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Impact of *Harsh* Environmental *Conditions* on Nodule Formation and Dinitrogen Fixation of Legumes

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1. Introduction

Dinitrogen (N₂) comprises almost 80% of the atmosphere. The triple-bonded molecule cannot be used directly by higher plant. The element nitrogen, or "azote," meaning "without life," as Antonie Lavoisier called it about 200 years ago, has proved to be anything but lifeless, since it is a component of food, poisons, fertilizers, and explosives (Schoot Uiterkamp 1990). The atmosphere contains about 10^{15} tonnes of N₂ gas, and the nitrogen cycle involves the transformation of some 3×10^9 tonnes of N₂ per year on a global basis (Postgate 1982). However, transformations (e.g., N2 fixation) are not exclusively biological. Lightening probably accounts for about 10% of the world's supply of fixed nitrogen (Sprent and Sprent 1990). The fertilizer industry also provides very important quantities of chemically fixed nitrogen. World production of fixed nitrogen from dinitrogen for chemical fertilizer accounts for about 25% of the Earth's newly fixed N₂, and biological processes account for about 60%. Nitrogen availability is probably the second most limiting factor in agricultural production, second only to water availability (Date 2000). The legume-Rhizobium symbiosis is the single most important source of biologically fixed nitrogen in agricultural systems (Graham and Vance 2000). Since it is a biological process, it does not depend on external sources of energy, except for free and renewable sunlight, and has few detrimental ecological effects (Phillips 1999).

2. Importance of biological nitrogen fixation by legumes

The legume-*Rhizobium* symbiosis allows many species to obtain their nitrogen nutrition from N_2 . This has been known since the Greeks discovered the value of legumes in enhanc-



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ing soil fertility, some 2300 years ago. Since 1888 it has been known that this is due to the nodules on legume roots and the bacteria in them (Date 2000). This nitrogen fixation is very hard to quantify in economic (Lanyon 1995) or ecological terms (Hoglund and Brock 1987), but is estimated to be responsible for between 45% (Danso 1995) and 85% (Vance et al. 1988) of the biological nitrogen fixation in agricultural areas. Biological nitrogen fixation (BNF) has the advantage of being environmental friendly and therefore would be ideal for sustainable agriculture. Biological nitrogen fixation, a key source of N for farmers using little or no fertilizer, constitutes one of the potential solutions and plays a key role in sustainable grain legumes production. Given the high cost of fertilizer in developing countries and the limited market infrastructure for farm inputs, current research and extension efforts have been directed to integrated nutrient management, in which legumes play a crucial role (Chianu et al. 2008). Certain microorganisms have the ability to use the renewable source of energy to fix atmospheric nitrogen under mild conditions, such as normal temperature and normal pressure. Nitrogen fixation is a key process in which molecular nitrogen is reduced to form ammonia, which is the form of nitrogen that is used by living systems for the synthesis of many bioorganic compounds. Biologically-fixed nitrogen could be directly "absorbed" by plants and keep the environment almost "untouched". Crop rotation with legumes has been recognized to increase soil fertility and agricultural productivity since ancient China and Rome (Cheng 2008). Rhizobia live in the rhizosphere of leguminous plants such as soybeans and faba bean, forming colonies that produce root nodules (Foth 1990). Biological nitrogen fixation is an oxygen dependent reaction and therefore is prevalent in legumes growing in aerated, upland soils. Currently, approximately 2 tons of industrially-fixed nitrogen are needed as fertilizer for crop production to equal the effects of 1 ton of nitrogen biologicallyfixed by legume crops. Therefore, biologically-fixed nitrogen influences the global nitrogen cycle substantially less than industrially-fixed nitrogen. One day, this situation needs to be changed. Research in this field has pivotal importance and would be significantly beneficial.

Inoculation with compatible and appropriate rhizobia may be necessary where a low population of native rhizobial strains predominates and is one of the solutions which grain legume farmers can use to optimize yields. It is critical for sustained yield in farmlands deficient in native rhizobia and where N supply limits production. Research on use of *Rhizobium* inoculants for production of grain legumes showed it is a cheaper and usually more effective agronomic practice for ensuring adequate N nutrition of legumes, compared with the application of N fertilizer (Chianu et al 2008). The major findings are: (1) complete absence of or very weak institutions, policy and budgetary support for biotechnology research and lack of its integration into wider agricultural and overall development objectives, (2) limited knowledge of inoculation responses of both promiscuous and specifically nodulating legume varieties as well as the other factors that inhibit BNF, hence a weak basis for decision-making on biotechnology issues, (3) limited capacity and lack of sustainable investment, (4) poorly developed marketing channels and infrastructure, and limited involvement of the private sector in the distribution of inoculants, and (5) limited farmer awareness about and access to (much more than price) inoculants. The lessons learned include the need: (1) to increase investment in *Rhizobium* inoculation technology development, and strengthen policy and institutional support, (2) for public private partnership in the development, deployment and dissemination of BNF technologies, (3) to develop effective BNF dissemination strategies (including participatory approach) to reach farmers, and (4) for greater emphasis on capacity building along the BNF value chain. According to an FAO report, production of N fertilizer for 2007 was 130 million tons of N, and this should further increase in the coming years (FAO 2008). This extensive use has certain drawbacks. A proportion of added fertilizer is lost as a result of denitrification and leaching of soil by rainfall and irrigation. In addition, leaching leads to water pollution is caused by eutrophication. As a consequence, extending application of biological nitrogen fixation by any means is an important issue.

The legume-Rhizobium symbiosis is the single most important source of biologically fixed nitrogen in agricultural systems (Graham and Vance 2000); although under some conditions free-living and associative systems are important (Phillips 1999). Since it is a biological process, it does not depend on external sources of energy, except for free and renewable sunlight, and has few detrimental ecological effects (Phillips 1999). Conversely, chemical nitrogen fixation depends on fossil fuels (generally natural gas), both as an energy source and as a reductant source (Braswell et al. 1997). In addition, extensive use of nitrogen based fertilizers tends to be inefficient, with absorption by the crop ranging from 20 to 50 % of applied nitrogen (Graham and Vance 2000). Much of the nitrogen not absorbed by crops can find its way into ground water, lakes and rivers, where it poses both health and environmental problems (Graham and Vance 2000). Since the legume-bacteria symbiosis is so important in agriculture, even a slight increase in its efficiency, in terms of total nitrogen fixation, would have a major impact a global nitrogen inputs, and increase the efficiency of production for the corresponding cropping system (Phillips 1999). The interaction between legumes (Leguminosae) and bacteria in the family Rhizobiaceae leads to the development of a nitrogen-fixing symbiosis (Ohyama et al. 2009). Several environmental factors can adversely affect the performance of symbiotic nitrogen fixation by legumes. These factors can intervene at the following levels: survival of rhizobia in the soil, the infection process, nodule growth and nodule function. Also, these factors can affect N₂ fixing performance indirectly through their effects on host plant growth.

3. Effect of harsh environmental conditions on survival of rhizobia in agroecosystems and process of nodulation and nitrogen fixation of legumes

Rhizobia is the common name given to a group of small, rod-shaped, Gram-negative bacteria that collectively have the ability to produce nodules on the roots of leguminous plants and belong to the family Rhizobiaceae, which are part of the α -proteobacteria. In early studies, the taxonomy of rhizobia was based on the rate of growth of isolates on laboratory media and their selective interaction with their plant hosts. It was soon established that no strain could nodulate all plants, but that each could nodulate some legumes though not others (Long 1989). This led to the concept of cross-inoculation groups, with organisms grouped according to the hosts they nodulated. Within the genus Rhizobium several strains nodulate a common host, but are distinct according to genetic and/or phenotypic properties and are therefore classified as distinct species (e.g. *R. tropici* and *R. etli*). For a time this was the basis on which rhizobia were identified. However, developments in molecular biology and advances in bacterial taxonomy (Graham et al. 1991) have resulted in a rhizobial taxonomy based on a wide range of characteristics and to the distinction of new genera and species. Rhizobia were confined to the Alphaproteobacteria, namely in the order Rhizobiales. The Alphaproteobacteria class comprises 11 genera that include bacteria able to induce nodule formation in legume plants: Agrobacterium, Allorhizobium, Azorhizobium, Bradyrhizobium, Devosia, Mesorhizobium, Methylobacterium, Ochrobactrum, Phyllobacterium, Rhizobium and Ensifer (Euzéby 1997). Agrobacterium genus has been proposed by Young et al. (2001) to be emended and reclassified as Rhizobium. The members of the genus Sinorhizobium were transferred to the genus Ensifer by decision of the Judicial Commission of the International Committee on Systematics of Prokaryotes (Tindall 2008). Three additional genera belonging to the Betaproteobacteria class were recently added to the list of genera containing rhizobia species, namely, Burkholderia (Moulin et al. 2001), Cupriavidus (Chen et al. 2001,2003) and Herbaspirillum (Valverde et al. 2003). It is noteworthy to mention that these 14 genera also include a variety of non-symbiotic bacteria. More recently a member of the γ -proteobacteria has been found that also nodulates legumes (Benhizia et al. 2004).

When studying any living organism, it is important to know how each species grows and responds to certain conditions that can be found in their natural environment. Ascertaining how bacteria respond to environmental signals, or stressful conditions, is a vital part to understanding how those microbes live, thrive and survive. Every bacterium has optimum conditions that make this process easier, however in order to survive in a changing environment (or some other form of stress) the bacteria must be able to adapt. This adaptation is a stress response. Two types of stress responses operate in microorganisms: the general stress response and specific stress responses. The general stress response is normally controlled by a single or a few master regulators (Bremer and Krämer 2000) and provides cross-protection against a wide variety of environmental cues, regardless of the initial stimulant (Hecker et al. 1996; Hecker and Völker 1998). This response is effective in allowing the cell to survive, but it may not be enough to let the cell grow under the stressful conditions (Bremer and Krämer 2000). Under prolonged stress conditions cells employ specific stress responses, which utilize highly integrated networks of genetic and physiological adaptation mechanisms (Bremer and Krämer 2000). Usually, there is also a complex relationship between cellular response systems and global regulators, adding another level of control to the cell's emergency stress response and long-term survival reactions (Hengge-Aronis 1999). Although the above description is usually what happens, not all general responses occur immediately on stressful stimuli as some activate on entry into stationary phase; likewise some specific stress response are induced as soon as stress is detected.

3.1. Temperature

3.1.1. Survival of Rhizobia

Soil environmental conditions are critical factors to the persistence and survival of rhizobia in the soil. The changes in the rhizospheric environment can affect both growth and saprophytic competence, which will influence competitiveness and persistence (Dowling and Broughton 1986). In arid and semiarid regions of the tropics, the soil temperatures near the surface can be very high. In Egyptian sandy soils, the temperature near the soil surface was 59 °C at the air temperature 39°C. However, the soil temperature decreased rapidly with depth, being moderate 35 °C, at 15 cm. It appears, however, that rhizobia are more resistant to high temperatures in soil than in laboratory medium (AbdelGadir and Alexander 1997). The study performed with Indian desert soils suggested that not the high soil temperature but the low organic matter and poor soil moisture were the major factors that reduced the numbers of different micro-organisms (Rao and Venkateswarlu 1983). Indeed, in droughtaffected Acacia senegal soils the numbers of culturable rhizobia were significantly reduced, approximately from 10⁷ to 10⁶ cfu g⁻¹. In conclusion, one can assume that during the dry season, the water deficit together with the high soil temperature will considerably decrease rhizobial numbers or cause a lack of rhizobia in the surface soils (0-10 cm). In order to improve the yield of legumes in more adverse environments, stress-tolerant cultivars should be combined with stress tolerant rhizobia. Studies on rhizobia biodiversity are an important approach to find more tolerant strains, even when non-adverse environments are sampled, since populations often contain tolerant strains to non-acting stresses, as resilience to respond to future problems (Giller et al. 1997). The ability of rhizobia to persist in the absence of their host plant is perhaps more dependent of their ability to endure adverse environmental factors than during symbiosis, where the nodule represents a protective environment.

Environmental stresses especially during the period between inoculation of seeds and germination impose severe problems to establishment of a successful symbiosis (Weaver and Holt 1990; Weaver et al. 1985). Every bacterium has its own optimum conditions, under which it grows at its best. For most rhizobia, the optimum temperature range for growth is 28 – 31 °C, and many are unable to grow at 37°C (Zahran 1999). Not only do the bacteria themselves have an optimum temperature range, but the processes within them do as well. Survival of Rhizobium leguminosarum, the microsymbiont of faba bean, may be affected mainly by extremes in temperature. Differences in adaptation to high temperatures have been demonstrated from different climatic zones. Eaglesham and Ayanaba (1984) reported that more than 90 % of cowpea rhizobia isolated from hot dry Sahelian Savanna in Niger were able to grow at 40 °C while the rhizobia isolated form cooler humid regions of West Africa did not grow at this temperature (Bowen and Kennedy 1956; Munevar and Wollum 1981). Some rhizobial strains isolated from nodules in arid environments are able to grow at 40 °C or even higher (Eaglesham et al. 1981). Kluson et al. (1986) reported differences in the tolerance of bradyrhizobia cultivated in liquid medium and in soil to temperatures within the range of 20 to 35 °C. Gewaily et al. (1991) similarly reported that survival of Rhizobium leguminosarum was significantly reduced at 40 °C when inoculated in sterile and non sterile soils. Baldani and Weaver (1992) attributed heat tolerance of *Rhizobium leguminosarum* biovar *trifolii* strains to cryptic plasmids. These plasmids induce the synthesis of heat shock proteins upon exposure of bacteria above normal growth temperatures (Sen et al. 1990). Abd-Alla and Abdel-Wahab (1995a) reported that Strain RCR 1001 was more resistant to heat and nodulated faba bean better than other tested strains. Although strain adaptation to high temperature has been reported, it has been associated with decreased effectiveness in establishing symbiosis, mostly due to plasmid loss (Hungria and Franco 1993). Additionally, heat stress has been shown to induce the synthesis of heat shock proteins in *Rhizobium* (Michiels et al. 1994) and to modify lipopolysaccharide patterns and bacterial mobility (Zahran et al. 1994). Studies on rhizobia biodiversity are an important approach to find more tolerant strains, even when non-adverse environments are sampled, since populations often contain tolerant strains to non-acting stresses, as resilience to respond to future problems (Giller et al. 1997). Temperature is often pointed out as the major factors in determining the bacterial community diversity (Fierer and Jackson 2006; Staddon et al. 1998).

The optimum temperature for rhizobia growth is 25-30 °C (Zhang et al. 1995), however in both saprophytic and symbiotic life rhizobia are often subject to temperatures out of this range. Most studies in rhizobial temperature stress tolerance focus soybean and commonbean microsymbionts. Soybean isolates grow weakly at 40 °C and no isolate was able to grow at 45°C (Chen et al. 2002). Rhizobia nodulating P. vulgaris can survive to 47 °C, but their symbiotic effectiveness is loss at high temperatures; while other isolates tolerant to 40 °C were able to remain infective at that temperature (Karanja and Wood 1988). Nandal et al. (2005) reported that mutants tolerant to high temperature (43 °C), obtained from a thermosensitive Rhizobium sp. strain, exhibited a different protein profile from that of the wild type at high temperature, namely the mutant strains showed overexpressed proteins and new proteins. A protein of 63-75 kDa was overproduced in all mutant strains, which probably corresponds to DnaK. In chickpea rhizobia, a 60 kDa protein that could correspond to GroEL was found to be consistently overproduced when isolates were submitted to heat stress (Rodrigues et al. 2006). Strains isolated from a chickpea wild relative (Cicer anatolicum), collected from high altitudes, were successful in nodulating chickpea at low temperatures, what represents an alternative source of chickpea nodulating rhizobia with potential use as inoculants (Ogutcu et al. 2008). The changes in the population occupancy of some bradyrhizobia tested were in accordance with the geographical distribution of indigenous soybean-nodulating bradyrhizobia (Saeki et al. 2006). Saeki et al. (2010) reported that the occupancies of bradyrhizobia in soil microcosms after long-term incubation may change depending on the incubation temperature. They suggest that soil temperature may be one important environmental gradient determining bradyrhizobial niches from northern to southern regions of Japan. Temperature stress is generally divided into two classes: heat shock and cold shock. Bacterial heat shock is the more characterised of the two (Phadtare et al. 2000). The heat shock response is very similar to the acid stress response, in that many proteins with a similar mode of action are synthesised. Heat shock proteins contribute to heat tolerance by conferring heat protection on the bacteria but do not alter the internal temperature of the cell (Yura et al. 2000). Like acid shock protein, there are two main types of heat shock protein: chaperones and proteases. These work in the same way as the cid shock proteins. Heat shock proteins, and their regulation, structure and function, have been studied in great detail. Their function appears to be highly conserved between both prokaryotes and eukaryotes (Netzer and Hartl 1998). Some of these proteins are also vital under normal (non-heat shock) growth conditions (Münchbach et al. 1999; Lentz 2004).

Cold shock is essentially the opposite of heat shock. Instead of proteins misfolding and denaturing, cells undergoing cold shock have to contend with a loss of membrane and cytosol fluidity and with the stabilisation of secondary structures of RNA/DNA (Phadtare et al. 2000). RNA/DNA stabilisation leads to a decrease in the efficiency of translation, transcription and replication. Bacterial cold shock response is an immediate and transient response to the temperature downshift. This is followed by low temperature adaptation that allows continued growth at low temperatures (Panoff et al. 1997). Generally, bacteria overcome loss of fluidity by increasing the amount of unsaturated fatty acids in the membrane phospholipids (Phadtare et al. 2000). Cold shock response also leads to the production of many cold shock proteins (CSPs). Just like ASPs and HSPS, these too are mainly chaperones and proteases (Phadtare et al. 2000). However, instead of protecting against the misfolding of proteins, the CSP chaperones are primarily used to bind to RNA/DNA to prevent stabilisation and allow translation and transcription to proceed as usual (Phadtare et al. 2000). CspA is an RNA chaperone and a major CSP found in many bacteria (Jiang et al. 1997). A CspA homologue is present in Sinorhizobium meliloti and is induced following a temperature downshift from 30 to 15 °C, along with the three rRNA (rrn) operons. It is unknown what function the genes and products of the rrn operons or CspA have in response to cold shock, as mutations made in these genes showed no change in cell phenotype at 15 °C compared to the wild-type (O'Connell et al. 2000; Gustafson et al. 2002). TypA is also required for growth at low temperatures and is believed to act as a regulator by controlling the phosphorylation of proteins (Kiss et al. 2004). Expression of several heat shock operons, mainly coding for small heat shock proteins, is under the control of repression of heat shock gene expression in various rhizobial species (Nocker et al. 2001). The molecular bases of temperature stress tolerance in rhizobia were investigated, by comparing the expression of chaperone genes dnaKJ and groESL in thermotolerant and thermosensitive isolates. Tolerance to cold, heat and heat shock was evaluated for 53 mesorhizobia (Alexandre and Oliveira 2011). The analysis of the dnaK and groESL expression by Northern hybridization, using isolates from three species groups, showed an increase in the transcripts levels with heat, but not with cold stress. Laranjo and Oliveira (2011) reported that under heat stress a protein of approximately 62 kDa was newly detected in Mesorhizobium chacoense. The over expression of a 62 kDa protein in Mesorhizobium huakuii and Mesorhizobium septentrionale was controlled groEL expression and increased upon heat shock. Interestingly, a 62 kDa protein, the NdvCprotein, has been described as important in osmoregulation, associated with symbiotic systems in B. japonicum (Bhagwat et al. 1996). Further studies are required to elucidate the function of overexpressed genes in order to clarify their role in environmental stress tolerance of rhizobia, as well as their contribution to symbiotic effectiveness (Alexandre and Oliveira 2012).

3.1.2. Rhizobium- Legume molecular signalling exchange

Molecular signalling in the rhizosphere controls the nature of relationships between plants and other soil organisms. For instance, legume-derived signals, such as betaines and isoflavonoids, function by chemoattracting rhizobia and trigger the events that lead to endosymbiosis. The molecular signalling exchange between both microsymbiont and legume is fundamental for an effective legume-rhizobium symbiosis and can determine the specificity of this symbiotic relationship. Production of Nod factors or lipo-chito-oligosaccharide signalling molecules by the prokaryotic partner is activated by the release of plant phenolic signals, mainly flavonoids, into the rhizosphere. The phenolic flavonoid compounds partly determine the specificity of the symbiotic relationship as each Rhizobium species responds to specific flavonoids. Another determinant of host symbiont specificity is attributed to the different Nod factors substituents attached to the oligosaccharide backbone (Dénarié et al. 1996; Oldroyd 2001). Production of Nod factors or lipo-chito-oligosaccharide signalling molecules by the prokaryotic partner is activated by the release of plant phenolic signals, mainly flavonoids, into the rhizosphere. The phenolic flavonoid compounds partly determine the specificity of the symbiotic relationship as each Rhizobium species responds to specific flavonoids. Another determinant of hos symbiont specificity is attributed to the different Nod factors substituents attached to the oligosaccharide backbone (Dénarié et al. 1996; Oldroyd 2001). Most rhizobia species interact with only a few select legumes, but some have been shown to have a broad host range (Pueppke and Broughton 1999). For example, the strain Ensifer sp. NGR234 is able to nodulate over 120 plant genera, including the non-legume Parasponia andersonii. This feature may depend up on the family of Nod factors secreted, which are more diverse than in all other rhizobia known (Schmeisser et al. 2009) and in the concentration of Nod factors released by the NGR234 that is much higher than usual. More recently, M. opportunistum WSM2075 was isolated from Biserrula pelecinus root nodules, but the symbiotic genes of this organism provide a broader range of hosts for nodulation, including also Astragalus adsurgens, A. membranaceus, Lotus peregrinus and Macroptilium atropurpureum (Nandasena et al. 2009). On the other hand, there are legumes species that can be nodulated by several rhizobia species and others that are very restrict for nodulation and only accept as microsymbionts a reduced number of species. For example, Phaseolus vulgaris is known as a promiscuous host, since it can be nodulated by rhizobia belonging to diverse genera (such as Bradyrhizobium, Rhizobium and Ensifer) while Cicer arietinum is considered a restrict host, because it is nodulated only by Mesorhizobium species. Nevertheless, the host range depends on the legume cultivar used and conditions tested (Martinez-Romero 2003).

Temeprature play a critical role on the exchange of molecular signals between rhizobia and their host, thus reducing nodulation. Low temperature inhibits inter-organismal signaling between the two symbiotic partners. It has been shown that low temperature inhibits the biosynthesis and rhizosecretion of plant to-bacteria signal molecules (for example genistein from soybean roots, which are necessary for the induction of the nod genes of *B. japonicum* (Zhang and Smith 1997; Abd-Alla 2001, 2011). Low temperature also inhibit the induction of bacterial nodulation genes (nod gene) required for the biosynthesis of bacteriato-plant signaling molecules, lipo-chitooligosaccarides (LCOs), the so-called Nod factors (Zhang et al.

1995). The disruption of Nod factor production/excretion at low temperature incubations has been previously reported in R. leguminosarum bv. trifolii (McKay and Djordjevic 1993), and in B. japonicum (Duzan et al. 2005). Low temperatures delay the onset of nodulation (Pan and Smith 1998) and reduce the rate of subsequent nodule growth, resulting in effects on small final nodule size (de Lira Juniora et al. 2005). The presence of appropriates flavonoids in root exudates is a critical factor in nodule formation (Richardson et al. 1988 a,b) and dictate rhizobia legume specificity. The nodulation status of the pea (Pisum sativum L.) was most profoundly improved via the addition of the flavonoid naringenin (Bandyopadhyay et al. 1996), possibly via the induction of nod gene expression in R. leguminosarum. In the soybean-B. japonicum symbiosis, Kosslak et al. (1987) reported that genistein was the most effective inducer for the expression of the nodYABC operon of B. japonicum. Exogenous application of genistein results in the short circuiting of plant-bacterium signaling, and has been confirmed as an effective means to mitigate the adverse effects of low temperature on nodulation and nitrogen fixation (Zhang and Smith 1997). Pre-incubation of B. japonicum with genistein hastened the onset of nitrogen fixation, and increased the number and size of the nodules and plant growth. Additionally, this beneficial effect of genistein increased with decreasing temperature (Zhang et al. 1995). Firmin et al. (1993) and Ovtsyana et al. (1999) observed that nodule formation on cv. Afghanistan inoculated with R. leguminosarum bv. viciae is at least partly controlled by Nod-factors that carry a modification on the reducing terminus (an acetyl group encoded by the acetyl transferase NodX) as well as by a single genetic locus sym2A in the host-plant. R. leguminosarum bv. viciae strains producing Nod factors lacking this modification cannot nodulate these pea plants at low temperatures. Somehow, blockage of nodulation is overcome at higher temperatures suggesting a temperature-sensitive gene-for-gene relationship between nodX and sym2A (Kozik et al. 1995; Lie 1984; Olsthoorn et al. 2000). There is, however, little information available about the effect of high temperature on Rhizobium- Legume molecular signalling exchange, additional studies are required.

3.1.3. Rhizobia-legume nodulation and nitrogen fixation

The interaction between *Rhizobium* and host legume results in the formation of root nodules. The relation between rhizobia and legume is a selective one: individual species of rhizobia have a distinct host range, from narrow to broad, allowing nodulation of a particular set of leguminous species. Also, a particular leguminous species can be infected by a certain range of rhizobia. Development of functional nodules requires temporally and spatially controlled activity of genes and gene products of both partners (Oldroyd et al. 2001). The *Rhizobium*-legume interactions are controlled by signal exchange between the two partners. Legumes secrete specific flavonoid-type molecules (Gagnon and Ibrahim 1998). These compounds are main components in the chemical communication between symbiotic legumes and different species of nitrogen-fixing bacteria leading to nodule formation and N₂ fixation (Werner 2001). Flavonoid perception attracts the specific rhizoba to the root hairs and activates *nodulation (nod)* gene expression, via the bacteria activator NodD (Lindström et al. 2002). NodD activates transcription of *nod* boxes promoters, and represents the first level of host-specific recognition (Schultze and Kondorosi 2008). The *nod* genes expression lead to the production

of strain-specific lipo-chito-oligosaccharides, also called as Nod factors (Spaink 2000). Nod factors are considered the second level of host-specific recognition (Perret et al. 2000). Nodfactors induce various plant responses, including root-hair deformation, cortical cell-division, 'pseudo-nodule' and nodule-formation (Broughton et al. 2003). On production of Nod factors, the rhizobia then surround and attach to the tip of root, causing the root to start to curl (Yao and Vincent 1969). Rhizobia trapped in a curled hair, or between a hair and another cell, proliferate and begin to infect the outer plant cells, which in turn stimulates plant cells to produce infection threads (Callaham and Torrey 1981). Rhizobia are released from infection threads into the cytoplasm of plant cells. The Rhizobia are surrounded by plasma membrane of plant origin and then briefly replicate their DNA and divide before stopping both processes (Robertson et al. 1978). The membrane-enveloped rhizobia continue to divide within the host cells before they differentiate into bacteroids and start to fix nitrogen (Roth and Stacey 1989a, b). The endosymbiotic forms of the rhizobia are referred to as bacteroids and begin to fix nitrogen by the action of the enzyme nitrogenase (Xi et al. 2000). Atmospheric nitrogen is converted into ammonia by bacteroids and is subsequently assimilated into the plant following its conversion to glutamine by glutamine synthase. Within the nodule interior and the neighboring plant cells, essential nutrients are exchange between bacteroids and plant cells through a symplastic and apoplastic pathway (Abd-Alla et al. 2000 a).

Temperature regulates the metabolism of the plant and the bacteria, as well as the plantbacteria association (Young et al. 2006). Sometimes, the sensitivity of the host toward low temperature affects nitrogen fixation severely, leading to an abrupt cut-off at temperatures where the bacterial cells can still grow and metabolize. Root hair infection is much more temperature sensitive than nodule development (Hafeez et al. 2000). Naeem et al. (2008) suggested that suboptimum temperatures affect the growth of bacteroids even within the nodule and also affect the cell-to-cell movement of the bacteroids. Most of the work related to low soil temperature effects on signal exchange and nodulation has been conducted by Smith group on soybean. The root zone ternperatures blow 17.5 °C cause reduction and delays nodule formation and the onset of nitrogen fixation (Zhang and Smith 1996). Soil temperatures below 25 °C are prevalent in Canada until mid-June/mid-July (Zhang and Smith 1994). This delay reduces the time during which there is nitrogen fixation by more than 10 days (Zhang and Smith 1995). This leads to decline in total nitrogen fixation and lower crop yield (Lynch and Smith 1994), and can be at least partially corrected by the addition of genistein (Zhang and Smith 1997; Pan and Smith 1998). It is known, however, that low soil temperatures affect nodule initiation (Cordovilla et al 1999) and function (Rice et al. 1995). A greater concen in the tropical zone, but also important during the peak of summer in temperate zones., is excessive soil temperature and its ability to be an important limitation to nodulation and nitrogen fixation (Hungria and franco 1993; Boddey et al. 1997; Graham and Vance 2000). Hungria and Vargas (2000) note that maximum soil ternperature in the tropics regularly exceed 40 °C at 5 cm below the soil surface and 50 °C at 1 cm. Such high temperatures can limit nodulation. The range for optimal growth of legurnes dependent on nitrogen fixation have been recognized to be narrower than for nitrogen-fed legumes since at least (Hungria and Vargas 2000). Elevated temperature inhibited plant growth by diminishing net photosynthesis and nitrogen fixation (Aranjuelo et al. 2007). The optimum temperature for nodule formation is ranged between 25 and 33 °C (Pankhurst and Sprent 1976), although decline in nitrogenase activity have been recorded above 28 °C (Hungria and Franco 1993). High temperature might affect N₂ fixation directly or indirectly. Direct inhibition by temperature is a consequence of decreased nodule development (Dart and Mercer 1965; Piha and Munns 1987), activity (Meyer and Anderson 1959; Piha and Munns 1987; Hernandez-Armenta et al. 1989) and accelerated nodule aging. Indirect inhibition is related to temperature effects on root hair formation depression, decrease of nodulation sites (Frings 1976; Jones and Tisdale 1921), and modified adherence of bacteria to root hairs (Frings 1976). Moreover, Aranjuelo et al. (2007) observed that high temperature negatively affected bacteroid enzyme activity regardless of water availability, although the inhibitory effect on the bacteroid fraction was even greater. The decrease in malate dehydrogenase activity was observed in plants exposed to elevated temperature suggests that malate should also be reduced. Malate depletion implies that less carbon is redirected to mitochondria and that there is less energy available for N₂ fixation. The observed decrease in enzyme activity could be related to a reduction of the total soluble protein of the bacteroid. The large (70%) reduction in soluble protein could affect nitrogenase protein responsible for N₂ fixation activity. These results show that the nodule bacteroid fraction was more sensitive to drought and elevated temperature than the nodule plant fraction. Relatively high temperature has also been shown to influence infection, N₂-fixation activity, and legume growth (Mohammadi et al. 2012) and has a strong influence on specific strain and cultivar interactions (Arayankoon et al. 1990). Reduced nitrogenase activity in such nodules can also be explained on the basis of these effects. It has been reported that there is considerable variation in the rhizobia strains in terms of survival and nodulation ability at high temperature (Michiels et al. 1994). Karanja and Wood (1988) found that a high percentage of the strains that persisted at 45 °C lost their infectiveness. They attributed these losses to plasmid curing. It appears that every legume and Rhizobium combination has an optimum temperature relationship, which is around 30 °C for clover and pea, between 35 to 40 °C for soybean, peanut and cowpea, and between 25 to 30 °C for common bean (Long 2001).

3.2. Desiccation

3.2.1. Survival of Rhizobia

One of the most severe and widespread problems facing the crops production is the degradation of soil quality due to desiccation and salinity. In fact, almost 40% of the world's land surface is affected by salinity-related problems (Vriezen 2007). These two harsh environmental conditions can have a dramatic impact on the endogenous soil bacteria (Fierer et al. 2003; Han et al. 2005). Water, and its availability, is one of the most vital environmental factors to affect the growth and survival of micro-organisms (Potts 1994). Desiccation is one of the most common stresses soil microorganisms have to face so often. The responses of bacterial cells to desiccation can be: shrinkage of the bacterial cytoplasm and capsular layers, increase in intracellular salt levels, crowding of macromolecules, damage to external layers (pili, membranes), changes in ribosome structure, and decrease in growth. Reactive oxygen species can also damage proteins and DNA, leading to accumulation of mutations (Potts 1994). Death of rhizobial cells during desiccation was suggested to associate with changes in membrane integrity (Bushby and Marshall 1977). It has been hypothesised that during dehydration, the removal of water hydrogen bond to the phospholipid head groups of the membrane decreases the spacing between adjacent lipids. Then, the membrane is converted from the liquid crystalline into the gel phase already at room temperature. Subsequent rehydration results in a further phase transition of the membrane back to the liquid crystalline phase. As consequence, the membrane barrier is disrupted, leading to leakage of membranes (Potts 1994: Welsh 2000). Desiccation tolerance is the ability of cells to undergo nearly absolute dehydration through air drying, without being killed. This is the most severe water deficit stress since the removal of the cell-bound water imposes such structural, physiological and biochemical stresses that cells must adapt or die (Billi and potts 2002).

Of particular importance to the crop production is the impact of these harsh environmental conditions on the persistence and survival of rhizobia. The ability of rhizobia, to survive desiccation depends on their ability to cope with radiation stresses, reactive oxygen species, certain salts and solutes, and temperature extremes (Potts 1994; Billi and potts 2002; Ramos et al. 2001). Desiccation stress can be differentiated into three main stages: drying, storage and rehydration. These phases can be manipulated in several ways, namely, by the severity and the speed of drying and rehydration and by the duration of storage. The consequences of drying are fourfold: (i) the accumulation of salts and solutes, (ii) hyperosmotic stresses, (iii) the distribuance of metabolism when a certain water activity has been reached, and (iv) the accumulation of damage when the aqueous monolayer is removed from macromolecules. The accumulation of damage during storage is comparable to that caused by ionizing and UV radiation and damage by reactive oxygen species (Mattimore and Battista 1996; Shirkey et al. 2000) when organisms are not metabolically active and thus unable to repair any damage (Potts 1994). Finally, during rehydration, hypoosmotic stresses and the appearance of reactive oxygen species affect survival (Stead and Park 2000). A low survival of rhizobia occurs when drying was rapid (Bushby and Marshall 1976; Antheunissen and Arkestein-Dijksman 1979). These results were confirmed by Mary et al. (1985, 1986) who used Sinorhizobium meliloti RCR2011 to test survival after slow and fast drying with and without the addition of salts. It was recorded that fast drying process resulted in a decrease in survival of rhizobia in mineral soil (Chao and Alexander 1984). The reduction of water content causes the accumulation of salts and other compounds that produce osmotic and salt stress. High concentration of these compounds may be toxic and decrease the viability of cells (Steinborn and Roughley 1975; Vriezen et al. 2006). Conversely, the accumulation of certain compounds, including osmoprotectants and compatible solutes, may increase desiccation survival (Fougere and Le Rudulier 1990; Gouffi and Blanco 2000; Madkour et al. 1990). Osmoprotectants are exogenous solutes that stimulate bacterial growth in an environment with high osmolality, whilst compatible solutes are specific organic osmolytes that accumulate in high amounts within a cell to counter a hyper-osmotic gradient, but do not conflict with cellular functions (Miller and Wood 1996). Several compatible solutes have also been shown to stabilise enzyme stability in cells under stressful conditions (Poolman et al. 2002). Some compounds can function as osmoprotectants and compatible solutes, whilst some can only function as one of these groups.

Many osmoprotectants are transported into the cytoplasm where they act as, or are converted into compatible solutes (Hirsch 2010). Compatible solutes can be collected in high concentrations (Bremer and Krämer 2000). Since only a limited number of compounds meet the required criteria, the same compatible solutes are employed against hyper-osmosis throughout various bacteria (Braun 1997). Different compatible solutes work more effectively than others within their bacteria; e.g. glycine betaine is more effective in S. meliloti and E. coli than it is in Bacillus subtilis (Botsford and Lewis 1990); whilst proline is a compatible solute in E. coli but not in rhizobia (Gloux and LeRudulier 1989). Also, the strength of hyper-osmolarity can determine how the bacteria respond and what osmoprotectants are used (Breedveld et al. 1990; Gouffi et al. 2000). In a similar way, the compound used to bring about hyper-osmosis can stimulate a stronger stress response compared to others; e.g. generally sodium chloride (NaCl) induced hyper-osmosis causes a stronger stress response then sucrose induced hyper-osmosis, due to the ionic nature of NaCl (Gloux and Le Rudulier 1989). Compatible solutes can either by synthesized *de novo*, when required by the bacteria, or they are accumulated from the environment, depending on the situation. Under conditions where osmotic upshift is severe and immediate, cells do not have the time required to synthesise compatible solutes and so must acquire them from their environment. Reduction of water activity below 0.53 inhibited the function of RNA polymerase (Brown 1990). The storage phase is characterized by a slow decline in viable counts in rhizobia after slow drying. Mary et al. (1985, 1986) noted that the better survival of Sinorhizobium during storage under desiccation conditions from 22% to 67% than at 3% and 83.5% relative humidity. Similar trend have been recorded in Bradyrhizobium (Mary et al. 1994; Boumahdi et al. 1999). Cell death occurs at 83.5% relative humidity due to failure of intracellular enzymes. Antheunissen et al. (1981) showed that when dried occur at slow rate, rhizobia can survive desiccation for up to 4 years. These long-term storage studies are rare, but they show that in the family Rhizobiaceae, sinorhizobia can survive desiccation for years. A decline in viable cells during longterm storage under desiccation conditions can be explained by the accumulation of oxygenand radiation-induced damage (Vincent et al. 1961; Mary et al. 1994). The rate of rehydration has important consequences for repairing of accumulated damage and survival of bacteria. Combined process of drying and fast rehydration resulted in rupture of the cell envelope. Cells ruptured on the subpolar region which is also the region from which flagella originate. A suggestion is made that the point where a flagellum emerges from the cell is a point of weakness (Bushby and Marshall 1977; Salema et al. 1981). Slow rehydration of S. meliloti, R. leguminosarum, and Pseudomonas putida from dried inoculant formulations provide higher viable counts than did rapid rehydration (Kosanke et al. 1991). Chen and Alexander (1973) reported that a higher percentage of drought-tolerant than drought-sensitive bacteria was able to grow at low water activities. When these bacteria were grown in media with high salt concentrations, bacteria generally became more tolerant of prolonged drought and they persisted longer. In general, rhizobia do not, or cannot, synthesise their own solutes so use uptake systems to accumulate them (Gloux and Le Rudulier 1989). However, Streeter and Gonez (2006) reported that many rhizobia accumulate various carbohydrates such as trehalose, a disaccharide made up of two glucose molecules joined together by an α , α -1,1 linkage, is employed by many organisms to protect membranes and proteins from desiccation stress. Rhizobia also accumulate trehalose, among other carbohydrates, and also betaine and proline, in response to desiccation. Trehalose and sucrose are the only carbohydrates that are synthesized de novo in response to stress. Bacteria can produce trehalose from glucose 6-phosphate and UDP-glucose (the OtsA–OtsB pathway), from glycogen-like α (1-4)-linked glucose polymers (the TreY-TreZ pathway) and from maltose (the TreS pathway). The TreYZ pathway is common to many rhizobia (Streeter and Bhagwat 1999), whereas the OtsAB and TreYZ pathways are found in *R. leguminosarum* bv. trifolii strain NZP561 (Mcintyre et al. 2007). Cultured Bradyrhizobium japonicum USDA110 and B. elkanii were found to have three enzymes for trehalose synthesis: trehalose synthase (TS), maltooligosyltrehalose synthase (MOTS), and trehalose-6-phosphate synthetase (Streeter and Gomez 2006). Trehalose at relatively high concentrations is also present in *B. japonicum* bacteroids residing within nodules, suggesting that these differentiated nitrogen-fixing cells are under stress. Addition of trehalose to culture medium of B. japonicum at the time of desiccation stress had a significant positive effect on survival (Streeter 2003). Although it may not be practical to use trehalose as a carbon source in inoculant production, it may be possible to engineer greater trehalose accumulation in rhizobia to enhance their survival in response to dryness. Trehalose may protect desiccated cells by their ability to form glasses under dry conditions, in this way maintaining the native conformation of proteins and other macromolecules (Ramos et al. 2001). Trehalose levels also increase in R. leguminosarum bv. trifolii TA1 cells as they encounter osmotic stress (Streeter 1985; Breedveld et al. 1993). R. leguminosarum bv. trifolii strain NZP561 accumulates trehalose upon entry into stationary phase (Mcintyre et al. 2007), but in this rhizobial strain, trehalose synthesis is constitutive and modified post transcriptionally rather than induced as in other rhizobia. Mutations in otsA or treY individually in strain NZP561 did not dramatically affect trehalose accumulation, but double otsA treY mutants did not accumulate trehalose and were more sensitive to desiccation. They were also less competitive with regard to occupying nodules than were wild-typestrains (Mcintyre et al. 2007). The transcriptional analysis of the genome of B. japonicum subjected to desiccation stress indicated that genes critical for pilus are upregulated (Cytryn et al. 2007). Pili, especially type IV pili, are often important for biofilm formation (Shime-Hattori et al. 2006; Jurcisek and Bakaletz 2007). It is extremely likely that desiccation-stressed B. japonicum cells show some of the same patterns of gene expression, as do cells in biofilms. Similarly, S. meliloti cells grown under salt and osmotic stress (Dominguez-Fererras et al. 2006) upregulate some of the same genes uncovered in transcriptome arrays of biofilm cells of other bacteria (An and Parsek 2007). Biofilms are one of the many ways that bacteria use to protect themselves from desiccation and it is well known that exopolysaccharide, an important component of the biofilm matrix, protects bacteria from drought stress. Loss-of-function EPS mutants of many bacteria show impaired biofilm formation (Yildiz and Schoolnik 1999; Danese et al. 2000; Whiteley et al. 2001; Matsukawa and Greenberg 2004), as do S. meliloti exoY loss-of-function mutant cells (Fujishige et al. 2006) and exopolysaccharide mutants of M. tianshanense (Wang et al. 2008). Nevertheless, it is not known whether exopolysaccharide-deficient mutants that are incapable of biofilm formation are less capable of surviving desiccation stress under field conditions. Vanderlinde et al. (2010) demonstrated the important role for exopolysaccharide in desiccation tolerance in *Rhizobium leguminosarum* bv. *viciae* 3841 and identify a novel genetic element (ABC transporter) required for biofilm formation. Identification of a novel genetic element required for desiccation tolerance, and proper biofilm formation. An increased tolerance to desiccation can enhance the survival of rhizobacteria within the soil considerably (Rokitko et al. 2003). This is of particular interest with nitrogenfixing rhizobia because desiccation is a major cause of the poor on-seed survival rates of commercial inoculants and the subsequent poor performance of rhizobial inoculants in the field (Deaker et al. 2004). To further our understanding of how bacteria persist and survive in the soil environment, and improve on-seed survival of rhizobial inoculants, a complete understanding of the mechanisms used for desiccation tolerance is necessary.

3.2.2. Rhizobium-Legume molecular signalling exchange, noduation and nitrogen fixation

To the best of our knowledge, there is no information available addressing the impact of drought on molecular signals exchanges between the two partners. Further research is needed to clarify the impact of drought on Rhizobium-legume molecular signaling exchange. However, several studies both on herbaceous and woody legumes have shown that drought cause deleterious effect on nodulation and nitrogen fixation (Singleton and Bohlool 1984; Zahran and Sprent 1986; Arayangkoon et al. 1990; Marcar et al. 1991; Purwantari et al. 1995; Hatimi 1999). If the stress conditions are long or/and strong enough, formation of nodules will cease completely. It is generally believed that the infection process is the most sensitive phase during nodule development (Singleton and Bohlool 1984). Once nodules have formed, they are less affected by stress factors than the initial nodule formation process (Purwantari et al. 1995). Studies with herbaceous legumes having the root hair infection mode indicated that drought and salinity stresses cause changes in root hair morphology and decrease the numbers of markedly curling hairs. In mildly water-stressed Vicia faba nodules, the loss of turgor induced a reduction in the peribacteroid space, the vesicle membrane became sinous, and the cell cytoplasm appeared granular. Under more severe water stress, a total disappearance of peribacteroid spaces was associated with the rupture of vesicle membranes. The bacteroid content appeared to be very heterogeneous (Guerin et al. 1991). A shortage of water supply can slow the growth of the nodule and accelerate its senescence. Nitrogenase activity is decreased significantly, accompanied by the decrease in respiratory activity of the soybean and common bean nodules (Weisz et al. 1985; Gerosa-Ramos et al. 2003). A limitation in metabolic capacity of bacteroids and oxidative damage of cellular components are contributing factors to the inhibition of nitrogenase activity in alfalfa nodules (Naya et al. 2007). In addition, the transport of fixed nitrogen out of the nodule is decreased, possibly due to an insufficient supply of photosynthates in stems and leaves under stress (Huang et al. 1975).

The major pathway of water movement into nodules is through vascular connections with the root. The surface of the nodule is the main area for gaseous exchange and is therefore more adapted to water loss than uptake. Nodules always need an efficient water supply to export the product of fixation. Water-stressed plants transpire at a lower rate than unstressed plants. A lower rate of water movement out of the nodule during drought stress may restrict export of N₂ fixation products, thus inhibiting nitrogenase activity via a feedback mechanism (Serraj et al. 1999). Water shortage may induce oxidative stress in nodules. This leads to general decrease of antioxidant activities that are associated with nodule senescence (Hernandez-Jimenez et al. 2002; Porcel et al. 2003). It was found that water stress imposed during vegetative growth was more detrimental to nodulation and nitrogen fixation than that imposed during the reproductive stage (Pena-Cabrales and Castellanos 1993). The mechanisms of desiccation tolerance could be attribitted to the ability to limit to a minimum the cell metabolism, the increased catalase activity and the presence of specific plasmids for drought tolerance. Some experiences have shown that working with legumes and rhizobia strains selected for desiccation tolerance, a symbiotic interaction can take place (Soria et al. 1996). Abd-Alla and Abdel-Wahab (1995b) observed that nodulation and nitrogenase activity were significantly decreased by increasing drought stress. Leghaemoglobin and protein contents of nodule cytosol were also severely inhibited by drought stress. This decline was attributed to the induction of protease activity. However, carbohydrate contents of the nodule cytosol increased significantly. This accumulation was attributed to a sharp decline in invertase activity and low use of sugar by the bacteroids. This study indicated that harmful effects of water deficits can be alleviated by increasing K⁺ supplementation (Abd-Alla and Abdel-Wahab 1995b). Water stress is quickly reflected as changes in hormonal content (Hsiao 1973). The nodules are an active site of synthesis of auxins and cytokinins. Therefore, it is likely that nodules, besides the supply of organic N, are a source of cytokinins that makes the plant more tolerant to water stress (Phillips and Torrey 1973).

Many legumes are very sensitive to excess water. Nodule development and function are usually more affected than the infection itself, and some effects such as decreased nitrogenase activity may be even more intense than in the case of water deficit. Reduced to zero, the contribution of O_2 to the nodule appears to be the main problem of the effect of waterlog-ging. The diffusion of O_2 within the nodules is in part regulated by a physical barrier located in nodular parenchymal cells (Andres et al. 2012). Under stress conditions, the diffusion resistance increases by identifying a lack of O_2 inside the nodule, leading to inhibit its activity (Day and Copeland 1991). The ability of aerobic bacteria to utilize nitrogenous oxides, as terminal electron acceptors, enables them to survive and grow during periods of anoxia. This may be advantageous for the survival of rhizobia in soils (Zablotowicz et al. 1978).

3.3. Salinity stress

3.3.1. Survival of Rhizobia

The world's demand for food is increasing at such a rate that the ability to meet anticipated needs in the next several decades is becoming questionable. Irrigated agriculture presently accounts for about one-third of the world's production of food and fibre; it is anticipated that it will need to produce nearly 50 percent by the year 2040 (Rhoades et al. 1999). This will likely be difficult, because extensive areas of irrigated land have been and are increasingly becoming degraded by salinization and waterlogging resulting from over-irrigation

and other forms of poor agricultural management (Ghassemi, et al. 1995). Soil salinity affects about 800 Mha of arable lands worldwide (Munns and Tester 2008), and this area is expanding. Salinity affects agricultural production in arid and semiarid regions, where rainfall is limited and is not sufficient to transport salts from the plant root zone (Tester and Davenport 2003). The damaging effects of salt on organisms are caused not only by osmotic forces, but also by toxic levels of sodium and chloride. Salinity stress is important factor limiting the productivity of leguminous crops. Soil salinity reduces survival and growth of rhizobia in the soil and inhibits rhizobia-legume symbiosis, resulting in lower productivity of legumes (Abd-Alla 1992; Abd-Alla et al. 1998). Rhizobia are known to be more salt tolerant than their respective plant partners. Maximal limit of tolerance to salinity is superior in rhizobia as compared to their host plant which frequently constitute the limiting factor in saline soils (Kassem et al. 1985). In some cases bacteria that are highly salt tolerant as freeliving cells produce ineffective nodules, showing reduced symbiotic efficiency and/or low rates of nitrogen fixation (Chien et al. 1992, Abd-Alla and Abdel-Wahab 1995a). Some bacterial strains of various species are able to grow within the range of 300-700 mM NaCl (Mpepereki et al. 1997). Many authors reported that some rhizobial strains can persist and survive in the saline soils (ElSheikh and Wood 1989, 1990a; Ishaq et al. 1989), although the majority of rhizobia are not capable of tolerating the harmful effects of high osmolarity (Soussi et al. 2001). The ability of rhizobia to tolerate salt stress depends also on the species and even the strain of rhizobia studied (Bernard et al. 1986). Fast growing rhizobia are generally considered to be more tolerant to saline stress than bradyrhizobia (ElSheikh and Wood 1990b) and strains isolated from saline soils are typically more tolerant (Hua et al. 1982). Nevertheless, many fast growing rhizobia are very salt sensitive and some rhizobia isolated from saline soils are sensitive (Zahran 1999). The ability of rhizobia to adapt to fluctuations in the osmolarity of their surrounding is of fundamental importance for their survival. Osmotolerant rhizobia use a variety of accumulated or non-accumulated osmoprotectants, including betaines, amino acids and sugars as a strategy to counter the high oscillations of their environment osmolarity (Bernard et al. 1986; Gouffi et al. 1999). Osmoregulation is the main strategy employed by rhizobia to cope with salt stress (Ghittoni and Bueno 1996), including altered polysaccharide production upon salt treatment (Lloret et al. 1998). Survival and growth in saline environments are the result of adaptive processes, such as ion transport and compartmentation, osmotic solute synthesis and accumulation, which lead to osmotic adjustment and protein turnover for cellular repair (Munns and Termaat 1986; Paul and Cockburn 1989).

Bacteria grown in saline environments must maintain positive turgor across the membrane by allowing influx of salt or solutes or by exclusion of salts via the production of compatible solutes or other organic osmolytes. Bacteria prefer uptake over *de novo* synthesis of organic osmolytes when present in surrounding environment. Exogenous osmolytes that improve cell growth under adverse osmotic conditions are referred to osmoprotectans (Galinski 1995). Under elevated salinity *S. meliloti* can accumulate K⁺ ions (Miller and Wood 1996) and an organic anion, glutamate, and synthesize the following compatible solutes: N-acetylglutaminylglutamine amide (NAGGN), trehalose, and glycine betaine, if the medium contains its precursor, choline (Hua et al. 1982; Botsford and Lewis 1990; Smith et al. 1994; Talibart et al. 1994, 1997). Recent data indicate that the set of endogenous osmolytes produced by rhizobia can vary at least according to the species level and NAGGN has been found only in *S. meliloti* (Smith and Smith 1989; Smith et al. 1994; Talibart et al. 1994, 1997). The types of the accumulating osmolytes also depend on the stress level and on the growth phase of the cell culture. In *S. meliloti*, glutamate accumulated at low salt concentrations, but at higher levels, glutamate and NAGGN were observed. All three osmolytes, glutamate, NAGGN, and trehalose accumulated only at extremely high NaCl concentrations (Smith et al. 1994). When glycine betaine was exogenously supplied in the growth medium, it accumulated as the major osmolyte during the lag and early exponential phases, whereas glutamate and NAGGN prevailed at the late exponential phase (Talibart et al. 1997). There was no improvement in the growth of chickpea rhizobia with the addition of glutamate to 340-400 mmol⁻¹ NaCl in the medium (Botsford 1984; Elsheikh and Wood 1989; Gonzalez-Gonzalez et al. 1990).

• Trehalose was the major osmolyte of the stationary phase (Smith et al. 1994, Talibart et al. 1997). A number of different environmental factors, such as the level of osmotic stress, growth phase of the culture, carbon source and osmolytes of the growth medium control the combination of naturally occurring endogenous osmolytes in rhizobial cells (Smith et al. 1990, 1994). Bacteria prefer uptake of compatible solutes over synthesis de novo. The transport systems for external osmolytes (osmoprotectants) are relatively unspecific, and bacteria accept components of plant and animal origin (Galinski 1995). The enhancement of growth of rhizobia from different species, resulting from added glycine betaine and other betaines under saline conditions, has been recorded (Sauvage et al. 1983, Bernard et al. 1986). S. meliloti and many other rhizobial species can use glycine betaine as a C and/or N source under low osmotic stress but as an osmoprotectant in high osmolarity. Contrary, Bostford (1984) observed that exogenous application of glycine betaine did not ameliorate inhibition of the growth of S. meliloti in NaCl. Only the slow-growing B. japonicum, which is considered as an osmosensitive species, is incapable to transfer glycine betaine and its precursor choline (Boncompagni et al. 1999). Under low osmotic stress glycine betaine is degraded via successive demethylation to glycine, while at elevated osmolarity the catabolism is blocked and glycine betaine is accumulated in cells (Sauvage et al. 1983; Bernard et al. 1986; Smith et al. 1988; Boncompagni et al. 1999). Furthermore, Exogenous application of calcium alleviated the inhibitory effect of NaCl on growth of rhizobia and induced NaCl tolerance (Chien et al. 1991; Howieson et al. 1992; Reeve et al. 1993). There is an increasing demand for identifying rhizobial species that could also work under stressed soil environment so that the productivity of the inoculated legumes does not suffer under derelict soils.

3.3.1.1. Rhizobum-legume signalling exchange

Nodulation (nod) factors are products of nod gene exerssion of rhizobia. Nod factors are signals known to be used in the communication with leguminous plants in the process of root nodule formation. Nod factors are major host-specificity determinants (factors that determine which rhizobium nodulates with a specific host plant) and trigger the nodulation program in a compatible host (Mergaert et al. 1997). The influence of salt (NaCl) stress on the expression of enzymes involved in Nod factor production, and on the huge variety of Nod factors produced by growing *R. tropici* have been investigated (Estevez et al. 2009). In surprising study by Guasch-Vidal et al. (2013) observed that, in the absence of flavonoid inducers, high concentrations of NaCl induced nod genes and the production of Nod factors. The higher transcriptional activity at the nod region in the presence of NaCl, as revealed by the increased the β-galactosidase activity of a *nodP*:lacZ fusion, that lead to a higher and detectable production of Nod factor. However, a high concentration of salinity had adverse effect on Nod factor activity and decreased root hair deformation in soybean (Duzan et al. 2005). Evidence existed that introduction of exogenous nod gene inducers increases nodulation and nitrogen fixation of some legume species (Abd-Alla et al. 2013a). Pre-treatment of Bradyrhizobium japonicum with genistein increased nodulation and nitrogen fixation of soybean and common bean (Zhang and Smith 1996; Abd-Alla 2001, 2011); and pre-induction of Rhizobium leguminosarum with hesperetin and naringenin was found to stimulate nodulation and plant dry matter accumulation of pea and lentil plants (Begum et al. 2001). Flavonoid inducers act in low concentrations and the pre-activation of rhizobia used as inoculants (biofertilizers) in undoubtedly economically justified. Pre-activation of strains might increase rhizobial competitiveness in the soil environment (Hungria and Philips 1993). Indeed, it has been shown that flavonoid pre-activated B. japonicum increased soybean nodule quantity and weight (about 30%), the seasonal level of N₂ fixation (35%) and yields (10-40%) when compared to conventional inoculants (Zhang and Smith 2002). Likewise, field pea and lentil plants displayed increased nodulation and biomass production when inoculated with Rhizobium leguminosarum preinduced with hesperetin (Begum et al. 2001). In common bean (Phaseolus vulgaris L.), a negative effect of NaCl on the expression of nod genes by Rhizobium tropici and Rhizobium etli and on nodulation factors' pattern was observed (Dardanelli et al. 2008). The preincubation of B. japonicum with the signal molecule genistein, under saline conditions, was described as a method to alleviate the stressful effects of salt on soybean-B. japonicam symbiosis (Miransari and Smith 2009). Efforts to overcome the inhibitory effect of salinity on nodulation of feungreeck by pre-inocubation of Rhizobium tibeticum with hesperetin and apaginein were successful (Abd-Alla et al. 2013a). It is supposed that flavonoids have selective value in plant-microbe interaction under salinity stress. Improving legume inoculation efficiency is extremely important to improve legume production under drastic conditions.

3.3.1.2. Rhizobium-legume nodulation and nitrogen fixation

In legumes salinity can result in a root system devoid of root hairs and a mucilaginous layer, and incapable of forming an infection thread (Singleton and Bohlool 1984; Zahran and Sprent 1986). Unsuccessful symbiosis may be attributed to failure in the infection process due to poor establishment of *Rhizobium* (Rai and Prasad 1983). The reduction of nodulation in soybean under saline conditions was attributed to shrinkage of the root hairs (Tu 1981). Yousef and Sprent (1983) showed that NaCl affected nodulation and they concluded that there may also be effects on infection. In salt-treated faba bean plants, total nodule weight per plant was reduced with increasing amounts of salt given. A reduction of 16.7, 41.2, 50.8 and 72.6% in nodule weight was caused by salinity levels of 5.8, 8.8, 11.6 and 14.6 dSm⁻¹, respectively. Total nodule weight per plant followed a similar pattern to nodule number per

plant (Abd-Alla 1992). The detrimental effects of salinity on nodulation parameters were most pronounced for NOD1-3 and Williams 82, intermediate for DR-1, and less marked for PI416937. Self-grafted NOD1-3 plants showed 50 to 62% inhibition in nodulation responses (activity, number, mass) while grafting of PI416937 scions to NOD1-3 roots resulted in less than 7% inhibition by salt. Nodule number on PI416937 roots was greater when grafted to NOD1-3 scions (relative to self-grafted PI416937 plants), confirming a shoot role in autoregulation of nodule number (Abd-Alla et al. 1998). These results indicated that shoot factors are of primary importance in determining salt-tolerance of the PI416937 genotype and that hypernodulation expression in the mutant is negatively affected by salt treatment.

The salt injury on the symbiotic interaction not only inhibits the formation of the nodules, but also thereby leads to the inhibition in nitrogenase activity and reduction of the growth of the host plant. Other effects of salinity on the nodulation, includes formation of non-functional nodules with abnormal structure, and degradation of peribacteroid membrane (Bolanos et al. 2003). Tu (1981) observed that the interaction of *Bradyrhizobium japonicum* to the root hairs under increasing concentrations of NaCl in the range from 0% to 1.8% in the culture solution was affected. At a concentration of 1%, inhibition was evident in the bending of the hairs, a phenomenon markedly accentuated as rising concentrations. At 0.2% NaCl, growth was slow and at 1.2% nodulation did not occur. Results of some experiments on Vicia faba and Vigna unguiculata showed that the application of different concentrations of NaCl at the time of root hair formation significantly reduced root colonization, root hair curling, shrinking root hairs and hypodermic cells (Zahran and Sprent 1986; Georgiev and Atkins 1993). Several hypotheses have been suggested to explain the negative effects of salt on nitrogen fixation in plant legumes. It is likely that the depression in specific nitrogenase activity was due to salt reducing the protein, leghaemoglobin and carbohydrate contents of both the cytosol and the bacteroids. Salinity levels at 11.6 and 14.6 dSm⁻¹ induced a 37 and 59% decline in carbohydrate and 41 and 53% in leghaemoglobin and 19 and 53% in protein content of nodules, while nodule C_2H_2 reduction (specific nitrogenase activity) showed a 55 and 61% decreases, respectively (Abd-Alla 1992). Abd-Alla and Omar (1997) reported that 0.5% NaCl significantly reduced nodulation of fenugreek and addition of wheat straw and cellulytic fungi minimize the adverse effect of salinity on nodulation.

Protein synthesis is readily inhibited by water stress because of the decreases in the level of polyribosomes (Bewley 1981). Thus, the decline of nodule soluble protein may result from a general reduction of protein synthesis and from an increased protease activity in the cytosol (Becana et al. 1986). The disappearance of nodule soluble protein induced by plant water stress can be compared to the situation naturally occurring during nodule senescence (Pladys and Rigaud 1985) or after feeding the plant with nitrate (Ohyama and Kumazawa 1981, Nishiwaki et al. 1997; Ohyama et al. 2011; Abdel-Wahab and Abd-Alla 1995a,b, 1996). In both cases, the presence of active proteases was responsible for the protein digestion, and it seems likely that these enzymes may also be induced under water restricted conditions (Guerin et al.1990; 1991). It is clear that most biochemical parameters of the bacterial and plant components of the nodules (cytosol) were affected by high levels of salinity of 100 and 125 mM NaCl (Abd-Alla 1992). Both the protein and carbohydrate contents of nodule cyto-

sol were more sensitive to salinity than the corresponding bacteroids at the same levels of salinity. The negative effects of salinity on the oxygen diffusion barrier, which normally would reduce the O₂ flux into the nodule was also demonstrated (Soussi et al. 1998; Serraj and Drevon 1998). The decline in nitrogen fixation can be attributed to a reduced carbon supply to bacteroids, mainly in the form of malate limitation and likely a result of the salt induced inhibition of nodule carbon metabolism through the inhibition of sucrose synthase activity (Ben Salah et al. 2009). The accumulation of solutes such as proline, sucrose, and Dpinitol has been described in nodules of some legumes such as alfalfa and cowpea, and they exert an osmoregulatory function in situations of salinity (Irigoyen et al. 1992). Miransari and Smith (2007) indicate that under high levels of stress plant spent most of its energy to inhibit or alleviate the stress rather than developing a symbiosis with N-fixing bacteria. Miransari and Smith (2008) reported that genistein application into rhizobia inoculant improves plant growth through improved nodulation and nitrogen fixation in both normal and salt stress conditions. Inhbition of N2-fixation of legumes under salt stress seems to some extent to be due to nodule senescence. More work is required to elucidate the mechanisms by which salinity interferes with normal nodule physiology and function to alleviate the inhibitory effect of salinity in crop legume production.

3.4. Soil pH

3.4.1. Survival of Rhizobia

Agricultural soils are either alkaline or acidic that effect on rhizobial growth, survival and subsequent formation of nitrogen-fixing symbiosis with a legume host. Global warming and agricultural practices cause increase in the amount of soil affected by acidity, and thus limit legume crop productivity. Worldwide, more than 1.5 Giga hectares of acid soils limit agriculture production (Graham and Vance 2000) and as much as 25% of the agriculture land is impacted by problems associated with soil acidity (Munns 1986). Each Rhizobium has its own optimum pH, under which it grows at its best. Although neutral conditions are generally optimum for bacteria, different species of Rhizobium display varying degrees of pH resistance as measured by their ability to grow (not just survive) (Glenn and Dilworth 1994). Rhizobia can be more sensitive to acidic condition than their legume host. Indeed, it is in many cases the inability of the rhizobia to persist and survive under acidic conditions that reduces the effectiveness of the symbiosis. Therefore, the selection of rhizobial strains tolerant to acidic conditions may improve the acid tolerance of the legume through an efficient symbiotic nitrogen fixation under acidity conditions. However, the relationship between low pH of soil and rhizobia competitiveness, and ability to presist under acidic stress is not always straight forward. Chen et al. (1993) reported that mutants of R. leguminosarum have been able to grow at a pH as low as 4.5. Moreover, Foster (2000) recorded that S. meliloti was viable only below to pH 5.5. Some rhizobia have wide range of pH such as S. fredii can grow well between pH 4 – 9.5 but B. japonicum cannot grow at the extremes of that range (Fujihara and Yoneyama 1993). These values are the extremes and hindered the growth and survival of bacteria between 1 and 2 pH units (Richardson and Simpson 1989). Although some acidtolerant rhizobia strains have been selected (Wood et al. 1988; Vinuesa et al. 2005; Laranjo and Oliveira 2011), the mechanisms that employ to survive and grow under acidic conditions have not been fully elucidated and therefore the molecular basis for differences in pH toleranceamong strains of rhizobia is still not clear. Several genes, such as actA, actP, exoR, lpiA, actR, actS, and phrR, were shown to be essential for rhizobia growth at low pH (de Lucena et al. 2010). Kurchak et al. (2001) have been identified 20 genes in *R. leguminosarum* that are responsible for acid stress namely as *act* genes (acid tolerance).

Root nodule bacteria employ mechanisms for maintenance of intracellular pH (pHi) are crucial. Like other Gram-negative bacteria, root nodule bacteria show an adaptive acid tolerance response, with growth at moderately acidic conditions protecting against an extreme acid shock. Variations in acid tolerance within species of root nodule bacteria imply a genetic basis to low pH tolerance and studies of acid-sensitive mutants suggest that as many as 20 genes could be involved (Glenn and Dilworth 1994). Sensing mechanisms are composed of two components: a sensor and a regulator, and one has been found in *S. meliloti*; the genes actR and actS encode for the regulator and sensor respectively (Tiwari et al. 1996b). ActS is the membrane bound product of actS that, on detection of external acidity, activates ActR (product of *actR*) via phosphorylation. ActR then goes on to activate the transcription of other acid response genes within the bacterium (Tiwari et al. 1996b). The membrane bound product of actA is basic and responsible for maintaining internal pH at around 7, when the external pH drops below 6.5 (Tiwari et al. 1996a). They demonstrated that mutants defective in this gene are unable to maintain intracellular pH and cannot grow at a pH lower then 6. Tiwari et al. (1996a) indicated that calcium involves in acid tolerance mechanism S. meliloti. Riccillo et al. (2000) reported that glutathione play a key role in acid tolerance of Rhizobium tropici. Glutathione also provides protection against chlorine compounds in E. coli and against oxidants in E. coli and Rhizobium leguminosarum bv. phaseoli (Crockford et al. 1995). The activation of glutathione synthesis might be essential for tolerance to acid stress (Muglia et al. 2007). TypA is act as a regulator by controlling the phosphorylation of proteins and required for growth at acidic condition (Kiss et al. 2004). Reeve et al. (1998) show that, in addition to the genes like actA, acts and actR that are absolutely essential for growth of S. meliloti at low pH, there is phrR gene which, while not essential for growth, appears to be induced by exposure to low pH. Cunningham and Munns (1984) found that Rhizobium produce greater amounts of exopolysaccharides are able to survive in acidic conditions more successfully than *Rhizobium* that can only produce smaller amounts.

Many agricultural fields are alkaline with an average pH above 7.0 to 8.5. A major problem in alkaline soils is reduced nutrient availability. Alkalinity stress can also retard *Rhizobium* from growing and subsequent establishment of a viable nitrogen-fixing symbiosis with a legume host. Therefore, it makes good sense agriculturally to select rhizobial isolates that are tolerant of alkaline conditions as well as capable of nodulating legumes (Abd-Alla et al. 2013b). Although *R. leguminosarum* bv. *trifolii* has been reported to colonize soil at a higher rate and produce nodulates at a higher frequency in alkaline conditions; it is also known to grow unaffected at pH 11.5 (Zahran 1999). Homospermidine, a polyamine present in 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). *Rhizobium*

etli strains EBRI 2 and EBRI 26 are more competitive than strain CIAT 899G in soils with high salt or alkaline conditions (Shamseldin and Werner 2004). These isolates can be use as inoculants for alkaline agriculture fields.

3.4.1.1. Rhizobium-legume molecular signalling exchange

Soil pH adversely affects several stages during the development of symbiosis, including the exchange of molecular signals between the legume and the microsymbiont (Hungria and Vargas 2000). Hungria and Stacey (1997) observed that release of isoflavonoids nod-gene inducers by soybean and common bean roots was less at pH 4.5 than at pH 5.8 with some nodulation genes, including nodA, switched off as the pH decrease (Richardson et al. 1988 a, b). At low pH, induction of nod gene expression in R. leguminosarum biovar trifolii is markedly reduced in the presence of flavone-inducer. Furthermore, inducibility of nod gene expression in R. leguminosarum by. trifolii is also affected by a net reduction in the concentration of nod gene-inducing factors present in the root exudates of clover seedlings grown in acidic conditions (Richardson et al. 1989). Depression in soybean and common bean nodulation under acidic conditions may be partially alleviated by supplementation with flavonoid nod-gene inducers (Hungria and Stacey 1997), with studies to examine varietal differences in response or to maximize the expression of rhizobial nod-genes under acid conditions still needed. Also, acidic soil can affect the production and excretion of nodulation factors of *R. leguminosarum* bv. trifolii (McKay and Djordjevic 1993). Soil pH has been shown to effect on the profile of Nod factors secreted by R. tropici CIAT899, which is tolerant of acidic conditions (Morón et al. 2005). At least seven different classes of Nod factor structures were identified. More than 50 different Nod factors were detected at pH 4.5, and greater induction of the nod genes also occurred at this pH. This diversity of Nod factor structure may facilitate nodulation of bean at an acidic pH. Angelini et al. (2003) observed that Initial root colonization by rhizobia was advesly affected when both acid-tolerant and acid sensitive (pH 5.0) peanut rhizobia were grown at low pH. This effect could be attributed to deformation produced by acidic pH on the microsymbiont rather than on host legume. At acid pH, very low nodC gene expression was observed in acid-sensitive isolates, while a change in the flavonoids inducer effectiveness was determined in acid-tolerant isolates. There is no information available on the of effect alkalinity on the exchange of molecular signals between the legume and the microsymbiont and additional studies are needed.

3.4.2. Rhizobium-legume nodulation and nitrogen fixation

The availability of some essential nutrients such as calcium, magnesium, phosphorus, and molybdenum is low in acid soils, whereas high levels of aluminum and manganese can become toxic to plants and rhizobia (Coventry and Evans 1989). Soil pH can be distorted and this depending on the type of plant nitrogen nutrition and host legume. Thus, the N₂-fixing plants released protons, which lead to lower the soil pH. The buffering capacity of certain agriculture field can avoid major changes in pH, but in soils with low cation exchange capacity the problem may be significant. It is clear that N₂-fixing plants are more sensitive to acidity than plants of the same species that feed on mineral nitrogen (Andrews 1976).

Growth of rhizobia in acidified culture media has proved useful for selecting strains with an ability to colonize the rhizosphere and to nodulate their host plant in acid soils (Cooper 1988). A major problem is to distinguish between the effects of low pH and toxicity of some minerals, especially aluminum. In acidic soils with pH of >5.0, where heavy metal activity is relevant, the presence of available aluminum inhibits nodulation.

The information available on alkalinity is scarce compared to that on acidity. Singh et al. (1973) observed that the number of nodules on alfalfa root can be significantly decreased in culture solutions containing 0.1% Na₂CO₃ and NaHCO₃. Apparently, soil alkalinity significantly inhibited nodulation and nitrogen fixation of faba bean inoculated with *R. leguminosarum* bv. *viciae* STDF-Egypt 19 (HM587713). However, dual inoculation of *R. leguminosarum* bv. *viciae* STDF-Egypt 19 (HM587713) and AMF improve the nodulation status and nitrogen fixation of faba bean grown under alkalinity stress (Abd-Alla et al. 2013b). AMF colonization generally enhances rhizobial nodulation and N₂ fixation (Barea and Azcon-Aguilar 1983; Hayman 1986; Fitter and Garbaye 1994; Chalk et al. 2006). Many authors have now reported the enhancement of rhizobial nodulation in roots colonized with AM fungi (Meghvansi and Mahna 2009; Badri et al. 2010; Wang et al. 2011; Sakamoto et al. 2013).

3.5. Pesticides

3.5.1. Suvrival of Rhizobia

Over the past 60 years pesticides have been used increasingly in the environment. Pesticides are massive applied to agriculture land to produce a larger yield and high quality of crop as well as reduce the input of labor and energy into crop production (Ayansina 2009). Worldwide, about 3 billion kg of pesticides are applied each year with a purchase price of nearly \$40 billion per year (Pan-UK 2003). These compounds pose great threats to the varied agroecosystems. Although the application of these chemicals has been banned or restricted in many countries especially the developed ones, some developing countries are still using these compounds because of their low cost and versatility in industry, agriculture and public health (Tanabe et al. 1994, Sarkar et al. 1997). These pesticides can enter the soil environment either by direct application or via plant root exudates. This input of pesticides can affect many soil organisms in different manners. Information from previous studies indicated that some soil bacteria are able to tolerate or degrade some pesticides as sole carbon or nitrogen source (Dick and Quinn 1995; Liu et al. 1991; Sarnaik et al. 2006) bacteriostatic and lethal effects can also occur (Fox et al. 2007). Some pesticides were toxic and inhibited rhizobial growth and survival (Graham et al. 1980; Mallik and Tesfai 1983; Singh and Wright 2002; dos Santos et al. 2005).

The effect of pesticides on rhizobia will depend upon the species and strain (Sawicka and Selwet 1998; Singh and Wright 2002; Zablotowicz and Reddy 2004), type of pesticide applied, and the dose applied (Kaur et al. 2007; Zawoznik and Tomaro 2005).

A *Rhizobium* sp. strain, named PATR, was isolated from an agricultural soil and found to actively degrade the herbicide atrazine (Bouquard et al. 1997). They showed that the hydroxylation of atrazine due to presence of constitutive enzyme consist of four 50-kDa subunits. Drouin et al. (2010) showed that fungicides had the highest deleterious effects on the rhizobia, followed by herbicides and then insecticides. Tolerance or resistance of rhizobia against pesticides is a complex process and is controlled by physiological/genetic level of microorganism. These microorganisms developed such tolerance or resistance have the ability to degraded or change the configuration of pesticide (Kumar et al. 1996; Andres et al. 1998a, Ortiz-Hernández and ánchez-Salinas 2010). The resistance or tolerance against pesticides might be attributes to physiological activities that induce the microbial metabolism for the formation of a new metabolic pathway to bypass a biochemical reaction inhibited by a specific pesticide (Bellinaso et al. 2003). Dominant resistance of rhizobia to specific pesticide depends upon genetic modifications, inherited by the subsequent generation of microbes (Johnsen et al. 2001; Herman et al. 2005). It well established that pesticides not only have toxic impact on rhizobial growth and survival but also have deleterious effect on their ability to produce plant growth promoting substances. Further studies are required to addressing the toxicological effects of pesticides on survival of rhizobial and their plant growth promoting activates at molecular level to fortify the effective implementation of this approach to protect the soil ecosystem from pesticide hazard.

3.5.2. Rhizobium- Legume molecular signaling exchange

Published studies regarding pesticides' effects on Rhizobium-legume molecular signaling exchange are usually very scattered and done with few pesticides on a limited number of same genus (Fox et al. 2001, 2004). They reported using in vitro assays that 30 different pesticides and environmental contaminants specifically disrupted crucial symbiotic signaling between flavonoid phytochemicals and S. meliloti NodD receptors. Fox et al. (2007) reported in vivo evidence that a subset of organochlorine pesticides and pollutants inhibit symbiotic signaling between alfalfa and S. meliloti, resulting in delayed symbiotic recruitment, reduced symbiotic nitrogen fixation, and a decline in alfalfa plant yield. Pesticides may also disrupt symbiosis by altering the array of flavonoid phytochemicals a plant produces or by reducing the overall flavonoid secretion pattern, thereby disrupting plant-rhizobial signaling. Pesticides may affect the plants in a manner that the bacteria-induced root hair deforming factors, similar to Nod Rm-1 and related compounds (Truchet et al. 1991), are incapable to influence morphogenic activity of the plants. Andres et al. (1998b) reported that alfalfa and soybean seed and root exudates treated with fungicide (thiram) inhibit the expression of rhizobia nodulation genes. Temporal and chemical specificity of symbiotic signaling is crucial for coordinating the actions of host legume and microsymbiont necessary for symbiotic nitrogen fixation. In the dynamic soil environment, rhizobia are exposed to a mixture of agonistic and antagonistic phytochemicals, and the degree to which its NodD receptors are able to interpret these signals to locate its symbiotic partner. This will most likely determine the efficiency of symbiotic nitrogen fixation in that particular soil environment.

3.5.3. Rhizobium-Legume and nodulation and nitrogen fixation

Pesticides are essential for controlling plant pests, and accordingly, improve the productivity of major crops including legumes. The presence of residual pesticides in the rhizosphere may interfere with the infection process by inhibiting bacteria-induced root hair deformation and nodule formation. The infection process may be changed either by pesticide effects on the virulence of the attacking bacteria or by effect on root fibers of plant in which the infection occurs (Kumar 1981). Nodulation inhibition with pesticides may also be due to alteration of root hair morphology or to alteration in the quality and quantity of root exudation (Ratnayake et al. 1978) including isoflavonoid compounds and lectin that play an important role in the attraction and attachment of rhizobia to root hairs (Hansen 1994). Pesticides applied to leguminous crops constitute a potential hazard to growth, nodulation and nitrogen accumulation (Abd-Alla and Omar 1993). Abd-Alla et al. (2000 b) indicated that root nodule formation was inhibited in cowpea, common bean and lupin, the effect being most evident in the case of cowpea. Reduction in the number of nodules on soybean was recorded following trifluralin application at rates of 0.74 and 1.1 kg⁻¹ (Kust and Struckmeyer 1971). Nodule dry mass of soybean was also decreased in five soil types treated with pesticides (Dunigan et al. 1972). Nodulation of broad bean was inhibited by the herbicides trifluralin and metribuzin (Bertholet and Clark 1985). Similar results were also obtained by Eberbach and Douglas (1991) using the herbicides paraquat and glyphosate. Abd-Alla and Omar (1993) recorded a significant reduction in nodule formation by faba bean plants grown in herbicide-treated pot soil. Curley and Burton (1975) attributed the inhibition in nodulation, concomitant with pesticide application, to the inability of rhizobia/bradyrhizobia to multiply in the presence of pesticides. Pesticides may inhibit nodulation through their effect on cellulolytic and pectolytic enzyme production by rhizobia, as occurs in other micro-organisms (Mahmoud and Omar 1995). Production of these enzymes by rhizobia is essential for root hair penetration (Hansen 1994). Reports on the effect of agrochemicals on symbiotic attributes of legumes are, however, contradictory. For example, Miettinen and Echegoyen (1996) observed a substantial decline in nodulation in legume crops grown in soil amended with imidacloprid. Pesticides may influence on the nodule development and effectivenss of nitrogen fixation through the effect inside the host plant or nodule structure (Rup 1988). Aggarwal et al. (1986) evaluated the effect of carbamate on nodulation in Pisum sativum and Vigna sinensis. They observed that the low doses of the insecticides had little effect on nodulation whereas the higher concentrations adversely affected it. In a similar study, Alonge (2000) evaluated the phytotoxicity of imazaquin on the growth of soybean plants and found that chlorophyll content in the leaves, root nodules, shoot growth, whole plant dry weight, and grain yield were reduced. Pesticides can reduce the symbiotic efficiency of nitrogen fixing bacteria and the host leguminous plants. Pesticides, which are either applied to crops or found as contaminants in the soil, significantly disrupt symbiotic nitrogen fixation and subsequently lower plant yields. The decrease in the nitrogen content of plant tissue is mainly due to the side effects of pesticides on rhizobia/bradyrhizobia.The decrease in nodule number on pesticide-treated plants may explain the decrease in tissue N (Abd-Alla et al. 2000 b). Similarly, Anderson et al. (2004) claimed that insecticides (chlorsulfuron) negatively affect on nodule formation and nitrogen fixation of chickpea. A comparable observation on the effect of insecticides (fipronil) on legumes has been reported. For example, the effect of insecticide fipronil on growth and nodulation of chickpea, pea, lentil and green gram were determined by Ahemad and Khan (2011a, b). They showed that Fipronil displayed varying degrees of toxicity to the tested legumes. The highest toxicity of fipronil was observed on shoot dry biomass, leghaemoglobin, and chlorophyll content and the seed protein in chick-pea, nodule numbers and nodule biomass in pea.

3.6. Heavy metals

3.6.1. Survival of rhizobia

Heavy metals are most important inorganic pollutants such as Cu, Ni, Cd, Zn, Cr, Pb. Heavy metals enter soil from industrial operation; animal manures and sewage sludge application after these elements enter to the soil they remain for several thousands of years. Accumulations of these metals in various ecological systems cause a massive threat to the varied agroecosystems (Abd-Alla et al. 1999; Ceribasi and Yetis 2001; Cheung and Gu 2007). Recently there has been increasing concern with heavy metal contamination, not only due to their toxic impact on living organisms, but also due to their irreversibly immobilized in soil components (McGrath and Lane 1989). Some metals such as Zn, Cu, Ni and Cr are essential micronutrients for living organisms at very low level. Optimum level of physiological function can be achieved in suitable environment and adequate nutrient supplies. Some elements, such as heavy metals, though essential for organisms, are harmful if present in excess. The impact of certain metals such as Cd, Hg and Pb on biological and physiological functions of living organisms is still obscure. However, all these metals could be toxic at relative low concentrations (Gadd 1992). Soil microorganisms are very sensitive when subjected to moderate heavy metal concentrations (Giller et al. 1998). High levels of heavy metals in soil cause dramatic changes in microbial composition and their activities (Baath et al. 1998; Lakzian et al. 2002; Khan and Scullion 2002, Paudyal et al. 2007; Wani et al. 2008a; Khan et al. 2009; Krujatz et al. 2012) resulting in microbial populations with higher tolerance to metals, but with lower diversity, when compared to unpolluted soils (Baath 1992; Baath et al. 1998). These changes in ecosystem led to losses in soil fertility. Wood and Cooper (1988) reported inhibition of multiplication of rhizobial strain caused at 50 µM Al concentration. Al toxicity is great problem under acidic medium as solubility of free Al ion (Al3+) increases rapidly under acidic condition (McLean 1976). Fast growing acid producing bacteria, it promotes Al to show its full toxicity. Since the distribution of various ionic species of Al is pH dependent (Martin 1991), slight change in pH may significantly affect the relative concentration of the various charged Al species and their ligands and hence the toxicity of aluminium. Further, slight change in pH alone can significantly affect the growth of root nodule bacteria (Thornton and Davey 1983; Richardson and Simpson 1989). In combination with acidity, Al is detrimental to root nodule bacteria in those cases where growth occurs to longer log periods, decreases growth rates and lowers final cell densities (Whelan and Alexander 1986). The toxic effect of aluminium is due to its effect on bacterial DNA (Johnson and Wood 1990; Martin 1988). The deleterious effects of metals on rhizobial composition within agroecosystem and different legume genotypes, however, have been contradictory (Wani et al. 2007a, b, 2008a, b). To validate this concept of conflicting effects of metals on rhizobia, Paudyal et al. (2007) conducted an experiment which revealed that rhizobia grew poorly in culture medium supplemented with even lower concentration of aluminium, while rhizobial growth was completely inhibited at 50 mM Al concentration (Wood and Cooper 1988; Chaudri et al. 1993; Broos et al. 2004). Hirsch et al. (1993), in a study, demonstrated that the persistence of *R. leguminosarum bv. trifolii* was drastically altered by long-term exposure to heavy metals, and this *Rhizobium* lost their infectability to form functional nodules. In a similar study, Chaudri et al. (2000) reported that the irrigation for long term with sewage sludge containing Zn or Cu or mixture of Zn and Cu significantly decreased growth and survival of *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii*, in soils. On the contrary, Angle and Chaney (1991) indicated that even in highly contaminated soils, metal activity was not high enough to exert an antagonistic influence on the soil rhizobial population or the symbiotic association between alfalfa and *R. meliloti*. Arora et al. (2010) investigated the impact of aluminium and copper, iron and molybdenum on growth and enzyme activity of fast and slow-growing rhizobial species. They indicated that *Sinorhizobium* BMP1. However, these strains were very sensitive to Al than other metals. In addition, Al was significantly depressed the various enzymatic activities of *Sinorhizobium meliloti* strains RMP5 and BMP1.

The lack of adverse effects of metals on soil populations of *R. meliloti* is contrary to several previous reports with other species of *Rhizobium*. Many studies indicated that *R. leguminosarum* biovar *trifoli*, or some portion of the symbiotic process, was adversely affected by metal contamination of soil (McGrath et al. 1988; Giller et al. 1989; Mcilveen and Cole 1974; Rother et al. 1983). Tong and Sadowsky (1994) found that fast-growing rhizobia were more susceptible to Zn and Co (20-40 μ g ml⁻¹) than slow-growing (80-480 μ g ml⁻¹), and less susceptible to Mo. The discrepancy is related to the differential metal tolerance of the rhizobial species (Abd-Alla et al. 2012). A higher protein expression was usually related to tolerance mechanisms in *Rhizobium* (Saxena et al. 1996, Pereira et al. 2006).

3.6.2. Rhizobium-Legume molecular signalling exchange, noduation and nitrogen fixation

During the last decades, a plethora of reports have been published concerning the impact of heavy metals on survival of rhizobium and nitrogen fixation. However, there is minor information about the effect of heavy metals on molecular signals between rhizobia and their host legumes. Recently, Pawlak-Sprada et al. (2011a, b) have found that treatment of soybean (*Glycine max*) and yellow lupine (*Lupinus luteus*) with cadmium (Cd²⁺) or lead (Pb²⁺) trigged an increase of phenylalanine ammonia-lyase mRNA level and phenylalanine ammonia-lyase activity. Phenylalanine ammonia-lyase is a key enzyme of the phenylpropanoid pathway. Several products of this pathway such as flavones and isoflavones are strongest inducer of rhizobial nodulation genes (Firmin et al. 1986; Redmond et al. 1986). The total amount of isoflavonoids in soybean and yellow lupine were significantly increased by about 15 % in cadmium- and 46 % in lead-treated plants. High concentration of heavy metals may reduce the isoflavonoid exudate and therefore inhibits the induction of *nod* genes. Some reports are available dealing with the toxicity of different heavy metals on nodulation and nitrogen fixation (Bhandal et al. 1990; Hung et al. 1974; Paivoke 1993a, b; Yakolevya 1984). Conversely, field studies by Heckman et al. (1984) failed to detect adverse changes in either plant growth or N₂-fixation in the sludge-amended soils. Borges and Wollum (1981) added Cd salts to the soil and reported that N₂-fixation of soybean was not affected. The deleterious effects of high levels heavy metals on nodulation and N₂ fixation of *Rhizobium*-fab bean symbiosis are probably due to their inhibitory effects on the growth and activity of both symbionts (El-enany and Abd-Alla 1995). Wetzel and Werner (1995) reported that 75 mg kgsoil as CdCl₂ significantly decreased nodulation of alfalfa roots and shoots dramatically increased. Younis (2007) reported that when 50-200 mg kg-1 soil of Co, Cu, Cd and Zn was added deliberately to soils used for Lablab purpureus cultivation, these metals significantly decreased growth, nodulation and nitrogenase activity of plants in both pot and field experiments. Sepehri et al. (2006) observed that 2 mg Cd/kg soil had a variable effect on symbiotic properties of S. meliloti strains and consequently on S. meliloti-alfalfa symbiosis. A decreasing effect of cadmium concentration on root nodules and N concentration in plants inoculated with sensitive rhizobial strains in comparison with plants bacterized with tolerant strains was 68% and 41%, respectively. The effects of metals on rhizobial composition within soil or nodule environment and different legume genotypes, however, have been contradictory (Wani et al. 2007a, b, 2008a, b). Mandal et al. (2011) reported that nitrogenase activity was reduced to almost 2 fold in plants with Arsenate-treated soil but not abolished in root nodule of blackgram. Their microscopic observations of the cross section of root nodules revealed that the effective areas of N₂-fixing zone were reduced in plants grown in arsenic contaminated field. Studies examining the effect of Mesorhizobium metallidurans and leguminous Anthyllis vulneraria symbiosis in soil that was contaminated with Zn, Cd and Pb showed a significant increase in the total N levels in both plants and soil (Frerot et al. 2006; Mahieu et al. 2011). Cupriavidus metallidurans is another bacterial species isolated from mining areas that tolerates high concentrations of heavy metals, and this organism has been deemed a model of tolerance to metals (Mergeay et al. 2003; Vaneechoutte et al. 2004) and efficiently nodulates Mimosa pudica (Chen et al. 2008). Recently, a symbiotic capacity for N₂ fixation and a high tolerance to heavy metals was demonstrated for some Cupriavidus necator strains (Ferreira et al. 2012; Silva et al. 2012; Avelar Ferreira et al. 2013).

Metalloprotein (Cd-binding protein) was isolated from nodules of faba bean, grown for 58 days at 100 ppm Cd and 0 Cd by fractionation on a sephadex G-100. The major cadmium containing protein eluted with a molecular weight higher than 67 kD, close to the void volume of the column (200 kD). This protein constituted 38% of the total Cd-binding protein (metallothioneins). A second peak was noticed in protein fractions of a molecular weight of about 67 KD. This fraction contained about 53% of Cd-binding protein. A very small amount of cadmium (ca. 3.4%) was eluted with proteins of 8-17 kD. Apparantly, the cadmium-binding protein of 67 kD it appears that Cd-binding proteins play an important role in the detoxification of excess Cd and determining resistance of faba bean-Rhizobium symbiosis to cadmium toxicity was synthesised only in the presence of appreciable cadmium concentrations (El-enany and Abd-Alla 1995). The assessment of variation in protein profile is considered a good indicator to estimate the level of stress imposed on *Rhizobium*-legume symbiosis exposed to heavy metal contamination.

4. Conclusion

Impact of harsh environmental conditions play an essential role in the control of legume-rhizobia interactions. They can arrest the growth, multiplication and survival of rhizobia in soil rhizosphere. The harsh environmental conditions may also have depressive effect on the steps involved in legume-*Rhizobium* symbiosis such as molecular signaling, infection process, nodule development and function, resulting in low nitrogen fixation and crop yield. Selection of hosts and their nitrogen-fixing endosymbionts that are tolerant to a broad range of environmental stresses is important for agriculture system. Understanding the key molecular factors and steps in rhizobia-legume interaction is of crucial importance for the development of *Rhizobium* strains and legume cultivars with high N₂-fixation potential. Prevelence and aboundance of rhizobia species vary in their tolerance to major environment factors; consequently, the selection of resistant strains is an important option. Better N_2 fixation can be achieved by selecting tolerance or resistance rhizobia from soil subjected to environmental stress. The selection and characterization of harsh conditions-tolerant strains with efficient symbiotic performance may be a strategy to improve *Rhizobium*-legume symbiosis and crop yield in adverse environments. Environmental stress severely affected on various metabolic activities of legumes including, nod gene expression, photosynthesis, synthesis of proteins, enzymes and carbohydrates. Therefore, understanding the environmental stressrhizobia-legume interactions is urgently required for growing legumes under harsh environmental stress. Research into these areas is currently underway in several research groups throughout the world and it is anticipated that this research will provide beneficial outcomes resulting in improved sustainability and productivity in agricultural systems.

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