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Review

Environmental stress conditions affecting the N_2 fixing *Rhizobium*-legume symbiosis and adaptation mechanisms

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Rhizobia are bacteria which fix atmospheric nitrogen in association within the root or the stem nodules of legume plants and transform atmospheric nitrogen to ammonia. Biological nitrogen fixation is an important process for sustainable land management, because nitrogen is the principal crop production's limiting factor. However, several environmental conditions such as salinity, temperature, acidity/alkalinity, drought, heavy metals, etc., are critical factors which can have detrimental effects on the steps involved in *Rhizobium*-legume symbiosis as infection process, nodule's development and function, resulting in low nitrogen fixation and crop yield. The presence of *Rhizobium*- legume symbioses able to fix appreciable N_2 amounts under unfavorable conditions is very interesting, because these symbioses represent the best source of nitrogen especially in arid and semi-arid regions, where they contribute to land stabilization and fertilization. Hence, the better understanding of rhizobial physiological responses to different intrinsic and extrinsic stresses factors is very important to improve crop production by harnessing biological nitrogen fixation process.

Key words: *Rhizobium*, legume, symbiosis, environmental stress, heavy metals, soil fertility.

INTRODUCTION

Nitrogen is a major limiting factor in agricultural production even if it represents almost 80% of the atmosphere (Abd-Alla et al., 2014). This paradox is due to the high stability of the nitrogen molecule (N_2) and to the fact that only some prokaryotic organisms are able to reduce it in an available form.

The biological nitrogen fixation (BNF) is a natural phenomenon consisting on the conversion of atmospheric nitrogen into ammonia by the nitrogenase enzyme complex. This biological reduction of N_2 to NH_3 is a highly endergonic process with a minimum energy requirement of Ca.960 KJ mol^{-1} N-fixed (Sprent and Raven, 1985). Nitrogenase function requires ATP and electrons, supplied respectively by respiration and electron carriers, usually ferrodoxin. Nitrogenase catalyzes the reduction of several substrates, including H^+, N_2 and C_2H_2. The principal

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reaction for dinitrogen reaction is as follows:

$$N_2 + 16\ MgATP + 8\ e^- + 8\ H^+ \longrightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16\ Pi$$

The energy requirements in this symbiosis are provided to bacteria by carbonaceous substances resulting from plant photosynthesis. *Rhizobium* can infect some root cortex cells of leguminous plants and initiate the formation of a new plant organ, the root nodule. These bacteria proliferate within root nodule cells then differentiate into a nitrogen fixing form called a bacteroid, which can fix the atmospheric nitrogen (Chanway et al., 2014).

The *Rhizobium*-legume symbiosis presents many advantages for both host plant and rhizobial bacteria by stimulating plant growth in nitrogen-deficient soils, offering the major success factor of the legume's family as compared to other plants and offering an adequate bacterial microhabitat necessary for nitrogen fixation (Noel, 2009).

Furthermore, this symbiosis is the result of a balance between environmental factors affecting both plant and bacteria. So the success of the legumes infection and nodulation depends on environment factors and *Rhizobium* survival. Environmental stress impose a major threat to both symbiotic nitrogen fixation and agriculture which can be limited by soil and climatic factors such as salinity, drought and temperature. For this reason, the *Rhizobium*'s tolerance to different environmental stresses is a desired property for use in nitrogen-depleted soils.

This review is focused on the study of the physiological responses to different stresses factors that can affect the rhizobial survival and the symbiotic nitrogen fixation in a perspective to understand the limiting factors of this symbiotic association and to better harness this biological process.

STRESS FACTORS AFFECTING SYMBIOSIS AND NITROGEN FIXATION

Various factors such as the soil physico-chemical composition can interfere with the infection process and nodulation, or can influence the activity of nitrogen-fixation during the symbiosis (Kinkema et al., 2006).

Salt and osmotic stress

Salinity is one of the major factors threatening agriculture in arid and semi-arid areas. Nearly 40% of the world's land surface can be categorized as having a potential salinity problem (Zahran, 2001; Niste et al., 2013). The main cause of salinity is the nutrient imbalance in the soil, which is considered as a constraint influencing the N_2 fixing symbiosis and the survival of both partners (Mohammadi et al., 2012; Niste et al., 2013). Salinity is concentration of dissolved mineral salts comprising cations and anions present in the soil (soil solution) and in water. The principal cations in solution consist of Na^+, Ca^{2+}, Mg^{2+} and K^+ and the major anions are Cl^-, SO_4^{2-}, HCO_3^-, CO_3^{2-} and NO_3^- (Aggarwal et al., 2012).

The response to saline stress varies among free rhizobia for which the growth is inhibited at 100 mM NaCl, and symbiotic rhizobia, such as *Sinorhizobium meliloti* found to be tolerant to NaCl concentrations ranging from 300 to 700 mM (Zahran, 2001). Some rhizobia isolated from Acacia trees seem to be highly salt tolerant and can grow at a concentration of 500-850 mM NaCl (Zahran, 2001).

Rhizobial strains differ in their ability to tolerate osmotic stress and can use different adaptation mechanisms such as intracellular accumulation of low-molecular-weight organic solutes (Zahran, 1999) including amino acids such as glutamate, N- acetylglutaminyl - glutamine, sugar and polyamines or the accumulation of ions such as K^+. Rhizobia subject to salt stress may undergo morphological alterations, leading to changes in cell morphology and size or modifications in the pattern of extracellular polysaccharides and lipopolysaccharides (Ventorino et al., 2012). These compounds may have an impact on symbiosis because of their implication in the initial steps of the symbiotic interactions. Moreover, some authors have reported that tolerance to salinity may be due to a plasmid-mediated resistance since salt resistance can be rapidly transferred from tolerant to sensitive bacteria, thus extra chromosomal genes can contribute to survival in saline soil (Pereira et al., 2008). Changes in the gene expression appear also to be among the rhizobial adaptation mechanisms to tolerate hyperosmotic stress (Lapez-Go'mez et al., 2013).

Temperature stress

High soil temperature is one of critical factors which can prevent the development of a nitrogen-fixing association between the two symbiotic partners especially in arid and semi-arid regions. The survival of rhizobia in soil is more affected by high temperatures than by low temperatures because it can be deleterious (Niste et al., 2013). In arid regions, high soil temperature affect lives of both free and symbiotic rhizobia (Zahran, 1999). Most *rhizobia* have an optimum growth temperature at 28-31°C and many of them are unable to grow at 38°C (Graham, 1992). However, some rhizobial strains isolated from Acacia have the ability to grow at high temperatures which can reach 44°C (Zahran et al., 1994). Temperature can influence not only the survival of free rhizobia, but also the exchange of molecular signals between the symbiotic partners (Sadowsky, 2005). High temperature can induce an inhibiting effect on bacterial adherence to root hairs, on bacteroïd differentiation, on nodule structure and on legume root nodule's functioning (Zahran, 1999; Alexandre

and Oliveira, 2013). Sudden temperature changes induce synthesis of heat shock proteins (HSP) which can play a protective role and contribute to heat tolerance with no alteration of the internal cell temperature (Yura et al., 2000). Most bacteria have only a small number of HSP but rhizobia seem to present an exception (Alexandre and Oliveira, 2013). The HSP include some proteins such as IbpA and IbpB that show similarity to *Esherichia coli* and other proteins more different in sequence and phylogenetic origin (Alexandre and Oliveira, 2013).

The molecular bases of temperature stress tolerance in rhizobia were studied by comparing the expression of chaperone genes *dnaKJ* and *groESL* in thermotolerant and thermosensitive isolates. These chaperones are characterized by their role as folding modulators, in sequestering and stabilizing a wide range of polypeptides presented in wrong conformational structure (Alexandre and Oliveira, 2013). Nandal et al. (2005) reported that mutants tolerant to high temperature, obtained from a thermosensitive *Rhizobium sp.* strain, exhibited a different protein profile from the wild-type at high temperature and showed overexpressed proteins as well as new proteins. This protein overproduction was confirmed by other studies in mutant strains as DnaK (Alexandre and Oliveira, 2013; Abd-Alla et al., 2014), in chickpea rhizobia as GroEL (Rodrigues et al., 2006) and also in *Mesorhizobium* strains (Laranjo and Oliveira, 2011). *Bradyrhizobium japonicum* shows a total of five *groESL* operons, which only $groESL_{1,4,5}$ are heat inducible and are differently regulated. The $groESL_1$ is σ^{32}-dependent and is highly induced by heat shock. The sigma factor σ^{32} is involved in the control of the heat shock response at the transcriptional level in many bacteria. Unlike GroESL system, DnaKJ system is far less studied but it was characterized in *B. japonicum* and was proved to be under the control of σ^{32} factor (Alexandre and Oliveira, 2013).

The expression of groESL genes from psychrophilic bacteria allowed the increase of *E. coli*'s tolerance to low temperature and decreased the growth temperature's lower limit (Ferrer et al., 2003). Another study reported that thermotolerance was improved by overexpression of native groESL system in *E. coli,* and which may be caused by the folding or refolding activity of the chaperone proteins to misfolded cellular proteins under thermal stress (Kim et al., 2009). The misfolding of intracellular proteins is recognized as a key factor for microorganism's inactivation under thermal stress. The chaperone system like GroEL-GroES has a major role in the defense system; it not only directly interacts with a number of intracellular proteins but also affects some transcriptional networks under stress condition. In fact, this complex forms an enclosed environment for the correct folding of approximately 50% of intracellular proteins under conditions of cellular stress (Kim et al., 2009).

At low temperatures, the cellular membrane rigidity presents a major problem for bacteria, in addition to a decreased rate of enzymatic reactions and the instability of single stranded DNA and RNA (Horn et al., 2007). Some rhizobia strains isolated from wild relative chickpea (*Cicer anatolicum*) collected at high altitude, have demonstrated their ability to nodulate chickpea (*Cicer arietinum*) at low temperatures (9-15°C) (Alexandre and Oliveira, 2013).

Bacterial cold shock response is an immediate and transient response to the temperature downshift and is followed by low temperature adaptation that allows continued growth at low temperatures. Arctic strains of rhizobia respond to cold shocks by synthesizing proteins under their minimal growth temperatures at freezing temperatures as low as -10°C (Cloutier et al., 1992). Proteins induced after cold shocks are designated as cold shock proteins (CSP). These low molecular mass proteins, usually nucleic acid-binding, are well characterized in *E. coli* but poorly studied in rhizobia. A homolog to the *E. coli CspA* gene was detected in *S. meliloti* and reported to be induced by temperature downshift; moreover this *CspA* is known to interact with mRNA, stabilizing the molecule in order to allow translation (O'Connell and Thomashow, 2000).

pH Stress

Either alkaline or acidic agricultural soil has a great influence on the survival or multiplication of rhizobia and can affect both the symbiosis partners. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic N_2 fixation (Zahran, 1999). The optimum pH for rhizobial growth is considered to be between 6.0 and 7.0 (Hungria and Vargas, 2000). In fact, at pH of 5.0-5.5, the nodulation in Acacia trees was absent (Brockwell et al., 2005). The rhizobial strains vary widely in their acidity tolerance. *Rhizobium tropici* and *Mesorhizobium loti* are considered as highly acid tolerant strains (Graham et al., 1994). Some rhizobial strains can withstand and survive even in a very low pH (about 3.5). Alkalinity is less harmful to the survival of rhizobia. Jordan (1984) showed that the majority of these bacteria can tolerate up to pH 9. The same result was found among strains nodulating Acacia (Zerhari et al., 2000) which showed remarkable and sometimes quite extraordinary tolerance to alkaline conditions (Brockwell et al., 2005). For example, rhizobial strains isolated from *A. farnesiana* have shown an ability to adapt and grow at pH 12.0 (Brockwell et al., 2005).

The physiological and biochemical mechanisms of rhizobial adaptation to acidic conditions are various (Graham et al., 1994). These mechanisms include among others the exclusion and expulsion of protons H^+ (El-Hilali, 2006), the increase of potassium and glutamate contents in the cytoplasm of stressed cells (Aaron and Graham, 1991), the change in the lipopolysaccharides

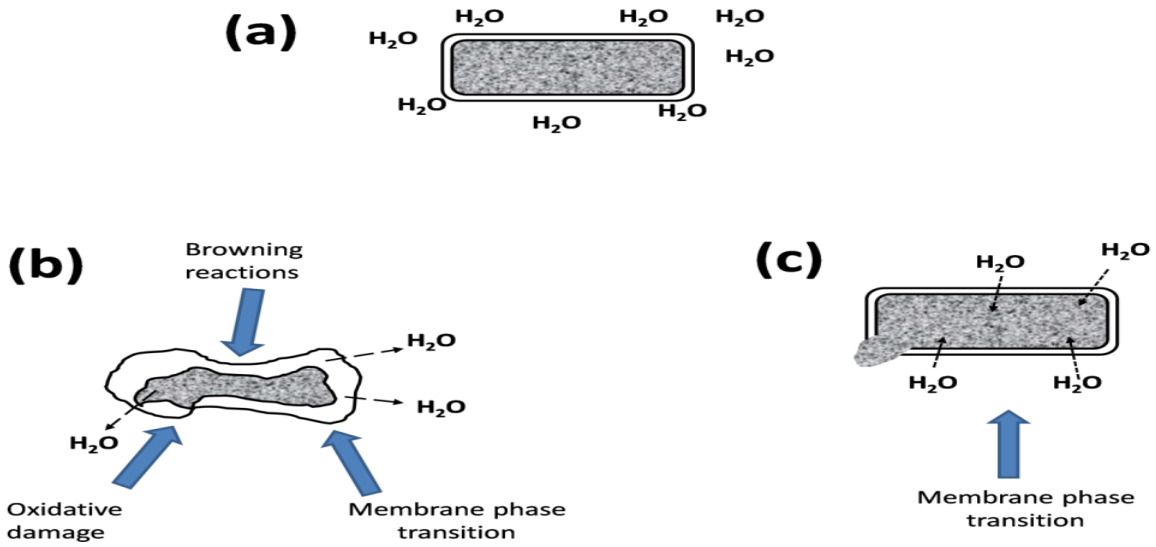

Figure 1. Schematic representation of: (a) hydrated bacterial cell, (b) dehydrated cell exposed to stress damage and (c) dehydrated cell upon rehydration with loss of membrane integrity (Casteriano, 2014).

composition (Vriezen et al., 2007), and the accumulation of polyamines (Fujihara and Yoneyama, 1993). The production of acid shock proteins (ASPs) is another common response contributing to this stress tolerance by conferring acid protection on the bacteria with no alteration of the cellular pH (Foster, 1993). Furthermore, several genes, such as *actA*, *actP*, *exoR*, *lpiA*, *actR*, *actS* and *phrR*, were shown to be essential for rhizobial growth at low pH (Abd-Alla et al., 2014).

However, the negative effect of the alkaline soil's conditions is the unavailability of essential minerals for both rhizobia and host plant such as iron and manganese (Farissi et al., 2014). High pH can also influence the growth of *Rhizobium* and its undergoing nodulation, although some rhizobial species such as *R. leguminosarum bv. trifolii* has been reported to colonize soil at a higher rate and produce nodules at a higher frequency in alkaline conditions (Zahran et al., 1999). Homospermidine, a polyamine present at high concentrations in root nodule bacteria, is also known to accumulate in *B. japonicum* in alkaline conditions, although its function is unknown (Fujihara and Yoneyama, 1993).

Drought stress

Drought stress can present a major agricultural problem which occurs when the available soil water is reduced and the atmospheric conditions induce continuous loss of water by transpiration or high evaporation (Jaleel et al., 2009). The cells under drought conditions are also susceptible to chemical damage as a result of water removal and exposure to the atmosphere (Figure 1). During dehydration, the formation of certain molecules particularly hydroxyl and peroxyl radicals can induce the lipids peroxidation, proteins denaturation and nucleic acid damage (Casteriano, 2014). Reducing sugars may covalently react with the amino side chain of amino acid residues via non-enzymatic browning or Maillard reaction, causing protein damage (Casteriano, 2014).

Drought effects on rhizobial persistence and survival in the soil, on root-hair colonization and on infection by rhizobia can consequently limit the nodulation (Zahran, 1999; Mhadhbi et al., 2011). However, some rhizobial species have shown an ability to tolerate and survive in drought conditions at -3.5 MPa (Abolhasani et al., 2010). The efficiency of these rhizospheric bacteria to persist in severe water deficit conditions can be used to ameliorate drought impact on plants and to help them to tolerate stress by producing physical and chemical changes (Yang et al., 2009). Many species of rhizobia can support severe drought conditions by various adaptive strategies including production of chaperones and sugars, synthesis of stress enzyme 1-aminocyclopropane 1-carboxylic acid, production of exopolysaccharides (Hussain et al., 2014), production of low molecular weight organic compound like trehalose, phosphate solubilization, improved nutrient availability, production of siderophores and phytohormones (Hussain et al., 2014). Under dryness conditions, the aerobic bacteria have shown their ability to use nitrogen oxides as terminal electron acceptors which can help them to survive and grow during periods of anoxia. This may present a great advantage for the survival of rhizobia in soil (Abd-Alla et al., 2014).

Soil fertility

Soil fertility can also affect the biological nitrogen fixation

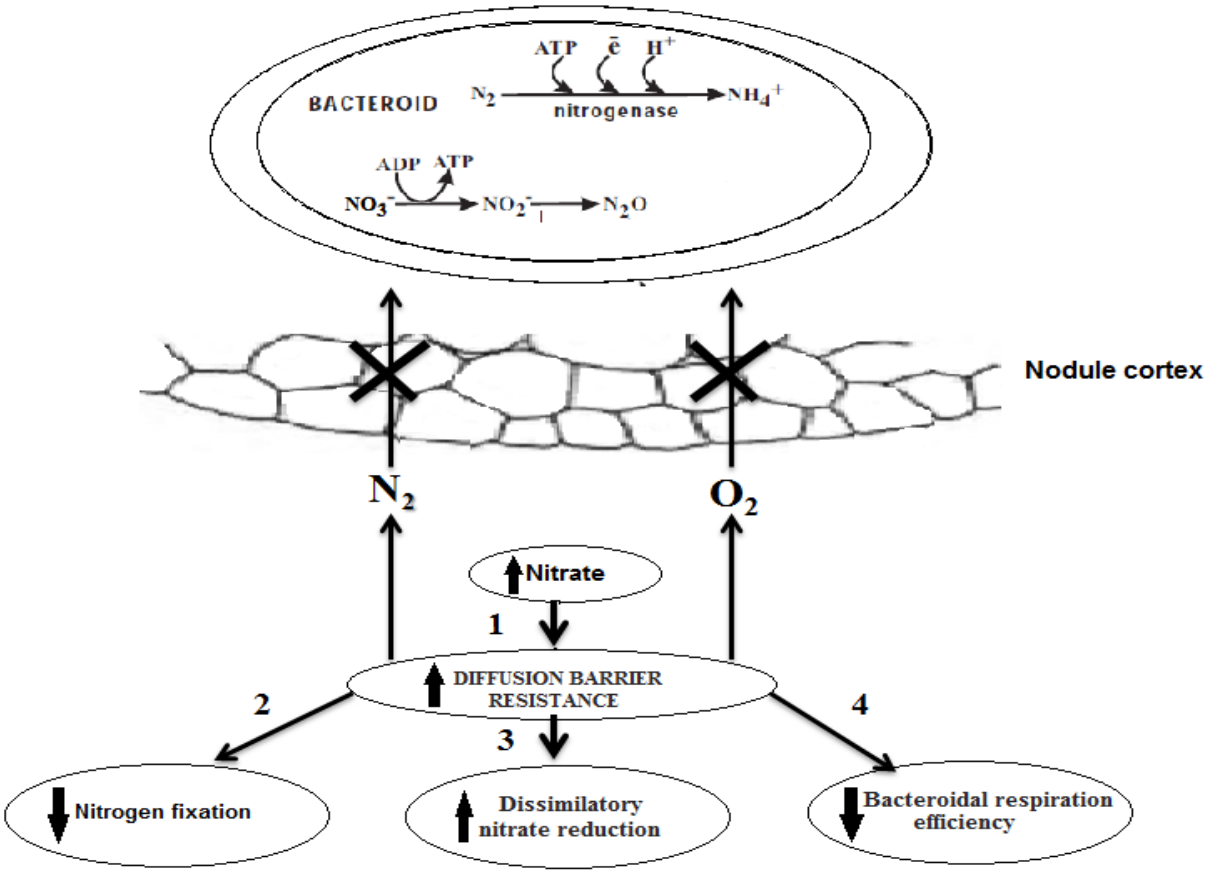

Figure 2. Schematic representation of the effect of the gas diffusion barrier in nodule cortex on bacteroid nitrogen fixation and dissimilatory nitrate reduction (Modified from Luciński et al., 2002). The nitrate availability in the soil induces: 1. Increase of diffusion barrier resistance. 2.4. Decrease of nitrogen fixation and bacteroidal O2 respiration. 3. Lowering of nodule oxygen conditions stimulate simultaneously dissimilatory nitrate reduction.

in *Rhizobium*-legume symbiosis. In fact, an excess of nitrates may cause an inhibitory action on nodulation and N_2 fixation activity (Luciński et al., 2002). The process of this inhibition is not fully understood, although several hypotheses have been proposed (Luciński et al., 2002). Some studies have concluded that legume plantation in soils containing a significant quantity of nitrates can have negative effect on the symbiosis induced by rhizobia (Luciñski et al., 2002) and can inhibit nodulation and nitrogen fixation of acacias (Brockwell et al., 2005). The plant-available N in soil reduced the inoculation response for *A. auriculiformis, A. mangium and A. mearnsii* in pot experiments (Turk et al., 1993). It has been showed in previous studies that the presence of NO_3^- ions reacts negatively on root infection (Wahab et al., 1996), nodule development and nitrogenase activity in legume plants because of the accumulation of nitrite (Luciñski et al., 2002). In the same context, it was demonstrated that the addition of NO_3^- (5-16 mM) to the alfalfa seedlings growth medium reduced significantly the number of rhizobial cells adhering to the alfalfa seedling roots (Zahran et al., 1999). It is also known that the free oxygen concentration inside the nodules is among the major factors that can induce changes in the nitrogenase activity. Oxygen availability in the infected zone nodule is limited, among others, by the gas diffusion resistance in nodule cortex. The presence of nitrate can directly or indirectly influence the effectiveness of resistance to gas diffusion which adversely affects the nodulation and the nitrogen fixation (Luciñski et al., 2002). In the presence of nitrate, both the energy cost of the nitrogen fixation process and the gas diffusion resistance increases, whilst the efficiency of the bacteroid respiration decreases (Figure 2).

Several species of rhizobia can resist to the presence of nitrates during infection and nodulation to a certain degree by induction of hydrogenase expression. This membrane enzyme is characteristic of some diazotrophs and can help some strains to be more tolerant to nitrates (Serrano and Chamber, 1990). Moreover, it was found that hydrogenase contributes to the formation of H^+ gradient

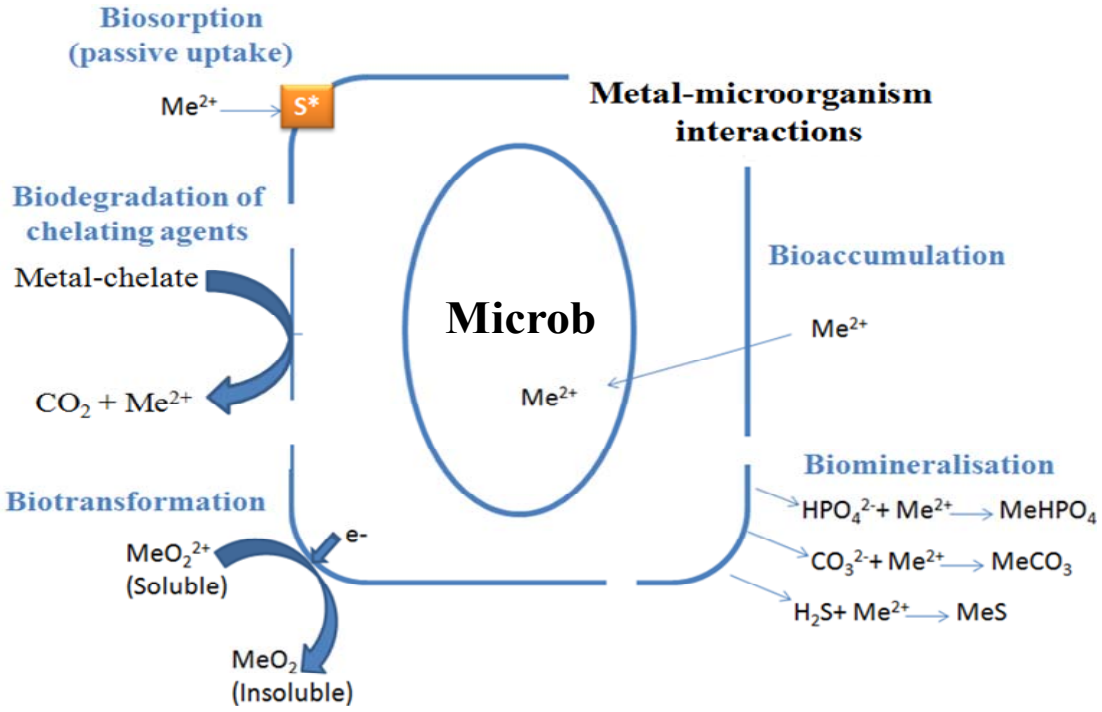

Figure 3. Schematic representation of the metals-microorganism interactions (Modified from Ledin, 2000). Me^{2+}: metal cation. * Functional groups present on the cell wall: carboxyl, phosphodiester, amines, hydroxyls etc.

across bacteroid membrane that enables ATP synthesis (Luciñski et al., 2002).

Heavy metals

Heavy metals are known as the most important inorganic pollutants which persist in the soil over long periods and have ecotoxicological effects on plants and soil micro-organisms. Some metals such as Zn, Cu, Ni and Cr are essential for growth of both rhizobia and their host plants, whereas others such as Cd, Hg and Pb seems to be not beneficial and could be toxic even at relatively low concentrations (Gadd, 1992). When exposed to moderate heavy metal concentrations, soil microorganisms were found to be very sensitive (Giller et al., 1998). Rhizobial response to different types of heavy metals depends on the applied concentrations (El-Hilali, 2006). Hence, cadmium even at considerably low concentration was found toxic for the microsymbiont, inhibited the nitrogenase activity and adversely affected the metabolic activities such as legume's photosynthesis (Ahmad et al., 2012). In contrast, nickel can induce a significant increase in the activity of hydrogenase in bacteroids (El-Hilali, 2006).

Microorganisms have developed resistance mechanisms to support high heavy metals concentrations while ensuring the maintenance of the biological role of essential ions (Figure 3). *Rhizobium* is able to produce huge amounts of extracellular polysaccharide and lipopolysaccharide which sequester most of the extracellular metal and play a role as first-defense barrier against heavy metal stress. However, they were not sufficient to support the highest levels of stress imposed (Mandal and Bhattacharyya, 2012). One of the most common resistance mechanisms is the extrusion of heavy metals from bacterial cell, avoiding accumulation to levels that possibly inhibit growth, or cause cell death (Pajuelo et al., 2011). This mechanism can be complementary to other resistance mechanisms (such as efflux mechanisms) avoiding reentry of expulsed metal, especially in extreme situations. Some of the efflux resistance systems are ATPases and chemiosmotic ion/proton exchangers (Silver and Phung, 2005). In addition, accumulation and complexation of the metal ions inside the cell, biotransformation of toxic metal to less toxic forms, methylation, precipitation and chelation with S-rich ligands like metallotioneins, glutathione, etc. are other metal detoxification mechanisms used by microorganisms (Gusmão et al., 2006). Gram negative bacteria can also synthesize proteins that adhere to the metal and store it in the periplasm in order to keep metals out of the cytoplasm and plasma membrane where the important reactions take place (Pajuelo et al., 2011).

Table 1. Principal mechanisms adopted by rhizobium to tolerate stress factors.

Stress factors	Mechanisms	References
Salinity	- Intracellular accumulation of organic solutes	Zahran (1999)
	- Cell morphology and size changes	Ventorino et al. (2012)
	- LPS and EPS structure changes	Ventorino et al. (2012)
	- Plasmid-mediated resistance	Pereira et al. (2008)
	- Gene expression changes	Lapez-Go'mez et al. (2013)
Temperature	- Synthesis of Heat shock proteins (HSP)	Alexandre and Oliveira (2013), Abd-Alla et al. (2014)
	- Synthesis of cold shock proteins (CSP)	Cloutier et al. (1992), O'Connell and Thomashow (2000)
pH	- Exclusion and expulsion of protons H^+	El-Hilali (2006)
	- Increase of potassium and glutamate level in the cytoplasm of stressed cells	Aron and Graham (1991)
	- LPS structure changes	Vriezen et al. (2007)
	- Accumulation of polyamines	Fujihara and Yoneyama (1993)
Drought	- Production of acid shock proteins (ASP)	Foster (1993)
	- Production of chaperones, sugars, EPS and synthesis of stress enzyme	Hussain et al., (2014)
	- Production of trehalose, siderophores and phytohormones	Hussain et al. (2014)
	- Phosphate solubilization	Hussain et al. (2014)
Nitrate	- Utilization of nitrogen oxides as terminal electron acceptors	Abd-Alla et al. (2014)
	- Induction of hydrogenase expression	Serrano and Chamber (1990)
Heavy metals	- Production of LPS and EPS	Mandal and Bhattacharyya (2012)
	- Extrusion of heavy metals from bacterial cell	Pajuelo et al. (2011)
	- Efflux mechanisms	Silver and Phung (2005)
	- Accumulation of the metal ions inside the cell	Gusmão et al. (2006)
	- Bioreduction of the metals toxicity	Gusmão et al. (2006)
	- Methylation, precipitation and chelation	Gusmão et al. (2006)
	- Synthesis of adhesion proteins	Pajuelo et al. (2011)

These resistance mechanisms are not incompatible and several of them can act simultaneously.

Hence, this review show clearly that even if environmental conditions such as salinity, temperature, acidity/alkalinity, drought, heavy metals, etc. are critical factors affecting different symbiotic steps of the *Rhizobium*-legume association, some microsymbionts strains have developed several mechanisms to tolerate these stress factors and overcome hard environment conditions. Several adaptation mechanisms of rhizobia to persist and to survive under stress conditions have been previously proposed and discussed in other studies and are summarized in Table 1.

CONCLUSION

The environmental conditions play an essential role in the control of legume-*Rhizobium* interactions. They may affect the growth, proliferation, symbiotic process and nitrogen fixation by *Rhizobium* in association with legumenous plants. In this literature review several

symbiotic systems of rhizobia which are tolerant to extreme conditions of salinity, alkalinity, acidity, drought, metal toxicity, fertilizer, etc., were identified.

Under poor conditions, *Rhizobium*-legumes symbiosis is very important because it may be the only way to fix nitrogen; this is why the selection of symbiotic partners tolerant to broad range of unfavorable environmental conditions is essential for agricultural pastoral systems.

Rhizobium-legume response to different environmental stress is complex phenomena that require the intervention of many genetic and biochemical adaptation mechanisms which should be included in future studies. In fact, further knowledge on these mechanisms involved by rhizobia to cope with adverse conditions will allow us to better understand their physiology and to select efficient isolates that can be used in inoculation projects for promoting the plants growth or in engineering genetic.

Conflict of interest

The author(s) have not declared any conflict of interest.

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