1. Introduction and literature review

1.1 General introduction

Legumes are members of the family Fabaceae (Leguminosae) (Cronquist, 1981). Population migration, trade and wars enabled some species [e.g. common bean (*Phaselous vulgaris* L.), soybean (*Glycine max* (L.) Merr.), pea (*Pisum sativum* L.) and faba bean (*Vicia faba*)] to spread to numerous regions throughout the world (Werry and Gringac, 1989).

Legumes play a unique role in agriculture worldwide due to their ability to fix atmospheric nitrogen (N_2), and to their high protein content (Messina, 1999). Legumes are used as food or feed crops. Grain legumes are common food throughout the world. They are second only to cereals as a source of human and animal nutrition (Erdman and Fordyce, 1989). The importance of legumes as food lies primarily in their high protein content. Most dry legume grains contain 20-28% protein (Chitra et al., 1995).

On a world wide scale, legumes provide 22% protein, 32% fat and oil, and 7% carbohydrates in terms of human nutrition. In terms of livestock nutrition they provide 38% protein, 16% lipids and 5% carbohydrates (Werry and Gringac, 1989).

Grain legumes play an essential role in human nutrition, balancing the deficiencies of the basically cereal-based diet (Dart and Krantz, 1976; Messina, 1999). The protein of legume grains is rich in the amino acid lysine, whereas the sulphur-amino acids are the major deficiency. The reverse is true for the protein of cereal grains. Therefore, it is evident that legume grain protein is the natural supplement to cereal grain protein (Bressani, 1973). In addition to being rich in protein, legumes are also high in bone-building minerals (Ca, Zn and Fe) and vitamins (ribovalvin, folate) essential for good health (Messina, 1999).

The beneficial effects of legumes in agriculture have been recognized even before the principles of crop rotation were established (Herridge, 1982). Symbiotic nitrogen fixation in legumes in association with rhizobia is important in the development of sustainable agriculture. Using symbiotically fixed N instead of chemical fertilizers decreases the need for application of chemical nitrogen fertilizers to crops. As a result both economic and environmental costs of agriculture are reduced. Grain legumes are particularly well suited to increase crop production in low-N nutrient environments because they can be used both to produce a high N content seed crop themselves

and to generate high N content plant residue. Thus a crop rotation with cereals results in significant improvement in yield (Nambiar et al., 1982). However, the grain legumes residues are low in N content, since most of the plant N goes to the grains. Therefore, rotations that include a legume as a green manure crop would be more beneficial in increasing soil N fertility (Sinclair and Vadez, 2002). Furthermore, using perennial legumes in an agroforestry cropping system offers an opportunity for long-term N_2 fixation as well as a long growth cycle to recover P from the soil (Alley et al., 1999; Pannell and Ewing, 2004).

Considerable attention has been given to increase symbiotic N_2 fixation activity of legumes. However, much of the research, especially with the advent of molecular genetics, has focused on improving rhizobia.

Nevertheless, the performance of *Rhizobium* inoculants as biofertilizers is limited under field conditions, unless the rhizobia are stuck to the seeds in a carrier-formulated inoculant, where it can reduce the competition of indigenous rhizobia (Huang and Erickson, 2007).

Furthermore, attempts to increase N_2 fixation activity by selecting efficient host legume cultivars that result in greater nodulation have achieved only limited success. However, nitrogen fixation activity is related to whole plant activity and greater nodulation does not necessarily always mean higher symbiotic N_2 fixation or yield (Pracht et al., 1994). Nevertheless, a comparison of strongly contrasting lines of chickpea showed greater nodulation capacity was associated with higher N_2 fixation and grain yield (Rupela et al., 1997).

Phosphorus plays a vital role for legumes, because there is a substantial need for P in the N_2 fixation process. The high requirement for P in legumes is consistent with the involvement of P in whole plant growth as well as the high rates of energy requirement for symbiotic nitrogen fixation and nitrogen assimilation in the nodule (Israel, 1987).

Although the existence and importance of the process of symbiotic nitrogen fixation has been recognized for more than a century, scientific advances over the last few decades have altered radically our understanding of its nature and mechanisms that regulate its flow. A heightened awareness of these physiological mechanisms, by which the plant control its needs of fixed N in accordance to available N, other nutrients and environmental factors, is a key element in designing strategies to enhance

the productivity of crop legumes through breeding programs or genetic engineering (Schulze, 2004).

1.2 Symbiotic nitrogen fixation (SNF)

Symbiotic nitrogen fixation is one type of biological nitrogen fixation. Biological nitrogen fixation is the process by which atmospheric N_2 is converted to NH_3. The net biological reaction is the following:

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \text{ -----> } 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$$

Biological nitrogen fixation occurs only in some species belonging to both prokaryotic kingdoms, *Archaea* and *Eubacteria*. Some of these biological nitrogen fixers are free living diazotrophs (e.g., *Klebsiella*, *Azotobacter* and *Rhodobacter*), which live in close association with roots (e.g., *Azospririllum*) or form an intercellular symbiosis in many genera. The latter are collectively named as rhizobia and they constitute the family *Rhizobiaceae* (Fischer, 1994; Raymond et al., 2004).

The rhizobia-legume symbiosis is the most well known and studied example for SNF. This symbiosis is estimated to be responsible for up to 60% of the biologically fixed nitrogen on land.

The enzymatic reduction of dinitrogen is energetically expensive since the bacterial enzyme nitrogenase complex catalyses a reaction, in which at least 16 adenosine triphosphate (ATP) are reduced to adenosine diphosphate (ADP) (20-30 ATP under physiological conditions), while fixing one equivalent of molecular N_2. Nitrogen fixation uses as much as 20% of the ATP that the legume plant produces (Postgate, 1998).

The successful higher plant symbiont must pay the energetic price of the reduction reaction, conduct a complicated signal exchange with rhizobia (recognition, infection), and provide an anaerobic environment for the oxygen sensitive nitrogenase enzyme by forming a novel organ - the nodule. Nodules are formed by the two interacting genomes of the higher plant and the *Rhizobium*-bacteria, and operate through the two interaction sets of metabolic capabilities, in a system of specialised tissues,

cells and compartments (Brewin, 1991). The bacteria are structurally integrated into infected nodule cells by a cell organelle like structure, the symbiosome. This organelle like structure 'symbiosome', which comprises the differentiated form of *Rhizobium*, the 'bacteroids', and the symbiosome space, is enclosed by a plant-derived membrane, the peribacteroid membrane (Werner, 1992; Day et al., 2001).

During SNF the plant cell provides the bacteroids with energy in form of assimilate C (malate), and essential nutrients for both bacteroid metabolism and bacteroid growth. The bacteroids excrete fixed N (probably ammonium) in the surrounding plant cell cytoplasm, where the fixed N is assimilated into organic structures (Whitehead et al., 1995).

In summary, the legume nodule emerges as a physiological compartment for protecting nitrogenase from oxygen as well as for supplying bacteroids with assimilates. This symbiotic nitrogen fixation takes place only in nodules, rhizobia rarely, if ever, fix nitrogen while they are free-living (Postgate, 1998).

In an efficient functional symbiosis, the nutritional benefits of N_2-fixation presumably offset the costs associated with these traits (Postgate, 1998).

Early studies of the legume-*Rhizobium* association noted that only a certain *Rhizobium* specie is able to perform a symbiotic nitrogen fixation with a certain legume/s, which are referred to classically as cross inoculation groups (Fred et al., 1932). In 1982, two groups of root nodules bacteria were described: fast growing types assigned the generic name *Rhizobium* and slow growing types referred to as *Bradyrhizobium* (Jordan, 1982). In 1996, seventeen species, in four genera (*Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Mesorhizobium*), were described (Young and Haukka, 1996). Recently, in 2003, based on the comparative analysis of 16s rDNA sequences, 44 species in 12 genera have been confirmed as nitrogen fixing bacteria in a symbiotic association with legumes (Sawada et al., 2003). However, the term *Rhizobium* is still used for bacteria of all genera (Raymond et al., 2004).

1.3 Symbiotic N_2 fixation - a limiting process

Symbiotic nitrogen fixation is a very energetically costly process (Postgate, 1998). Therefore; if any fixed nitrogen is available to the roots, the leguminous plant can create various mechanisms to limit SNF.

Numerous empirical laboratory and field studies with crop plants and some undomesticated legumes have documented decreasing nodulation rates with increasing soil nitrogen availability (e.g. Singleton and van Kessel, 1987; Caetano-Anolles and Gresshoff, 1991; Lang et al., 1993; Rubio Arias et al., 1999; Thomas et al., 2000)

This observation suggests that the relative benefits of nodulation decline with increasing abundance of reduced nitrogen, which plants can obtain directly from the soil solution. Furthermore, not only high N availability results in restricted nodulation but many other types of environmental stresses, which limit plant growth like salt stress or inadequate supplies of mineral nutrients, especially phosphorus (Caetano-Anolles and Gresshoff, 1991; Tsai et al., 1993; Oliveira et al., 1998; Taiwo et al., 1999).

Basically, a leguminous plant allows the appropriate *Rhizobium* to enter and establish the symbiosis under the concept of mutualism only if there is a shortage in available reduced N. The regulation of nodulation in dependence on these factors occurs at various levels of the infection process (Vitousek et al., 2004).

The fact that under conditions of high nitrogen or low phosphorus availability, the plant adjust nodule number and morphology (in particular nodule size and location) to fit with its necessity to fix nitrogen illustrates that plants do not permit unlimited infection by compatible rhizobia. Indeed, in various legumes, the majority of root-hair infections by compatible rhizobia do not lead to nodule formation (Nutman, 1962). This regulation mechanism is created by the host plant as adaptation to distinct environmental conditions (Hartwig et al., 1997). Furthermore, the limitation of nodule number and the weak response or susceptibility to further inoculation following an initial inoculation, are various indications for such adaptation (Nutman, 1962; Bhuvaneswari et al., 1980; Bhuvaneswari et al., 1981; Pierce and Bauer, 1983; Heron and Pueppke, 1987; Malik and Bauer, 1988; Takats, 1990). It was shown in recent years that the nodulation is eventually controlled by a shoot borne factor that mediates the mentioned stress impact on plant growth (Searle et al. 2003).

In addition to this persistent adaptation in the establishment of N_2 fixing symbiotic schemes, there are many other flexible mechanisms existing in plants to adjust N_2 fixing (nitrogenase) activity in nodules to plant demand (Hartwig et al., 1997). Understanding such regulating mechanisms on the physiological level is a prerequisite to link physiological adaptations in plants and nodules with nitrogenase activity which will be reflected in the amount of fixed N. There are basically three factors that are

discussed as to be the primary mechanisms to adjust nitrogen fixation to plant demand (Schulze, 2004). While either assimilate supply to nodules or oxygen diffusion into nodules is thought to be the limiting factor of nodule activity, various experimental results indicate that some N feedback mechanisms prevents nodule activity from providing excess nitrogen.

1.3.1. Limitation of symbiotic nitrogen fixation by assimilate supply

The high energy demand to convert atmospheric N_2 to NH_4^+ and to assimilate fixed N with the link to the essential micro aerobic conditions for nitrogenase activity and the expression of genes connected with nitrogen fixation, gives assimilated C and its pathways in nodules a distinct character in that the C metabolism is shunted towards malate production, although sucrose is the primary translocated C compound into nodules (Vance, 2004). Malate formation in nodules occurs mostly through the combined activity of carbonic anhydrase (CA) (EC 4.2.1.1), phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) and malate dehydrogenase (MDH) (EC 1.1.1.82) (Schulze, 2004). Malate serves as the principle energy substrate for bacteroids and, after conversation into oxaloacetate, as carbon skeleton for amino acid synthesis (Schulze, 2004). Moreover, malate is thought to be involved in a possible osmocontractile regulation mechanisms of nodule O_2 permeability. Thus, the importance of malate in nodules has both functional and metabolic dimensions (Schulze, 2004). The concentration of malate in effective nodules is much higher than in ineffective ones (Vance and Heichel, 1991.

The role of assimilate supply in nodule functioning and activity regulation has long been the focus in nitrogen fixation research (Vance and Heichel, 1991). Decapitation or phloem-girdling interrupts the assimilate supply to the nodules and causes an often dramatic and quick reduction in their activity (Vance and Heichel, 1991). In spite of C supply importance, and its distinctive metabolic pathways, there are strong arguments that nitrogenase activity is not regulated or limited via carbon supply (Denison et al., 1992; Hartwig et al., 1996; Almeida et al., 2000). This argument is based on the fact that e.g. stored starch is detected in nodules even under stress (Serraj et al., 1995) and decreases in nitrogenase activity after defoliation can not be prevented by increasing carbon supply (Denison et al., 1992; Hartwig et al., 1996). However, the role of photosynthate supply can not be ignored, and several questions remain open, like if the nodules mitochondria are O_2 limited, the accurate contribution of nodule CO_2 fixation via the PEPC enzyme to the nodule carbon balance, and the accumulation of malonate without knowing its role (Vance and Heichel, 1991).