and programmed process in development, in which ROS, antioxidants, hormones and proteinases have key roles. Nodules have high levels of the redox buffers, ascorbate and glutathione, which are

important in the nodulation process and in senescence. These metabolites decline with N-fixation as the nodule ages, but the decrease in redox buffering capacity does not necessarily lead to enhanced ROS or oxidative stress. Despite the importance of the delay of nodule senescence in N_2 fixation efficiency, relatively few reports have studied the genetics and biochemistry that control nodule senescence, especially involving ROS metabolism.

The decrease in the amount of antioxidants parallels the detection of higher ROS levels in the senescent nodules; simultaneously, this phenomenon appears to be correlated with a general decrease in the N_2-fixing efficiency. This correlation is established during both natural and stress-induced senescence. In this context, the decline of ascorbate and glutathione was concomitant with a decrease in nitrogen-fixing efficiency during natural senescence in a large number of legumes, such as the soybean (Evans et al., 1999) or the common bean (Loscos et al., 2008).

ROS have been detected around senescing symbiosomes throughout the nodule, suggesting the involvement of nodule senescence with oxidative mechanisms (Alesandrini et al., 2003; Rubio et al., 2004). At the molecular level, a transcriptomic analysis of *Medicago truncatula* nodules showed that the transcriptomes of nodule senescence have a high degree of overlap, suggesting that the detoxification pathways are similar (Van de Velde et al., 2006). Thus, a combination of multiple factors, including ROS metabolism might be involved in the control of the events leading to the rupture of the symbiotic homeostasis (Puppo et al., 2005). In addition, Groten et al. (2005) suggested that ROS, ascorbate and glutathione decline in a regulated manner during nodule development, and that this phenomenon does not necessarily cause oxidative stress but rather points to a developmental shift in the redox cross-talk related to the development and aging processes.

5. Oxidative Stress in Legume Nodule under Abiotic Stresses Conditions

N_2 fixation in legumes is significantly inhibited by abiotic stress, and this reduction is often associated with oxidative damage. The drop in nitrogen fixation under abiotic stress appears to be correlated with a modification of the redox balance and a decline in the antioxidant defense of nodules (Escurado et al., 1996; Gogorcena et al., 1997; Jebara et al., 2005). Moreover, this decrease in nitrogen fixation is associated with a carbon flux shortage (Matamoros et al., 1999). There is a decline of sucrose synthase activity (SuSy), and this is one of the earliest events occurring in nodules exposed to either water stress (Galvez et al., 2005) or to other environmental stresses (Arrese-Igor et al., 1999). More recent studies point to an association of reactive oxygen species in the signaling processes leading to SuSy and nitrogen fixation down-regulation under adverse conditions (Marino et al., 2006). For instance, the exposure of soybeans to short periods of darkness is sufficient to cause a rapid decline in transcripts encoding key nodule proteins and in the activity of SuSy (Gordon et al., 1993).

The ROS are hazardous substances for aerobic organisms, strong oxidants to their cell constituents and they may be especially harmful in legume nodules (Becana et al., 2000). When legume roots are subjected to water stress, oxidative stress may occur in nodules and an antioxidant defense system is activated (Zabalza et al., 2008). As a result, nodules are

particularly rich in both the quantity and diversity of antioxidant defenses that protect the nodule structures against high rates of nodule respiration and preserve the nitrogenase complex activity (Blokhina et al. 2003; Jebara et al., 2005).

These antioxidant systems can be divided into two categories based on their enzymatic activities: the ones that react with ROS and keep them at low levels (peroxidases, superoxide dismutases and catalases), and the ones that regenerate the oxidized antioxidants, ascorbate peroxidases and glutathione reductases (Smirnoff, 1993). However, this defense system seems to give leak responses and sometimes it cannot neutralize an overproduction of ROS (Zabalza et al., 2008). Indeed, the decline of nitrogen fixation under water stress is frequently associated with the decline in the activities of the antioxidant enzymes followed by a reduction in the homoglutathione resources (Gogorcena et al., 1995).

For example, Jebara et al. (2005) investigated the nodular antioxidant enzyme expression of *Phaseolus vulgaris* roots in response to 50 mM NaCl stress. They found a remarkable decrease of symbiosis parameters, such as nodulation, nodule dry weight and nitrogen fixing activity, particularly in roots that were also affected by salt treatments. The reduction of growth parameters was reflected by the variability of the enzymatic response of the nodules; while the salt treatment increased the superoxide dismutase and peroxidase activities, it decreased the activities of ascorbate peroxidase and catalase. The authors simplify when they state that their data suggests that the guaiacol peroxidase plays an important role in the detoxification of H_2O_2 in nodules under salt stress. Alternatively, Zabalza et al. (2008) suggested that guaiacol peroxidase (induced by an exogenous ascorbate on nodules of pea roots) may play another role, since that enzyme is related mainly to developmental processes; their research results were consistent with the suggestion of ascorbate acting in the developmental orchestration (i.e., cell walls reinforcements) rather than in antioxidant protection (Bashor and Dalton, 1999).

In fact, the beneficial effect of ascorbate for nitrogen fixation in non-stressed plants is revealed by the nodule effectiveness being strongly correlated with the high activity of the Halliwell-Asada cycle (Dalton et al., 1993). Furthermore, Bashor and Dalton (1999) showed that an external ascorbate supply to non-stressed soybean plants led to improved nodule functioning, which was related to a striking increase in nitrogenase activity, nodule leghemoglobin content and ascorbate peroxidase activity. However, Zabalza et al. (2008) showed that the ascorbate supply did not recover the nodule from drought stress, because other nodule antioxidant enzymes, the nodule carbon and nitrogen enzymes, nor the nitrogen fixation were improved; it also failed to prevent the decline of nitrogen fixation and the reduction of carbon flux in pea nodules. Their results suggest that exogenous ascorbate has a limited role in preventing the negative effects of water stress on nodule metabolism and nitrogen fixation.

In transgenic alfalfa, nodules overexpressing superoxide dismutase isoenzymes, water stress caused a decrease in the enzyme activities that produce or remove H_2O_2, in contrast to the phenomena detected in the leaves. The decline in ascorbate peroxidase activities, and especially in catalase, may be the result of a general but reversible slowing down process of nodule metabolism, as similar decreasing trends were observed for superoxide dismutases, guaiacol peroxidases, and soluble proteins (Rubio et al., 2002). These authors conclude that, in general, there were no major differences between WT and transgenic alfalfa for most of the parameters indicative of water stress tolerance.

There have been active research project on the association between ROS and nodule senescence induced by the abiotic stress. Experiments using dark-stressed plants provided valuable information on the process of nodule senescence, which is commonly diagnosed by a decrease in nitrogen content, nitrogen fixation, leghemoglobin, and total cytosolic protein (Gogorcena et al., 1997). Dark stress is also thought to disturb the oxygen relationships in nodules because nitrogen fixation requires a fine adjustment of the oxygen flux to the bacteroids. This control is exerted at two levels: a variable oxygen diffusion barrier and leghemoglobin facilitated diffusion within the infected cells. Then, the oxygen is consumed at high rates by mitochondria and the bacteroids, and in both respiratory processes AOS, including the superoxide radical and H_2O_2 are inevitably generated (Halliwell and Gutteridge, 1989). The high concentrations of leghemoglobin (near 1-3 mM) in infected cells and the tendency of its oxygenated form to auto-oxidize (the autoxidation of oxygenated Lb to ferric Lb during senescence) are conducive to the production of superoxide radicals and hydrogen peroxide in the cytosol.

In nodule senescence, it was reported that certain short-term nitrate applications (up to 2 or 3 mM) have an oxidative detrimental effect on the symbiosis between *Bradyrhizobium* and legumes crop, because it inhibits several steps of the infection process, nodule growth, and nitrogen fixation. In pea root nodules exposed to drought stress and nitrate applications, Escuredo et al. (1996) have shown that the induction of senescence are both caused by a decrease in antioxidant defenses and related metabolites, as well as an increase in catalytic Fe and oxidized proteins. Therefore, the authors hypothesized that the decrease in antioxidants is not a specific effect of nitrate, but a feature that may be common to nodule senescence. Further, they stated that catalytic Fe is critical for *Fenton* reactions to proceed *in vivo*. They also proposed the hypothesis that the increase in catalytic Fe that occurs in drought-stressed nodules causes the accumulation of damaged proteins and lipids (Moran et al., 1994; Gogorcena et al., 1995). However, they presented an important difference between the effects of nitrate and drought. In the latter case, the total content of lipids decreased and the lipid peroxide content increased by almost 1.5-fold; meanwhile, nitrate did not affect the lipid content and even decreased that of lipid peroxides by 21%. A logical explanation for this discrepancy is that drought triggers a process of enzymatic, Fe-independent lipid peroxidation in nodules, whereas this process may be slow or virtually nonexistent in nitrate-treated nodules.

These typical responses had been investigated before in early plant dark-stress studies. Since the exposure of soybeans to prolonged darkness induced structural and catabolic changes in the nodules that mimic natural senescence, it was suggested that the mechanisms underlying both processes were related (Cohen et al., 1986). The nodules of dark-stressed plants showed decreased energy charge (Ching et al., 1975), altered composition of the bacteroid population (Paau and Cowles, 1981), and increased proteolytic activity (Pfeiffer et al., 1983).

Analysis of the expression of genes involved in enzymatic antioxidant defense was performed in PQ-treated root nodules to verify the levels of the oxidative stress. The authors concluded the isocitrate dehydrogenase has a key role in NADPH recycling in nodules under oxidative stress, and they suggested that $NADP^+$-dependent isocitrate dehydrogenase should be grouped with the classical components of the plant antioxidant defense together with other dehydrogenases from the pentose phosphate pathway.

The interaction between rhizobia and their hosts may undergo an unexpected and positive response when these microorganisms show a resistance to the specific abiotic stressors, as exemplified in the current work of Bianco and Defez (2009). In their paper, the abiotic stress resistance of an engineered derivative strain (RD64) of *Ensifer meliloti* (having both free-living bacteria and bacteroids) was compared with its WT strain (1021), regarding their responses to the accumulation of indole-3-acetic acid (IAA), which was correlated to an increase in tolerance to different stress conditions (55 ^{o}C, 4 ^{o}C, UV-irradiation, 0.5 M NaCl, and pH 3.0). An increase of the *in vitro* tolerance to salt stress was caused by an IAA-induced increase at the levels of the bacterial osmolyte threalose of the modified RD64 *E. meliloti* strains. Moreover, *Medicago truncatula* plants nodulated by RD64 (Mt-RD64) showed re-modulation of phytohormones, with a higher IAA content in nodules and roots and a decreased IAA level in shoots as compared with plants nodulated by the WT strain 1021 (Mt-1021).

The response of nodulated *M. truncatula* plants to salt stress when 0.3 M NaCl was applied was analyzed. For Mt-RD64 plants higher internal proline contents, almost unchanged hydrogen peroxide levels, and enhanced activity of antioxidant enzymes (superoxide dismutase, total peroxidase, glutathione reductase, and ascorbate peroxidase) were found compared with Mt-1021 plants. These results were positively correlated with reduced symptoms of senescence, a lower expression of ethylene signaling genes, a lower reduction of shoot dry weight, and a better nitrogen-fixing capacity. The authors suggested a system to enhance stress tolerance in higher plants based on transgenic plants over-expressing IAA or other hormones. The disadvantage is that such a system might have an imbalance that affects the development and differentiation of the plants, despite aiming to the improvement of resistance.

Previous studies supported those trends and showed the re-modulation of tolerance of plants exposed to salt stress through their respective rhizobia strain partnering. Mhadhbi et al. (2004) reported that chickpea plants nodulated by a *Mesorhizobium ciceri* strain had a better tolerance to salt stress, regarding plant growth and nitrogen fixation measurements. They found that the superoxide dismutase level in *M. ciceri* was similar both in the presence or absence of NaCl. This similarity might be explained by the reduction of oxygen supply, which is a consequence of the decrease of permeability of the nodular cortex by salinity that limits the main source of ROS production. In addition, they found a differential activation of guaiacol peroxidase concomitant with differential responses of symbioses to salt treatment. These results confirm those obtained by Sheokand et al. (1995) who reported an average increase of 2.5-fold of nodule peroxidase in chickpea plants inoculated with *M. ciceri* strains and treated with 50mM NaCl. Moreover, they suggested that peroxidase activation seems to play an important role in plant salt tolerance.

6. Biological Nitrogen Fixation as the Key for Ecological Success

Advances in agricultural sustainability will require an increase in the use of Biological nitrogen fixation **(**BNF) as a major source of nitrogen for plants. Long-term sustainability of agricultural systems must rely on the use and effective management of internal resources. The

process of BNF offers an economically attractive and ecologically sound mean of reducing external nitrogen input and improving the quality and quantity of internal resources. Clearly, it is unreasonable to consider sustainable agriculture on a broad scale without BNF; further research is needed to optimize the contribution of BNF to sustainable agriculture (Saika and Jain, 2007).

The microorganisms that promote BFN have great importance because nitrogen is an essential component of proteins, nucleic acids and other nitrogen compounds, and therefore it is vital for all living organisms (Döbereiner, 1997). In climax ecosystems, the microbiota is in balance with the soil, and it is able to maintain its biodiversity and sustainability; however, this balance can easily be broken by man or by natural phenomena (Döbereiner, 1992). Legumes are important both ecologically and agriculturally because they are responsible for a substantial part of the global flux of nitrogen from atmospheric N_2 to fixed forms, such as ammonia, nitrate, and organic nitrogen. Legume-rhizobia symbioses contribute at least 70 million tons of N per year, approximately half of which derives from cool and warm temperate zones and the other half derives from the tropics (Brockwell et al. 1995). Increased plant protein levels and reduced depletion of soil N reserves are obvious advantages of legume N_2 fixation. Deficiency in mineral nitrogen often limits plant growth, and thus symbiotic relationships have evolved between plants and a variety of nitrogen-fixing organisms (Freiberg et al. 1997).

Even though BNF was mainly detected in oceans and legumes, some plants of the family Gramineae show a significant potential for obtaining nitrogen through the action of nitrogen fixing bacteria (Baldani et al., 2002; Alves et al., 2006). These plants have a fascicule root system, and thus they extract water and soil nutrients from soil horizons other than those of legumes, which have a pivoting root system. Some grass species are widely used as food by humans. Therefore, even if only part of demand of N could be provided by the association with fixing bacteria, the reduction in the use of nitrogen fertilizers would be equal to or higher than that observed with legumes, which can be self-sufficient (Döbereiner, 1992; Boddey et al., 2003).

Actinorhizal interactions (*Frankia*-nonlegume symbioses) are major contributors to nitrogen inputs in forests, wetlands and disturbed sites of temperate and tropical regions (El-Lakany and Luard, 1982; Schwencke and Carú, 2001; Wall and Berry, 2007). These associations involve more than 160 species of angiosperms classified over six or seven orders. The contributions of fixed nitrogen in native as well as managed ecosystems by the actinorhizal symbioses are comparable to those of the more extensively studied rhizobia-legume interactions. The different N_2-fixing organisms and symbioses found in agricultural and terrestrial natural ecosystems are shown in Figure 3.

The study of the efficiency in the use of N yields multiple advantages, such as the mere reduction of doses of fertilizers to keep productive levels and even genetic improvements to adapt plants to nitrogen-poor soils. The study of the acquisition and use of N should be linked to the understanding of the absorption, assimilation and redistribution of this nutrient in the cells and its balance between storage and use (Majerowicz et al., 2002).

Currently, new methods designed to increase nitrogen use efficiency are being intensely studied, especially through the recognition of biochemical and molecular pathways of absorption and assimilation in plants. Agroecological methods, such as BNF, are proposed to allow the sustainable use of this nutrient without production loss (Pradella et al., 2001; Herridge et al., 2008).

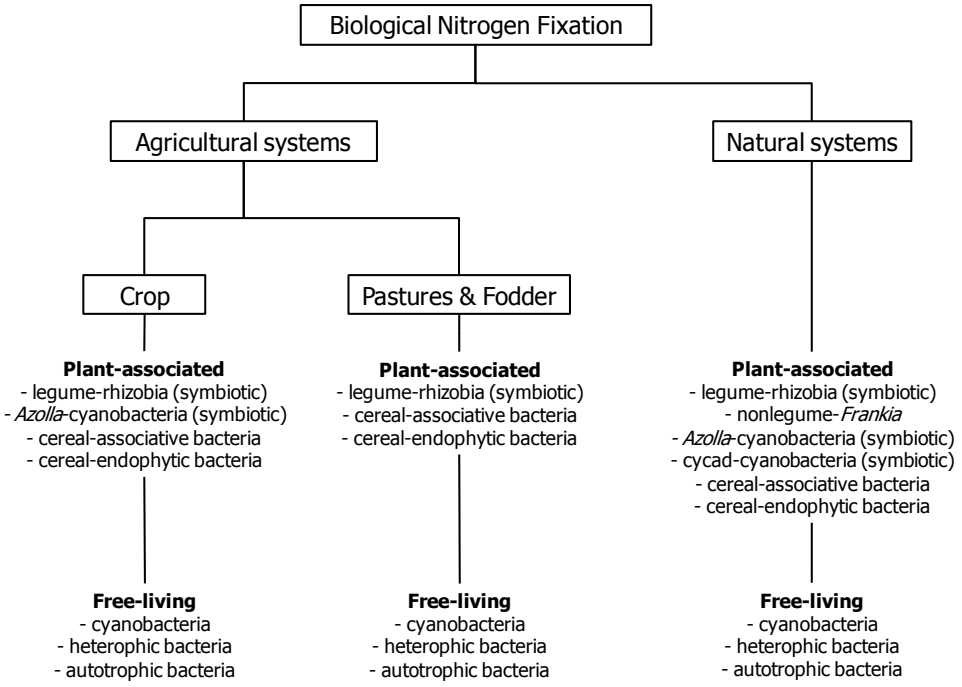

Figure 3. Biological N_2-fixing agents in agricultural and terrestrial natural systems (adapted from Herridge et al., 2008).

7. DIVERSITY OF BNF SYSTEMS

BNF is known to occur at varying degrees and in different environments, including soils, fresh and salty waters and sediments, on or within the roots, stems, and leaves of certain higher plants, and within the digestive tracts of some animals. The potential for nitrogen fixation exists for any environment capable of supporting the growth of microorganisms. Biological systems that are capable of fixing nitrogen are historically classified as non-symbiotic or symbiotic, depending on the involvement of one or more organisms, respectively (Hubbell and Kidder , 2003).

Until recently, it has been generally accepted that legumes (and the non-legume genus *Parasponia*) are nodulated exclusively by members of the family *Rhizobiaceae* in the α-proteobacteria, which includes the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* (Sprent 2001). In the last few years, however, several other species of α-proteobacteria have been shown to nodulate legumes (Moulin et al. 2002). These include strains of *Methylobacterium* that nodulate *Crotalaria* and *Lotononis* (Jaftha et al. 2002; Sy et al. 2001a and b); *Blastobacter denitrificans*, which nodulates *Aeschynomene indica* (van Berkum and Eardly 2002); and *Devosia* strains that nodulate *Neptunia natans* (Rivas et al. 2002). More controversially, a few members of the β -proteobacteria, such as *Burkholderia* sp. (originally isolated from *Aspalathus carnosa* and *Machaerium lunatum*, Moulin et al. 2001) and *Ralstonia taiwanensis* (isolated from *Mimosa pudica*; Chen et al. 2001) have been discovered in nodules of tropical legumes. The terms α and β -rhizobia were proposed to distinguish the rhizobial α and β -proteobacteria (Moulin et al., 2001). Phylogenetic analysis of available *nodA* and *nifH* genes from α and β -proteobacteria suggests

that β -rhizobia have evolved from diazotrophs through multiple lateral gene transfers (Chen et al., 2003).

The improvement in molecular biology methods to study the environment has contributed significantly to a major advance in the knowledge of microbial diversity. There is a growing interest in characterizing the diversity of these microorganisms in order to use their potential (Pittner et al., 2007). In general, studies on diversity are based on microbiological techniques and the subsequent characterization of isolates. However, the culture of microorganisms provides limited information about diversity, because most existing organisms are not easily isolated by conventional culture techniques.

Techniques of Random Amplified Polymorphic RAPD-DNA, Polymerase chain reaction-BOX-PCR, Amplified Fragment Length Polymorphism-AFLP and Amplified ribosomal DNA Restriction Analysis-ARDRA are extensively applied for assessing the diversity of microbial communities. The application of culture- independent techniques such as Denaturing Gradient Gel Electrophoresis-DGGE, the construction and analysis of clone libraries, quantitative real time-qPCR, and others molecular techniques are applied to study microbial communities (Andreote et al., 2008). For the evaluation of BFN diversity in different ecosystems, universal primers have been used to amplify the gene *nif*H via culture-independent techniques (Bashan et al., 2004). Such techniques allow a more complete characterization of the diazotrophic community than culture-dependent techniques (Roesch et al., 2007).

Different microorganisms have already been characterized by one or more of these methods (Oliveira et al., 2000, Depret and Laguerre, 2008, Monteiro et al., 2009, and many others). However, it is impossible to set up standardized conditions to accommodate the growth of all bacterial strains of all species for chemotaxonomic work, and a polyphasic approach is now imperative for a confident classification study. Polyphasic approach refers to the integration of genotypic, chemotypic and phenotypic information of a microbe in order to perform a reliable grouping of the organism (Colwell, 1970). For overviews of modern taxonomy, recent papers can be referred, such as Prakash et al. (2007), Rodríguez-Díaz et al. (2008) and Logan et al. (2009).

The preservation of microbial diversity is currently concentrated in institutions that hold collections of cultures available to the scientific community, such as the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ) in Germany, *Belgian Coordinated Collections of Microorganisms* (BCCM) in Belgium, *American Type Culture Collection* (ATTC) in the United States of America, *Embrapa Agrobiology Diazothrophic Microbial Culture Collection* (EMBRAPA), Brazil and *Japan Collection of Microorganisms* (JCM) in Japan (to access the culture collections in the world, go to http://wdcm.nig.ac.jp / hpcc.html). These institutes are also primarily responsible for bacterial identification and classification, including isolates obtained from the environment in groups already defined or in new taxa.

The results of independent studies of isolation and culture, based on the amplification and sequencing of fragments of the genes16S rRNA (16S rDNA), showed that microorganism diversity in environmental samples is substantially large (Head, 1998, Hunter-Cevera, 1998). The application of these methodologies in bacteria identification has allowed the discovery of an extensive number of new lineages in this group. Culture-independent methods tend to complement methods based on isolation and culture to survey and compare the composition, diversity and structure of microbial communities (Hugenholtz et al. 1998; Ranjard et al. 2000; Andreote et al., 2008). However, a question that still needs to be answered regarding

microbial communities in the rhizosphere is the relationship between the ecological function of communities and soil biodiversity (Barriuso et al. 2008). In spite of the lack of information about the importance of the diversity and species richness related to their ecological function (Freckman, 1994), soil organisms have been classified several times in different functional groups (Lavelle, 1996).

This lack of knowledge about bacterial diversity is partly owing to the high number of species present and is also because most bacteria are viable but not cultivable (Barriuso et al. 2008). The biological diversity of soil microorganisms has been expressed using a variety of indexes (Kennedy and Smith, 1995) and mathematical models (Tokeshi, 1993), but there is no accepted general model to describe the relationship among abundance, species' richness and dominance. It is therefore reasonable that the components of diversity are studied separately to quantify them (Ekschmitt and Griffiths, 1998).

8. STRATEGIES TO IMPROVE NODULATION

Co-inoculation studies with rhizobia and plant growth promoting bacteria (PGPR) have shown increased plant nodulation and N_2 fixation (Li and Alexander, 1988; Araújo and Hungria, 1999; Vessey and Buss, 2002; Silva, et al. 2006; Figueiredo et al. 2007, 2008). The effect of *Bacillus* with *Bradyrhizobium* sp strains on the symbiosis of the cowpea [*Vigna unguiculata* (L.) Walp.] stimulated the nodulation, and promoted a better fixation of N_2 during the symbiosis between *Bradyrhizobium* and cowpea (Figure 4). According to Vessey and Buss (2002), the application of *Bacillus* species to seeds or roots causes a variation in the composition of rhizosphere leading to an increase in growth and yield of different crops.

Figure 4. Root of cowpea (*Vigna unguiculata* [L.] Walp.) cv "IPA 205" co-inoculated with *Bradyrhizobium sp*. (EI 6) + *Bacillus sp.* (ANBE 31). (Experiment developed in Leonard jars under greenhouse conditions). (Courtesy of Figueiredo, M.V.B).

PGPR in combination with efficient rhizobia could improve the growth and nitrogen fixation by inducing the occupancy of introduced rhizobia in the nodules of the legume (Tilak et al., 2006). According to Saravana-Kumar et al. (2007), *Bradyrhizobium* prompted the nodulation and growth of legumes in combination with Active 1-aminocyclopropane-1-

carboxylate (ACC) deaminase containing PGPR. Moreover, certain rhizobacteria possess the enzyme ACC-deaminase that hydrolyses ACC into ammonia and α-ketobutyrate (Mayak et al., 1999). ACC-deaminase activity in PGPR plays an important role in the host nodulation response (Remans et al., 2007). PGPR containing ACC-deaminase could suppress accelerated endogenous ethylene synthesis and thus may facilitate root elongation and nodulation, which in turn improve the growth and yield of the plants (Zafar-ul-Hye, 2008).

The phytohormone ethylene acts as a negative factor in the nodulation process, but recent discoveries suggest several strategies used by rhizobia to reduce the amount of ethylene synthesized by their legume symbionts, decreasing the negative effect of ethylene on nodulation. At least one strain of rhizobia produces rhizobitoxine, an inhibitor of ethylene synthesis. ACC- deaminase has been detected in a number of other rhizobial strains. This enzyme catalyzes the cleavage of ACC to alpha-ketobutyrate and ammonia. It has been shown that the inhibitory effect of ethylene on plant root elongation can be reduced by the activity of ACC deaminase (Okasaki et al. 2007; Zafar-ul-Hye, 2008).

The positive role of rhizobitoxine production in nodulation has also been reported by Yasuta et al., 1999, Duodu et al., 1999, Yuhashi et al., 2000, Parker and Peters, 2001 and Ma et al, 2003. Recent studies have revealed that rhizobitoxine plays a positive role in establishing symbiosis between *Bradyrhizobium elkanii* and host legumes; rhizobitoxine enhances the nodulation process and nodulation competitiveness by inhibiting ethylene biosynthesis in host roots. In addition, the gene for ACC- deaminase was found in some rhizobia, such as *Mesorhizobium loti*, *Bradyrhizobium japonicum* and *Rhizobium* sp. ACC deaminase also facilitates symbiosis by decreasing ethylene levels in host roots (Okasaki et al. 2004). The accumulated evidence reveals general strategies by which rhizobia produce an inhibitor and an enzyme to decrease ethylene levels in host roots and thereby enhance nodulation (Okasaki et al. 2007). Reports on classical plant hormone effects on nodulation are often ambiguous and contradictory because (1) nodulation is the result of a fine balance between induction and repression of new nodule formation; (2) hormone requirements change with the varying stages of nodulation; (3) hormone levels and requirements change in different places in the shoot, root and nodule; (4) hormones interact with each other, leading to complex negative and positive feedback loops; (5) hormone requirements differ in different legume species, and (6) nodulation is regulated by both local and long distance signaling involving different actions of the same hormone in each regulatory pathway (Ferguson and Mathesius, 2003). The search for homologues of the recently discovered *Arabidopsis* hormone response genes in legumes and their silencing or over expression should help pinpoint the action of hormones during nodulation (Peng et al, 2009).

8.1. Mycorrhizal Infection of Legume Roots to Stimulate Nodulation

Mycorrhizal infection of legume roots was reported to stimulate both nodulation and N_2 fixation, especially in soils with low available phosphorous (Redecker et al., 1997; Bonfante and Anca, 2009). The majority of plants from terrestrial ecosystems interact with symbiotic mycorrhizal fungi (Smith and Read, 1997). There are different types of mycorrhizal symbiosis, of which the arbuscular mycorrhizal is the most common all over the world. The concept of ‘mycorrhiza helper bacteria’ (MHB), was introduced and discussed by Garbaye, (1994). It is thus opportune to revisit this particular field of research at the interface of plant

science, mycology, bacteriology and rhizosphere ecology, and which is generally related to the study of fungal–bacterial interactions in ecosystems (de Boer et al., 2005; Artursson et al., 2006). The MHB strains that have been identified to date belong to many bacterial groups and genera, such as Gram-negative Proteobacteria (*Agrobacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas*, *Klebsiella* and *Rhizobium*), Gram-positive Firmicutes (*Bacillus*, *Brevibacillus*, and *Paenibacillus*) and Gram-positive Actinomycetes (*Rhodococcus*, *Streptomyces*, and *Arthrobacter*). Many plant models have been used to study the MHB effect, including herbaceous and woody species mainly from temperate ecosystems. Only a few studies have focused on tropical plant species (Frey-Klett et al., 2007).

Some authors report that the effect of MHB stimulates root colonization by arbuscular mycorrhizal fungi (AMF) (Toro et al., 1996; Ratti et al., 2001; Vivas et al., 2006). According to Artursson et al. (2006), there is little information on the mechanisms controlling interactions of bacteria with AMF in the mycorrhizosphere. Artursson (2005) reported that some bacterial species respond to the presence of certain AMF and suggested the existence of a high degree of specificity between bacteria with the kind of AMF. Other authors demonstrated synergistic interactions between P-solubilizing bacteria and AMF (Kim et al., 1997). According to Artursson et al. (2006), Meixner et al., (2007) and Ikeda et al. (2008), although there are numerous studies on the interactions between AMF and bacteria, the underlying mechanisms of these associations are not well understood yet and the proposed mechanisms still need further experimental confirmation.

CONCLUSION

An adequate balance in the oxidative metabolism of legume nodules is essential to improve the symbiotic N_2 fixation efficiency, especially under adverse environmental conditions of soil and weather. However, this research issue has received less attention than others in the past few years. Therefore, more efforts are need especially on the biochemical and molecular aspects of oxidative stress and protection mechanisms associated with key processes of N_2 fixation efficiency, such as nitrogenase oxidation mechanisms, bacteroid oxidative metabolism and redox homeostasis, nodule-organelle interactions, leghemoglobin and nodule stress oxidative-induced senescence and aging. In general, the knowledge underling oxidative metabolism in legume nodules is incipient and fragmentary especially in relation to N_2 fixation efficiency under adverse environmental conditions like those of tropical regions.

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