An Insight into the Legume–*Rhizobium* Interaction

G. Yamal, Ankita Bidalia, Krati Vikram, and K.S. Rao

Abstract Active forms of nitrogen are limiting in soil, but the legume–*Rhizobium* interaction overcomes this barrier by biological nitrogen fixation and lessens the usage of fertilizers. An understanding exists between the two partners for symbiotic association to share their resources without either one becoming dominant. Certain compounds released by the host legume plants into the rhizosphere attract the rhizobia and activate the expression of rhizobial nod genes that in turn leads to the production and secretion of strain-specific NFs. NF signalling cascade and events of cell divisions in cortex and pericycle and bacterial infection occur in an orchestrated manner and give rise to a nodule. The nodule organogenesis can be studied under nodule formation and bacterial invasion. Depending on the persistence of meristem, nodules formed can be determinate or indeterminate, but ultimately it is the host plant species that determine the type of nodule formed. More than 90 % of arable land experience one or other kind of stress. Stress conditions affect the host plant, rhizobium and also the interaction between the two.

Keywords Legume • Rhizobium • Stress • Nodulation

1 Introduction

The earth's atmosphere consists of 78.1 % nitrogen gas, but the biologically active forms of nitrogen are limiting in soil and can restrict plant growth. Thus, it's imperative for plants to capture nitrogen, in the form of nitrates and ammonia, from the soil. Modern agriculture relies on application of industrially synthesized nitrogen fertilizers to maximize crop productivity. Production of nitrogen fertilizers is expensive and consumes a lot of fossil fuel. In addition to this, 30–50 % of applied nitrogen fertilizer gets leached out and leads to environmental problems. The reliance on chemical fertilizer can be reduced by biological nitrogen fixation (BNF), wherein the atmospheric nitrogen is converted to ammonia by an enzyme called nitrogenase. The

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process of BNF was discovered by Beijerinck in 1901 (Wagner 2011). This process has ecological and agronomical importance and accounts for 65 % of the nitrogen used in agriculture worldwide. It is estimated that roughly 200 million tons of nitrogen is fixed annually by the symbiotic association between rhizobia and legume (Graham and Vance 2003; Peoples et al. 2009).

Legumes are a large group of angiospermic plants skilled with the ability to establish symbiotic association with the nitrogen-fixing bacteria called rhizobia (including the genera *Azorhizobium*, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*) that are widespread under different edaphic and climatic conditions. The Fabaceae or Leguminosae family includes three subfamilies: Faboideae (or Papilionoideae), Mimosoideae and Caesalpinioideae. Members of these families can grow in nitrogen-poor soils; therefore, they play a crucial role in the sustainable development of the agriculture (Pislariu et al. 2015). One of the identified roles of BNF is in poverty alleviation. However, applying the knowledge of the mechanism of BNF and providing training to the farmers about the inoculation, providing the efficient strains at the cheaper cost to the farmers, could be a step towards sustainability (O'Hara et al. 2008).

The rhizobium and host legume develop a metabolic cooperation which forms an exchange-control system that enables the two partners to share their resources without either one becoming dominant (Lodwig et al. 2003). An intricate dialogue occurs between the two partners, and the compatibility is tested, after which the host plants and rhizobia exchange signals. The rhizobia enter and infect the roots of compatible legume plants either by the root hair or through cracks present in root epidermis. Rhizobial signalling to host root hairs then leads to root hair deformation, branching and curling, mitosis in the root cortex and ultimately the formation of specialized structures called nodules.

Thus, this two-way molecular conversation between the host legume and the bacteria leads to morphological, anatomical, physiological and cytological changes in plants. Depending on the shape and maintenance of a meristematic region, two kinds of nodules, viz. determinate and indeterminate, are formed by host plants upon infection by rhizobia, but ultimately it is the plant that determines the type of nodule formed. In general, the indeterminate nodules are formed by temperate legumes, and determinate nodules are formed by tropical legumes (Lotocka et al. 2012). So far various functional genomics resources have been developed for the two model legumes *Medicago truncatula* and *Lotus japonicus* (Pislariu et al. 2015).

Plants being sessile often have to face a wide variety of environmental conditions. Like other plants legumes also face one or other kind of stress as more than 90 % of arable land experience stress. The presence of stress effects the growth of the host plant, the rhizobia and their interaction. Thus, stress changes the physiological state of host plant, rhizobial survival in the soil, infection and nodule establishment and ultimately the nodule functioning. In the present chapter, effect of stresses such as soil pH, soil moisture, salinity, temperature, nutrients, predation, etc. on the legumes, rhizobium and legume–*Rhizobium* interaction has also been discussed. The present chapter is an attempt to give an overview of process of nodule formation and the changes that occur in the host plant, along with the different types of nodules formed and the effect of natural stresses on plant, rhizobium and their interaction.

2 Nodule Organogenesis

The history of the study of root nodule formation dates back to the sixteenth century in the drawings of Fuchs and Dalechamps; however, Malpighi proposed that the swellings (the word used by Malpighi for nodules) are due to insect larvae. Interestingly, the necessity of bacteria for nodule formation was demonstrated during the nineteenth century by Frank and Beijerinck, Frank showed that soil sterilization prevented nodule formation, and Beijerinck prepared pure cultures of nodule occupants and used them to infect legumes (Bond 1948; Pueppke and Broughton 1999). Root nodule is a structure, unique to leguminous plants and is of great interest for several reasons. The nodules can be formed only after bacterial infection and possess differentiated tissues in a definite arrangement. However, today it is well established that nodule formation involves a two-way dialogue between the host legume and the bacteria rhizobium. During the dialogue, various signalling molecules are exchanged that regulate the specificity of legume and rhizobium interaction for initiation, differentiation and functioning of nodules. This specificity has ecological and evolutionary importance as it allows infection by symbiotic friend and not a pathogenic foe (Long 2015). The nodule organogenesis can be studied into two subheadings: nodule formation and bacterial invasion.

2.1 Process of Nodule Formation

Plants release certain signal compounds (such as lectins, flavonoids) in the rhizosphere that favour the initiation of nodule development. In the 1970s, the lectin recognition hypothesis was proposed according to which the plant lectins and rhizobium exopolysaccharides (EPS) mediate specificity in symbiosis (Hirsch 1999; Long 2015). In the rhizosphere, the exuding phenolic flavonoid compounds from the roots of the host plant attract the rhizobia and activate the expression of rhizobial nod genes that in turn leads to the production and secretion of strain-specific NFs (Gage 2004; Ferguson et al. 2010). In the free-living rhizobium, nodD gene shows its expression, but the rest of the nod genes (nodABC, nodE, nodH, nodPQ) are expressed only in the presence of plant. There are ‘common’ nod genes, viz. nodABC, that are essential for nodulation and certain ‘host specific’ such as nodE (in *R. leguminosarum* for nodulation in peas or clover), nodH and nodPQ (in *R. meliloti* for nodulation in alfalfa). However, the ability of rhizobia to nodulate several legumes is linked not only to the presence of different nod genes in its genome but also with the legume promiscuity (Roche et al. 1991). Therefore, certain bacteria can nodulate only specific legume host, while others such as NGR234 and USDA257 (strains of genus *Ensifer*) have a broad host range (Pueppke and Broughton 1999). The infection of varied legumes by several strains of rhizobia occurs due the presence of several copies of the nodD gene, which in turn permit them to respond to different types of flavonoids produced and secreted by plants (Cooper 2007; Gibson et al. 2008).

The NFs are lipochitooligosaccharides (LCO), the derivative of chitin but differ in the number of GlcNAc units (2–4), in the length and degree of unsaturation of the fatty acid chain, as well as the presence of various substitutions on the oligosaccharide backbone with various substitutions at the (non)reducing-terminal and/or nonterminal residues (Haeze and Holsters 2002; Fliegmann and Bono 2015). These variations are characteristic for each rhizobium and are involved in the specific recognition between the legume plant and its symbiont (Ferguson et al. 2010). Nod factors are responsible for nodule organogenesis and control infection, leading to the formation of root nodules. Thus, it is the nod genes, NFs and EPS, that determine the host range.

Rhizobia enter into the plant root either by the root hair or through cracks present in root epidermis. However, root hair infection at the tips is the most common, because they have thinner and less cross-linked cell walls which further allow the rearrangement of underlying microtubules, change vesicle trafficking to the growing tip and allow easy penetration by microsymbiont. Root hair deformation gets started within 6–8 h after the attachment of rhizobia (Ferguson et al. 2010). Rhizobial signalling to host root hairs leads to root hair deformation, branching and curling and mitosis in the root cortex, which culminates in the formation of nodule primordium. The nodABC genes are essential for root hair curling as well as infection, for eliciting mitosis in the root cortex and for nodule formation (Haeze and Holsters 2002).

2.2 Process of Bacterial Invasion

Bacteria invade the plant cells through tubular structures termed ‘infection thread’. Infection thread is a composite structure, as it has parts contributed by the two symbiotic partners (Gage 2004). The infection thread is comprised of plant cell wall components (esterified and unesterified pectins, xyloglucans and cellulose) and encapsulates the dividing bacteria in small quantity, thus facilitating the passage of bacterial cell into the cortex. This microcolony of rhizobia contains high concentration of NFs and the cell wall-degrading enzymes. The rhizobial microcolony penetrates through the cell wall and the plasma membrane remains intact, which is followed by re-synthesis and redigestion (Gage 2004; Ferguson et al. 2010).

The increased NF levels produced by the invading rhizobia lead to the mitotic division of root cortical cells. These divisions of cortical cells in the roots result in the formation of nodule primordium. In the tip region of the extending thread, active cytoplasmic streaming along with plant cytoskeleton has been proposed to play a role in the growth of infection threads. The infection threads reach into the cortical zone of the root and then to newly induced dividing cells via root hair. Bacteria get released into an infection droplet in the host cell cytoplasm through the growing tip of the infection thread. The bacteria remain enclosed within peribacteroid membrane derived from plasma membrane of host plant; this structure is known as symbiosome (Udvardi and Day 1997). In symbiosome, these bacteria divide continuously even before they get differentiated into bacteria and start to fix nitrogen (Ferguson et al. 2010).

Reduction of nitrogen to ammonia occurs within bacteroids; this reaction is catalyzed by oxygen-sensitive enzyme nitrogenase. The level of oxygen in root nodules is regulated by leghaemoglobin, providing legume nodules a pink tinge. O'Brian and co-workers (1987) revealed that the host plant encodes the haemoglobin apoprotein, whereas the heme group is primarily synthesized by the bacterium. These researchers used a heme biosynthesis mutant strain LO505 of *Bradyrhizobium japonicum*. The mutant strain was deficient only in protoporphyrinogen oxidase activity and thus could not catalyze the penultimate step in heme biosynthesis. As a result of this, the mutant strain formed small root nodules. The bacteroids isolated from these nodules lacked protoporphyrinogen oxidase activity, and nodules contained no detectable leghaemoglobin in the nodule cytosol. These results suggest that bacterial heme synthesis is required for leghaemoglobin formation in soybean root nodules.

The leghaemoglobin remains localized within the host plant cytosol and not in the peribacteroid membrane. Thus, in nodules atmospheric nitrogen gets reduced to ammonia that subsequently assimilates into plants after its conversion into glutamine via glutamine synthase. Further, glutamate synthase enzyme converts glutamine into glutamate. The process of nodulation brings several changes in the root of the plants at morphological, anatomical, physiological and cytological levels (Ferguson et al. 2010; Gordon et al. 1992).

3 Changes in Root During Infection Thread Growth

Infection threads are considered to be tip-growing structures and develop from growing root hairs (Gage 2004). These probably elongate by using at least some of the machinery that was supporting root hair growth before infection took place. Since understanding the processes that contribute to tip growth in root hairs should enhance the understanding of the processes involved in infection thread growth, the same has been discussed in the following sections.

3.1 Cytological Changes During Root Hair Development

The root hair elongates through polarized secretion of vesicles to the tip region, with concomitant yield of the tip wall under the influence of internal turgor pressure (Hepler et al. 2001; Ketelaar and Emons 2001; Smith 2003; Rounds and Bezanilla 2013). Turgor pressure that provides the uniform stress or force in all the directions is a scalar property that irreversibly deforms the cell wall and ultimately leads to root growth but plays no role in determining the direction of growth (Kropf et al. 1998).

The directional growth of the cell depends on unequal mechanical properties of the cell wall which in turn depends on the unequal deposition of the wall matrix at certain sites of root while confining others and orientation of the cellulose fibrils (Kropf et al. 1998; Rounds and Bezanilla 2013). Under stress conditions the less viscous cell wall deforms at the faster rate in comparison to the more viscous cell wall. However, the viscosity and thickness of the wall at the tip of the cell result from several factors such as the net deposition, expansion and the variations in cross-linking within several components (Rounds and Bezanilla 2013). In root hairs, the vesicles that fuse at the tip are derived from Golgi bodies, located at a short distance behind the growing tip. These vesicles supply membrane and cell wall components that get incorporated into the plasma membrane, cell wall and extracellular matrix. During tip growth, vesicles and other organelles reach to the apical region of the cell by actin-dependent cytoplasm streaming. The cytoplasm typically moves towards the growing tip along the outside of the cell and then moves back towards basal regions via the centre region of the cell. This pattern of movement (most commonly observed in pollen tubes) is referred as ‘reverse fountain streaming’ (Iwanami 1956; Hepler et al. 2001; Rounds and Bezanilla 2013). The region which lies immediately adjacent to the tip does not show any cytoplasmic streaming and is devoid of organelles. This region termed as clear zone contains the vesicles that fuse with tip and provides material needed for growth. The transportation of vesicles from the base of clear zone to their site of fusion near the tip of the root hair is supposed to be mediated by diffusion, as the vesicles are delivered at the base of the clear zone and consumed at the apex (Miller et al. 1997; Lhuissier et al. 2001).

3.2 Roles of Actin and Microtubule Cytoskeleton in Tip-Growing Cells

Tominaga et al. (1997) experimentally revealed the mechanism involved in organization of actin filaments (AFs) in root hair cells (site of reverse fountain streaming) of *Hydrocharis*. Both microtubules (MTs) and AFs lie longitudinally within the cortical region of the root hair cell. However, in the transvacuolar strand, only AFs were present and MTs were entirely absent. The double inhibitor experiment using AFs inhibitor cytochalasin B, and MTs inhibitor propyzamide, showed that cytochalasin B reversibly inhibited cytoplasmic streaming while propyzamide alone had no effect. Removal of cytochalasin B after treating root hair cells with these inhibitors together failed to recover cytoplasmic streaming. However, after removal of propyzamide, both cytoplasmic streaming and original organization of AFs were recovered, suggesting that MTs play a vital role in the organization of AFs.

Similarly, in *Medicago truncatula*, the effects of microtubule stabilizing and destabilizing drugs on the morphology of the growing root hair have revealed the role of MTs in maintaining the normal structure of the subapical cytoplasmic dense region. These experiments imply that MTs play important role in maintaining the normal distance between the nucleus and the growing tip of the root hair during root hair growth and in actin maintenance (Lloyd et al. 1987; Tominaga et al. 1997; Sieberer et al. 2002).

3.3 Roles of Ca^{+2} in Tip-Growing Cells

Calcium is a versatile signalling component involved in root hair elongation (Robbins et al. 2014). Experiments conducted with ion-specific dyes have exhibited that root hairs show tip focus gradients of calcium like that of pollen tubes. The concentration of this ion at the tip of growing root hairs, just below the plasma membrane, is about 1 μM and varies up to a basal concentration of 100 nM within 20 μm, but the non-growing root hairs do not show such a gradient (Wymer et al. 1997). Necessity of calcium channel activity for root hair tip growth and maintenance was demonstrated by the use of calcium channel blocker, verapamil. 50 μM verapamil caused the dissipation of elevated calcium ion concentration and cessation of root hair growth. Re-establishment of calcium gradients shows that the highest calcium ion concentration promotes root hair growth. Change in the direction of root hair growth reorients the calcium ion gradient, and the gradient again changes/reverts when root hair growth is returned to the original direction.

4 Types of Nodules: Determinate and Indeterminate

According to the mode of development, nodules formed by *Rhizobium* are of two kinds, viz. determinate and indeterminate, but ultimately it is the host plant that determines the type of nodule formed (Franssen et al. 1992; Maunoury et al. 2008; Ferguson et al. 2010). In general, the indeterminate nodules are formed by temperate legumes, and determinate nodules are formed by tropical legumes (Nap and Bisseling 1990). The shape, site of first internal cell division, maintenance of a meristematic region and form of the mature nodule are the features that can be used to distinguish the two kinds of nodules (Newcomb and Peterson 1979; Ferguson et al. 2010). In general a nodule consists of an inner central region and an outer cortex that acts as an oxygen diffusion barrier (Witty et al. 1987; Parsons and Day 1990). Thus, the legume nodule anatomy is characterized by a central infected region, surrounded by a cortex of uninfected cells and a dichotomously branching vascular system. The vasculature system for sucrose/photosynthate delivery to nodule is present in cortex only. On the other hand, the central region of mature nodule consists of two types of cells: one those infected with rhizobia and other uninfected cells (which are generally less in numbers) (Gordon et al. 1992; Brown and Walsh 1994). Immunogold labelling studies using polyclonal antibodies to sucrose synthase were conducted by Gordon and co-workers (1992). They found a greater intensity of labelling in the cytosol of uninfected interstitial cells of the central nodule region compared with the cytosol of the infected cells. Thus, in indeterminate nodules starch is stored in both infected and uninfected cells, whereas it is rarely or never found in infected cells in determinate nodules (Gordon et al. 1992). In both determinate and indeterminate nodules, the epidermal responses are similar, but cortical responses are different, viz. in determinate nodules initials arise from outer/midcortical cells of the root, whereas in indeterminate nodules, the initials arise from inner cortical cells (Subramanian 2013).

The tissues that surround the infected tissue consist of three different cell layers. First layer is known as outer cortex, followed by nodule endodermis, which is one cell layered and inner cortex or 'nodule parenchyma' forms the third layer (Brown and Walsh 1994). Thus, the inner and outer cortex are separated by a 'common endodermis' termed as nodule endodermis (Frazer 1942; Bederska et al. 2012). The inner cortex or nodule parenchyma houses vascular bundles, each with its own endodermis (Brown and Walsh 1994). The xylem of the vascular bundles provide water and the photo assimilates are supplied to the nodule by phloem (Bederska et al. 2012). Thus vascular system guarantees import of nutrients from the host and export of nitrogenous products from nodules, as effective nitrogen fixation depends on the balance between the import of photo assimilates and the export of nitrogenous solutes (Walsh et al. 1989; Streeter 1993; Schulze 2004; Bederska et al. 2012). Inner cortex is also important to protect the nitrogenase (a key enzyme in nitrogen fixation, which is highly sensitive to oxygen) by forming a diffusion barrier. However, this barrier is strongly dependent on the nodule cortical anatomy (Brown and Walsh 1994; Sujkowska et al. 2011; Bederska et al. 2012). A physiological paradox occurs in the nodule where the aerobic requirements of bacteroid and the oxygen sensitivity of nitrogenase both have to be dealt with. Protection against oxygen is provided by the nodule environment through a cortical diffusion barrier. The main route of oxygen diffusion is through nodule apex, which generates a longitudinal oxygen gradient. As a result, the free oxygen concentration drops to less than 50 nM in the central nitrogen-fixing zone containing *Rhizobium* bacteroids. Interestingly, bacteroid respiration in the central zone is made possible by a high concentration of leghaemoglobin and induction of a high-affinity cbb3 oxidase. Therefore, these microorganisms fix nitrogen in a microaerobic, nitrogen-rich environment, and thus nif gene induction during symbiosis is regulated by N (Dixon and Kahn 2004).

The indeterminate nodules are generally elongated and have a persistent meristem, while the determinate nodules are globose or obovate and lack a persistent meristem (Walsh et al. 1992). Lotocka et al. (2012) suggested that the appropriate term for indeterminate nodules should be 'nodules with indeterminate growth meristem'. The first cell division occurs in the cortex in indeterminate nodules and is anticlinal, whereas in determinate nodules' first cell division takes place in outer cortex (Ferguson et al. 2010). In indeterminate and determinate nodules, the vascular elements are surrounded by one to several layers of pericycle and an endodermis, but in indeterminate nodules, the vascular elements continue to differentiate throughout the nodule life, towards the meristem (Walsh et al. 1992).

There are certain plants that have been used historically for studies on indeterminate and determinate nodules. These include *Medicago sativa* (alfalfa), *M. truncatula*, *Pisum sativum* (pea), *Vicia* sp. (vetches) and *Trifolium* sp. (cloves) for indeterminate nodule and G*lycine max* (soybean), *Vicia faba* (bean) and *Lotus japonicus* for determinate nodules (Cook 2000; Handberg and Stougaard 1992). However, *Lotus japonicus* and *Medicago truncatula* that develop determinate and indeterminate nodules, respectively, are considered as model organisms (Lopez et al. 2008). Both these plants had a common ancestor ~40 MY ago; still they

Table 1 Summary of differences between determinate and indeterminate nodules

Determinate nodules	Indeterminate nodules
Formed in tropical legumes	Formed in temperate legumes
Generally globose or obovate in shape	Elongated/cylindrical
No persistent meristem	Meristem is persistent, and the vascular elements continue to differentiate throughout the nodule existence
Starch is rarely or never stored in infected cells	Starch is present in both infected and uninfected cells
First cell division is periclinal and the initials arise from outer/midcortical cells	First cell division is anticlinal and the initials arise from inner cortical cells
No clear zonation	Five histological zones can be seen
Infected cells have minimal vacuolation	Infected cells are highly vacuolated
The bacteroids have high viability and are normal rod shaped and many per symbiosome	The bacteroids have low viability and are enlarged and branched and one per symbiosome
Bacteroids represent same genomic DNA content	Differentiation of bacteroids causes genome amplification
Model organism for studies *Lotus japonicus*	Model organism: *Medicago truncatula*

form different types of nodules (Lavin et al. 2005). Both these plants are diploid, have small genome and can be inbred to form genetically homogenous lines. Other characters which make these plants a favourite material are their short life cycle and prolific seed production. The genome sequencing has been done for both these plants, and thus mutants specific to symbiotic nitrogen fixation have been characterized, and the responsible genes have been isolated (Cannon et al. 2006). Table 1 summarizes the differences between determinate and indeterminate nodules.

4.1 Indeterminate Nodules

In the indeterminate nodules, the activity of meristem is persistent and new cells are constantly added to the distal end, forming cylindrical nodules. The nodules, growth and functioning occur simultaneously, and the differentiation can be observed in a single longitudinal section. During the development of indeterminate nodules, the first cell division is anticlinal and occurs in the inner cortex, and then in the endodermis and pericycle, the periclinal divisions take place and a primordium is formed. Being indeterminate nodules, the nodule meristem maintains its activity throughout the growth cycle and apical meristem continuously produces new cells that are being infected by bacteria, thus a gradient of developmental stages/zones can be identified while the nodule continues to grow. Five histological zones can be distinguished in the fully developed indeterminate nodules (Timmers et al. 2000; Ferguson et al. 2010; Bederska et al. 2012).

The apical part (Zone I) consists of meristem and produces cells of the nodule tissue, but no rhizobia. The distal part has an invasion or symbiotic zone. Adjacent to this part is the infection zone (Zone II) that has young symbiotic tissue. In this zone the cell division comes to an end and bacteria are released from the infection threads into the cytosol. These bacteria then undergo endocytotic internalization and differentiate into bacteroids to form symbiosomes. Bacteroid is different from the free-living, rod-shaped rhizobia (Paau et al. 1978, 1980; Vasse et al. 1990). This differentiation of bacteroids is linked to morphological and cytological changes, such as cell elongation, genome amplification, membrane permeabilization and loss of reproductive capacity (Mergaert et al. 2006).

Next to this is a transitional zone or interzone II/III with starch stored in cells, and here bacteroid-containing tissues undergo final differentiation. Bacteroid differentiation is orchestrated with dramatic changes in the invaded plant cells, which enlarge and are highly polyploid (Timmers et al. 2000). Zone III is the differentiated zone, where nitrogen fixation takes place. There is a senescent zone (Zone IV), where symbionts degenerate (Vasse et al. 1990; Hirsch 1992). The presence of a saprophytic zone (Zone V) was first time shown in alfalfa by Timmers and coworkers (2000). In this zone, rhizobia neither undergo differentiation into bacteroid nor they are surrounded by membrane envelope and possess features of free-living bacteria. This zone is of special interest as it forms an ecological niche where intracellular rhizobia take advantage of the interaction for their exclusive benefit and live as parallel saprophytic partners (Timmers et al. 2000). These rhizobia are returned to the soil after nodule decomposition (Lotocka et al. 2012).

4.2 Determinate Nodules

In determinate nodules, the nodule zonation is not clearly distinguished. The determinate nodules originate from mitotic activity of the root outer cortex. However, the mitotic activity stops during development, and increase in nodule size occurs mainly due to cell expansion. The inner region of determinate nodule has rhizobia-colonized cells where symbiotic nitrogen fixation occurs and relatively small rhizobia-infected (as yet uncolonized) cells in the surrounding region. During the development process, the infection thread branches and penetrates the cells of the central region, once the infection thread comes in contact with nodule primordia. The bacteria bud off from the tips of the infection threads into the plant cytoplasm and the bacteria become enclosed by the peribacteroid membrane. The bacteroids of determinate nodules represent the same cell size, genomic DNA content and reproductive capacity as the free-living bacteria. Thus at the end of symbiosis, these bacteria can return to a free-living lifestyle and recolonize the rhizosphere (Mergaert et al. 2006).

5 Molecular Conversation

Symbiotic nitrogen fixation genes can be broadly divided into nod, nif and fix genes (Fischer 1994). The nod gene products are required for the early steps in nodule formation (which is discussed subsequently). In free-living diazotrophs such as *Klebsiella pneumoniae*, nif genes exist and are structurally homologous to rhizobial nif genes. The conserved nif gene plays a similar role in rhizobia as in *K. pneumoniae* (Long 1989; Fischer 1994, Schmitz et al. 2002). Alternatively, the term 'fix gene' is used for genes that are essential for nitrogen fixation but do not have a homologous counterpart in *K. pneumoniae* (Long 1969; Fischer 1994). However, the homologues of some of the fix genes exist in bacteria that do not fix N (Dixon and Kahn 2004). Interestingly, both nif and fix gene mutants are able to cause nodule development, but the nodules do not fix nitrogen (Long 1969). Regulation of nif expression is a complex phenomenon, and the regulatory events that control the transcription of the nif genes in free-living and symbiotic diazotrophs have been well reviewed by Dixon and Kahn (2004).

5.1 NF Perception and Signalling Cascade

Roots of the host plant attract the rhizobia and activate the expression of rhizobial nod genes that in turn leads to the production and secretion of strain-specific NFs. There are two receptor-like kinases (RLK) that are located on the epidermal cells: LjNFR1 and LjNFR5 in *L. japonicus* and MtLYK3/MtLYK4 and MtNFP in *M. truncatula* (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003; Arrighi et al. 2006). These NF receptors comprise of an intracellular kinase domain, a transmembrane domain and an extracellular region having lysin motif (LysM) domains. These LysM domains mediate recognition of different NAG-containing ligands and facilitate microbial infection and symbiosis (Gust et al. 2012). Most of the NF receptors are characterized by the presence of activation loop (the site of phosphorylation). The NF receptors, LjNFR1 and MtLYK3/MtLYK4, have a typical serine/threonine kinase domain, but LjNFR5 and MtNFP do not have any activation loop (Limpens et al. 2003; Madsen et al. 2003; Radutoiu et al. 2003). The absence of an activation loop in the receptor of one of the kinase domains gives an indication that the two LysM RLKs form a heterodimeric receptor, with the active kinase domain functioning in downstream signal transduction. Thus, the NF that is perceived by two LysM RLKs leads to NF signalling cascade, cortical and pericycle cell division and bacterial infection events (Cardenas et al. 1998; de Ruijter et al. 1998).

The signalling cascade involves potassium ion (K^+) channel proteins located in the nuclear membrane (encoded by MtDML1, LjCASTOR and LjPOLLUX) (Ané et al. 2004; Imaizumi-Anraku et al. 2005; Riely et al. 2007), two nucleoporins (encoded by LjNup133 and LjNUP85) (Kanamori et al. 2006; Saito et al. 2007) and a calcium- and calmodulin-dependent protein kinase (CCaMK) (encoded by MtDM13/PsSYM9) (Lévy et al. 2004; Mitra et al. 2004). Rapid influx of Ca^{+2}, followed by membrane depolarization involving efflux of Cl^- and K^+ (Felle et al. 1999), induces oscillation in cytosolic Ca^{+2} concentration, which is known as Ca^{+2} ion spiking (Wais et al. 2000). Ca^{+2} spiking signals are perceived by CCaMK (Oldroyd and Downie 2004). Various mutation studies suggest that NF LRR RLK, the ion channels and the nucleoporins act downstream of NF perception, but upstream of Ca^{+2} spiking, and CCaMK acts downstream of Ca^{+2} spiking (Ferguson et al. 2010). Downstream of CCaMK, many transcription factors necessary for nodulation and nodule inception get activated. These include nodulation signalling pathway 1 (NSP1) (Smit et al. 2005), NSP2 (Kaló et al. 2005), Ets2 repressor factor required for nodulation (ERN) (Middleton et al. 2007) and nodule inception (NIN) (Schauser et al. 1999; Borisov et al. 2003). Mutational studies of NSP1 and NSP2 have shown that these mutants exhibit normal Ca^{+2} responses when treated with NFs but fail to initiate transcription of the early nodulation (ENOD) genes localized in the epidermis. NSP1 and NSP2 get activated after Ca^{+2} spiking (Catoira et al. 2000; Oldroyd and Long 2003), but probably downstream of CCaMK (Ferguson et al. 2010). In addition to these genes, the ENOD gene shows its expression in the epidermal cells. Experimental evidences suggest that expression of ENODs in the epidermis is an orchestrated effort of NSP1, NSP2, ERN1 and NIN (Andriankaja et al. 2007; Hirsch et al. 2009; Ferguson et al. 2010). NF signalling cascade, cell divisions in cortex and pericycle and bacterial infection, all these events occur in a coordinated way and give rise to a nodule.

5.2 *Epidermal and Cortical Responses During Early Stages of Nodulation*

During root hair invasion, bacterial infection is regulated by epidermis, whereas formation of a nodule is controlled by cortex. However, in case of crack invasion, the epidermis is breached and the bacteria gain direct access to cortical cells. The developmental processes in the cortex and epidermis are different, but coordinated such that a nodule primordium occurs close to the site of bacterial infection.

Essentiality of a cytokinin receptor for nodule development indicates cytokinin as a key player in nodule organogenesis. Indeed, cytokinin may be the mobile signal communicating epidermal perception of NF to the inner root (Subramanian 2013; Ferguson et al. 2010). Abscisic acid (ABA) has already been proposed as a mobile signal and is known to have a role in both the epidermis and cortex (Ding et al. 2008; Biswas et al. 2009; Ding and Oldroyd 2009). ABA is a negative regulator of nodule development and other plant hormones such as auxin, brassinosteroids and gibberellins acting as positive regulators. Like the hormones, there are certain factors and signals that are required both in the cortex and in the epidermis. For example, CCaMK is required in the epidermis and the cortex but involves entirely different pathways. Similarly, NSP1 and NSP2, which act downstream of CCaMK in the epidermis, and downstream of CCaMK and the cytokinin receptor in the cortex is thus required in both epidermis and cortex (Heckmann et al. 2006). Another transcription factor, NIN, also appears to have a role in both epidermal and cortical cells. NF perception in the epidermis causes rapid responses in the inner root. In *M. truncatula* within 16 h, cytoskeletal rearrangement takes place in pericycle cells (Timmers et al. 1999). Such rapid response in the inner root to rhizobia/NF suggests some form of signalling communication.

The activation of the mitotic cell cycle and regulators of the cell cycle in cortical cells play an important role during the formation of a nodule primordium. A cytokinin receptor, which has a histidine kinase domain (encoded by MtCRE1/LjLHK1), functions in the root cortex and is essential for cell division events (Gonzalez-Rizzo et al. 2006; Tirichine et al. 2007). Downregulation, or loss of function, of this receptor results in decreased nodule numbers, as nodule primordium is not formed by plant (Gonzalez-Rizzo et al. 2006; Murray et al. 2007). During such an event rhizobial infections take place, but the infection threads lose their direction and spread laterally rather than growing towards the root cortex (Murray et al. 2007). Thus, nodule primordia formation or the cytokinin receptor is not mandatory for bacterial infection events, but for guiding the growth of infection thread.

The loss-of-function Mtdmi3 mutants produce a non-nodulation phenotype as CCaMK activity is required in the epidermis. But gain-of-function mutants of CCaMK result in spontaneous nodulation due to controlled cell divisions in the cortex (Gleason et al. 2006; Tirichine et al. 2006). However, presence of functional copies of NSP1 or NSP2 is essential for nodulation in gain-of-function mutants of CCaMK (Gleason et al. 2006; Tirichine et al. 2007). Thus, NSP1 and NSP2 act downstream of CCaMK in the epidermis.

The transcription factor, NIN, has a role in both epidermal and cortical cells (Schauser et al. 1999; Borisov et al. 2003; Marsh et al. 2007). In the epidermis, the mutant NIN plants show show excessive ENOD11 expression (suggesting that NIN is not essential for NF-induced ENOD11 expression), excessive root hair curling, blocked rhizobial infection (Schauser et al. 1999; Marsh et al. 2007) and in the cortex, such mutants are unable to initiate cell divisions and nodule primordium formation (Schauser et al. 1999; Borisov et al. 2003). NIN acts as a negative regulator of NF signalling to regulate the spatial expression of ENOD11 in the root epidermis (Marsh et al. 2007). The expression of NIN is brought out by cytokinin or NF application (Gonzalez-Rizzo et al. 2006; Murray et al. 2007), further supporting the idea that NIN positively regulates cortical cell divisions.

6 Legume–*Rhizobium* Under Stress

Stress refers to any environmental condition that affects normal growth, metabolism and development of organisms. More than 90 % of arable land experience one or other kind of stress and causes more than 50 % of crop loss worldwide. In the symbiotic association of rhizobium and legume, it is important to discuss the factors that affect the microbe, the host plant and the functioning of the symbiotic association, as the N_2

fixation is strongly related to the physiological state of the host plant (Bordeleau and Prevost 1994; Zahran 1999). Stress factors may be from natural or anthropogenic sources (i.e. due to human activities). Natural stresses include soil pH, soil moisture, salinity, temperature, nutrients, predation, etc. Stress leads to the generation of reactive oxygen species (ROS). These ROS react with biomolecules like proteins, nucleic acids, membrane lipids, etc. and hamper their normal functioning in the cell. Plants respond to these stresses through synthesis of metabolites and antioxidant enzymes that enhance tolerance mechanisms in plants under stress (Latef and Ahmad 2015). The presence of stress changes the physiological state of host plant, rhizobial survival in the soil, infection and nodule establishment and ultimately the nodule functioning. In the following section, major environmental constraints and their effects on the legumes, rhizobium and legume–*Rhizobium* interaction are discussed.

6.1 Soil pH

Soil acidity alters the availability of phosphorous, calcium and molybdenum and determines the toxicity of iron, aluminium and manganese (Muthukumar et al. 2014). Highly alkaline soil has sodium chloride, bicarbonate and borate which are toxic to both legume and rhizobium. Neutral or slightly acidic pH in soil is required for most leguminous plants (Bordeleau and Prevost 1994). In both tropical and temperate areas, the acidic pH limits the growth and survival of rhizobium strains in soil and nodulation and ultimately constrains nitrogen fixation of legumes (Graham et al. 1994). In a study by Tang and Thomson (1996), they studied the effects of pH (4, 5, 6, 7 and 8) and bicarbonate (5 mM $KHCO_3$) on the growth and nodulation of 14 grain legume species supplied with N or reliant on N_2 fixation. Species shows a broader optimal pH range for growth when supplied with N, but showed sensitivity at low pH when reliant on N_2 fixation.

Thus, soil pH affects the number of nodules, the nitrogenase activity, the nodule ultrastructure and the fresh and dry weights of nodules to a greater extent (Vassileva et al. 1997; Zahran 1999). Generally, nodulation problems occur once the pH falls below 5.5, as the rhizobium attachment to root hair is hampered (Bordeleau and Prevost 1994; Zahran 1999).

Taylor et al. (1991) suggested that at low pH, the rhizobial population decreases. However, not all the strains exhibit pH sensitivity, and rhizobia appear to be more tolerant to alkalinity and acidity and then do their legume hosts, thus host legume is considered to be a limiting factor for creating rhizobium–legume symbiosis (Tang and Thomson 1996; Graham et al. 1994; Zahran 1999). *Bradyrhizobium* (slow-growing strain) is more tolerant to low pH than the fast-growing strains of rhizobium, with the exception of *R. loti* and *R. tropici*. In pH-tolerant strains, the cytoplasmic pH is not altered much by external acidity. Experimental evidences suggest that this tolerance can be attributed due to the differences in the lipopolysaccharide composition of strains, proton exclusion and extrusion, synthesis of acid shock proteins and high cytoplasmic potassium and glutamate levels (Aarons and Graham 1991; Bhat and Carlson 1992; Fujihara and Yoneyama 1993; Zahran 1999).

Like rhizobial species, the legume species also differ in their response to low pH (Tang and Thomson 1996). Muthukumar et al. (2014) suggest three possible mechanisms that enable plants to tolerate acidic conditions: (1) exclusion of toxic ions (such as Al and Mn) from the root apex, (2) tolerance to toxic levels of Al and Mn through detoxification in the plant symplasm and (3) enhanced efficiency in the uptake of limiting nutrients from acid soils (Kochian et al. 2005; Bhalerao and Prabhu 2013).

Soil acidity also limits the phosphorous availability, reduces legume growth and indirectly limits nodulation (Bordeleau and Prevost 1994). Phosphorous deficiency affects the growth of the legume host as well as of the symbiont. Rhizobial strains differ in their capacity to tolerate phosphorous deficiency, and generally slow-growing strains are more tolerant than the fast-growing ones (Zahran 1999). The presence of heavy metals such as aluminium further aggravates the problem of low pH, as under these conditions phosphorous is precipitated and becomes unavailable to plant and rhizobium in the rhizosphere. Thus, such conditions result in the stunted root growth, low calcium uptake by plant and reduced nodulation and nitrogen fixation (Zahran 1999; Azooz and Ahmad 2015). Wood et al. (1984) observed the symbiosis of *Trifolium repens* var Huia–*Rhizobium trifolii* strain HP3 in axenic solution culture system that under 10 mM phosphate and 50 mM Al, aluminium inhibits the root elongation at $pH < 6.0$ and root hair formation at $pH < 5$, while nodulation and rhizobium multiplication at $pH < 6.0$. Ferreira et al. (2012) demonstrated that the rhizobial strains of *R. tropici*, viz. UFLA04-195, UFLA04-173 and UFLA04-20, have greater efficiency for plant growth, shoot nitrogen content and nodulation when compared to strain CIAT 899.

6.2 Soil Moisture

Moisture regime determines the distribution, survival and activity of the rhizobium in their microhabitats (Orchard and Cook 1983; Zahran 1999). Waldon et al. (1989) isolated 74 rhizobial strains from the nodules of the desert woody legumes. Rhizobia isolated from surface and deep phreatic soil were compared. Jenkins et al. (1989) worked on three warm desert ecosystems such as sand dune, Chihuahuan Desert of New Mexico and Sonoran Desert of Southern California and observed that fast-growing rhizobia exists as free-living populations from 0 to 8 m depth, while slow-growing rhizobia dominated the surface 1 m of soil. However, this distribution of rhizobia was related to the concentration of total soil salts in the soils. Such studies show that rhizobia can exist in soil with low moisture contents but with low population density, while legumes are sensitive to extreme water regimes (Jenkins et al. 1989; Waldon et al. 1989; Tate 1995; Bordeleau and Prevost 1994). Faba bean and pea are known to be drought sensitive, whereas lentil and chickpea are known as drought-resistant genera (Azooz and Ahmad 2015). Tolerant legumes show osmotic adjustment (Ford 1984) by accumulating osmolytes such as glutamic acids (Botsford and Lewis 1990), trehalose, N-acetylglutaminylglutamine amide (D'Souza-Ault et al. 1993; Smith et al. 1994), proline (Kapuya et al. 1985) and pinitol (*o*-methylinositol) (Ford 1984).

Low moisture induces oxidative damage in legumes thereby effecting nodule performance (Azooz and Ahmad 2015). When 1-month-old alfalfa plants were inoculated with *Sinorhizobium meliloti* strains 102 F78 and subjected to drought, a decrease in plant growth due to decrease in leaf area and decrease in nodule dry mass was observed (Aranjuelo et al. 2007). Interestingly, Talbi et al. (2012) in their study observed that inoculation of *Phaseolus vulgaris* with a *R. etli* strain (which has enhanced expression of cbb 3 oxidase) increased the tolerance of *Phaseolus vulgaris*–*R. etli* symbiosis to drought and modulate carbon metabolism in nodules. The degree of water stress on the rhizobium and its activity also depends on the age and growth stage of the host plant. For example, in *Phaseolus vulgaris*, Pena-Cabriales and Castellanos (1993) demonstrated that water stress is more detrimental to nodulation at reproductive stage, rather than at the vegetative stage. Nodules initiated under sufficient water conditions show retarded growth, if exposed to dry conditions. In dry soil infection is restricted because of the short, stubby root hairs, which are inadequate for rhizobial infection (Bordeleau and Prevost 1994). Rate of nitrogen fixation in legume plants decreased under drought stress due to the accumulation of ureides in nodules and shoots and reduced shoot nitrogen demand, xylem translocation rate and metabolic enzyme activity (Azooz and Ahmad 2015).

6.3 Soil Salinity

Salinity is a major abiotic stress limiting agricultural production especially in arid and semiarid regions (Munns and Tester 2008). Rhizobial strains are found to be salinity tolerant than their partner legumes; the tolerance in this symbiotic nitrogen fixation depends on the plant as well as the rhizobium genotype (Dogra et al. 2013). Soil salinity adversely affects the microbial population mainly because of ion toxicity and osmotic stress (Tate 1995; Zahran 1999). The bacteria adapt to saline conditions by the intracellular accumulation of low molecular weight organic solute osmolytes (Csonka and Hanson 1991); increase in intracellular free glutamate and/or K^+ (Zahran 1999); release of osmoprotectants such as sucrose, ectoine, mannitol, lactose, etc. (Talibort et al. 1994; Ghittoni and Bueno 1995; Gouffi et al. 1999); accumulation of glycine betaine (Fougère et al. 1991); and increase in the content of polyamines (Fujihara and Yoneyama 1993). In a study by Sharma et al. (2013), the isolated rhizobial strains from the root nodules of three leguminous plants, namely, sesbania (*Sesbania sesban*), lablab (*Lablab purpureus*) and pigeon pea (*Cajanus cajan*), growing at a research farm in Dubai were able to nodulate in saline water 12 dS m^{-1} on 21-day-old seedlings.

Saline soils limit the productivity of legumes by adversely affecting the growth of the host plant, the symbiotic development of rhizobial root nodules and their nitrogen fixating capacity (Dogra et al. 2013). Some legumes, e.g. *Vicia faba*, *Phaseolus vulgaris* and *Glycine max*, are more salt tolerant than others, e.g. *Pisum sativum* (Zahran 1999). Increase in soil salinity causes water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization by displacing membrane Ca^{+2} by Na^{+}, reduction of cell division and expansion and genotoxicity in legumes (Azooz and Ahmad 2015). In another study by Latrach et al. (2014), salinity decreased the plant height, their dry mass and nodulation in the symbiotic combinations of two Moroccan alfalfa (*Medicago sativa*) populations (Demnate and Tata) and two rhizobial strains (rhLAr 1 and rhLAr 4). Oufdou et al. (2014) inoculated faba beans by rhizobial strains RhOF6 and exposed plants to 150 mM of NaCl. In the plant inoculated by RhOF6, glutathione S-transferase (GST) activity was generally increased while a decrease in glutathione peroxidase (GPOX), superoxide dismutase (SOD), ascorbate peroxidase (APOX) and monodehydroascorbate reductase (MDHAR) in roots of faba bean.

The initial steps of legume–rhizobium interaction are inhibited under salt stress (Zahran 1991). The legume–*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia. Tu (1981) demonstrated that failure of nodulation in soybean occurs due to decrease in rhizobial colonization and shrinkage of root hairs. Salt stress reduces nodule respiration and cytosolic protein production, especially leghaemoglobin, thus depressing nitrogen fixation (Zahran 1999).

6.4 Temperature

Ideally, 28–31 °C is found to be the optimal temperature for rhizobial growth, and they are generally unable to grow at and above 37 °C. Heat-tolerant rhizobia are likely to be found in environments affected by temperature stress. For example, rhizobial isolates from southern Nile Valley of Egypt were tolerant to 35–40 °C, although they formed less effective symbiosis with their legume hosts (Zahran 1999). At high temperature, the rhizobial strains become ineffective and outnumber the infective rhizobia in the rhizosphere. Zahran et al. (1994) reported that the heat stress changes the lipopolysaccharide pattern of some strains of rhizobia.

In legume host, elevated temperature negatively affects photosynthesis, respiration, water relations and membrane stability and also modulates levels of hormones and primary and secondary metabolites. The enhanced expression of a variety of heat shock proteins and production of ROS constitutes major plant responses to heat stress (Azooz and Ahmad 2015). Increased temperatures adversely affect root hair formation, adherence of bacteria to hairs, root hair infection, sites of nodulation, bacteroid differentiation and nodule structure and functioning, but accelerate nodule senescence (Roughley 1970; Roughley and Dart 1970; Pankhurst and Gibson 1973; Sutton 1983). Hungria and Franco (1993), in their study, screened strains of *Rhizobium leguminosarum* bv. *phaseoli* and observed that some of the strains were able to nodulate beans even when given a heat shock of 35 and 38 °C for 8 h in a day. However, the nodules formed were ineffective and nitrogen did not accumulate in plants. Thermal shocks of 40 °C at the time of flowering decreased the nitrogenase activity and nodule relative efficiency of plants but recovered only when new nodules were formed.

Michiels et al. (1994) compared heat-tolerant (CIAT899) and heat-sensitive (CNPAF512) strains of bean-nodulating rhizobia: 14 heat shock proteins were detected in CNPAF512 at 40 °C and 6 heat shock proteins in CIAT899 at 45 °C. In cowpea, Simões-Araújo and co-workers (2002) showed similarities in the transcripts – fragments derived after heat shock to those that encode for wound-induced proteins, disease resistance protein, heat shock proteins and xylan endohydrolase isoenzyme, as well as different housekeeping genes.

In temperate legumes, elevated temperatures delay nodule initiation and development, interfere with nodule structure and functioning, whereas nitrogen fixation efficiency is mainly affected in tropical legumes (Bordeleau and Prevost 1994). However, in a study on alfalfa (temperate legume) by Aranjuelo et al. (2007), elevated temperature decreased not only plant growth but also CO_2 and N_2 fixation rates and inhibited nodule activity. The results were obtained by inoculating alfalfa with *Sinorhizobium meliloti* strain 102 F78 grown under different temperature (25/15 or 28/18 °C, day/night) and water treatments.

6.5 Predation

Rhizobium bacteria have to adapt to soil conditions in the soil ecosystem, and spatial distribution of rhizobia in the soil influences their survival (Postma et al. 1990). The rhizobium is attacked by number of organisms such as insect larvae (for source of food), nematodes, bacteriophages, viruses, etc. (Andrés et al. 2012). In addition to the competition for resources with other organisms, rhizobia also have to cope with the predation by the protists (Jousset et al. 2006). This decreases bacterial number and also plays an important role in controlling bacterial populations (Jjemba 2001; Rønn et al. 2002). As a response to predation, bacteria have developed several adaptations such as morphological changes, increased motility, toxin production and membrane properties that make them unattractive (Jousset et al. 2006). In a study by Pérez et al. (2014), it was observed that in a cocultured medium of *Myxococcus xanthus* (a soil bacterium) and strains of *Sinorhizobium meliloti*, the predatory pattern is determined by the galactoglucan released by the rhizobial strain.

7 Epilogue

Understanding the role of biological N_2 fixation can help us to achieve agricultural sustainability worldwide. However, the relationship between plants, soil microorganism and soil is multifaceted. Plants and microorganisms, directly or indirectly, play a crucial role in the major ecological processes such as nutrient cycling, soil formation, improving soil fertility and BNF, etc. The process of nodulation involves two-way molecular conversation between the host legume and the bacteria. A number of natural and anthropogenic factors affect the legume–*Rhizobium* interaction. However, there are varieties of strains that can tolerate harsh environmental conditions. Optimum utilization of BNF can help us to achieve agricultural sustainability worldwide. The use of new inventions and its access to the farmers could be a step towards sustainability and will help in improving the economic status of the farmers. Therefore, more research program across the world to identify the superior legume varieties and rhizobial strains for the human welfare are required.

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