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Legume-bacterium (*Rhizobium*) Association-Symbiosis, A Marriage of Convenience, Necessary Evil *or* Bacterium Taken Hostage by the Legume

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Abstract: Nitrogen (N) is the key nutrient element, limiting crop production under most situations. A major reason for insufficient N supplies being its presence in soil in organic forms which must be mineralized before being used by the plants. However, leguminous plants are equipped with the facility to acquire a major portion of N directly from atmospheric N_2 through bacterial fixation (reduction). The bacteria (*Rhizobium* spp) reside inside the special structures on plant roots i.e., nodules and reduce atmospheric N at the expense of C supplied by the plant. This paper presents an analysis of the nature of association between the legume and bacterium.

Key words: Association, legume, bacterium

Introduction

In ancient times, the beneficial effects of leguminous species on soil fertility was well recognized. But it was not until the middle of the last century that this extraordinary capability of legumes to fix nitrogen by symbiotic bacteria, present in the root nodules was discovered. Liebig (1865) thought that legumes with their broad leaves were capable of absorbing NH_3 from the atmosphere and thus met much of their needs for nitrogen and also improved N economy of the soil. It was in 1886 that Hermann Hellriegel and Hermann Wilfahrt discovered that root nodules contain bacteria capable of fixing N_2 from the atmosphere (Bohm, 1986). Since then the value of leguminous species in crop rotation stands established beyond any doubt. On the global level, 172 million tons of N is added to the ecosystems through biological nitrogen fixation (BNF), an amount estimated to be 3 times that of industrially fixed N (Ishizuka, 1992). The amounts of N_2 fixed vary from 40 to 460 kg ha^{-1} depending upon the type of legume and the cultural conditions (Bothe *et al.*, 1983). Contribution of fixed N to the total plant N also varies widely and may reach 40-90% (Eaglesham *et al.*, 1977, Rennie *et al.*, 1982).

In most studies, the association of bacterium with the legume is termed as "symbiosis" while the later is regarded as host. Whether or not the association is of a symbiotic nature, provides the basis for this review.

What is symbiosis: Symbiosis is defined as any stable condition in which two different organisms live together in close physical association for their mutual advantage.

Nitrogen fixing symbioses found in nature – an evolutionary perspective: In essence, there are four kinds of N fixing symbioses, encountered in nature all involving prokaroytes as the microsymbiont, with bryophyte, gymnosperm or angiosperm as the macrosymbiont. Most primitive would appear to be the one involving *Azolla – Anabaena* (bryophyte and algae) followed by *Cycas – Nostoc* (gymnosperm and alga) *Casuarina – Frankia* (gymnosperm and actinomycete) may be regarded as primitive, compared to legume – bacterium (angiosperm and bacterium) association. Looking at these symbioses, it would appear that the two partners were almost un distinguishable from each other in *Azolla – Anabaena*; the later residing in special cavities. In case of *Cycas – Anabaena*, the microsymbiont appeared to have been localized in specialized coralloid roots and was no more embedded in the main body of the plant, unlike that in *Azolla*. In legume – bacterium association, the trend in localizing the later in still more specialized structures continued and the so-called nodules became characteristic of most legumes. Apparently, the association was progressing towards attempted extrusion of the microsymbiont from the host tissues, the process culminating in free-living N_2 fixers (e.g., *Azotobacter* and *Azospirillum*, *Bacillus* etc.) that were preceded by associative N_2 fixers (e.g., species of *Pseudopmonas*, *Enterobacter*, *Acetobacter*, *Beikerinckia* and *Kelbsiella* etc.).

Evolution from aquatic to terrestrial environments probably led to the free living N_2 fixation and plant systems free of N_2-fixing associations. For example, wheat being an upland crop reported to have associative abilities, has the least probability of harbouring N_2 fixers. On the contrary, significant associative and even endophytic N_2 fixation has been reported in graminaceous plants grown under relatively high moisture conditions (e.g., rice, sugarcane and kallar grass).

A critical evaluation of different N_2 fixing symbioses would therefore suggest that nodulation is a primitive character, at least when keeping in view the highly developed plant types (e.g., those belonging to the family Gramineae). In other words, the advanced plants acquired the ability to avoid microsymbiont. In the same perspective, legumes that do not nodulate can be termed advanced a reverse would be true for those that nodulate. Likewise, nitrogen fixation can be termed as a primitive character, while ability to nodulate is a recent feature (at least in comparison to bryophytes and cycads). Thus, the ability of rhizobia to nodulate their host is of a more recent origin than the ability to fix N_2. Interestingly enough, legumes that do not nodulate are also reported to fix N_2 (Bryan *et al.*, 1996).

The ancestors of both partners (legume and bacterium) are supposed to possess the pre-adaptations, which ensured a subsequent evolution of the symbiotic system (Provorov, 1998). The bacterial pre-adaptations might have been the ability to fix N_2 and to resist the plant defense reactions [then how it could be symbiosis which should in principle mean mutually accepted arrangement, while in this case, the association is thrust upon by bacterium onto the plant with consequent triggering of defense system and bacterial strategies to overcome the defense]. The plant pre-adaptation might have been spontaneous formation of the nodule-like structures and an ability to permit a persistence of potential symbionts in them. However, as would be argued later, this may not be the legume's ability to permit, but its inability to stop the entry of the bacterium into the root tissues. The plants with the ability to stop bacterial entry would thus be considered highly evolved. Indeed nodulation is a more ancient property than symiotrophic nitrogen nutrition (in non-legumes).

The ability to nodulate and form effective nitrogen-fixing symbiosis may have appeared at different stages of legume evolution. It seems likely that the ability to form legume nodules was not present in the common ancestors of all rhizobia but that the nodulation genes were transferred between phylogenetically distinct bacteria, so that the phylogeny of nodulation genes will probably differ from that of the bacteria harboring them. Nitrogen fixation genes are often linked to nodulation genes, but they need not have the same evolutionary history (Young and Haukka, 1996). Nevertheless, genes controlling nitrogen fixing symbioses of legumes with specialized bacteria are probably the products of many millions of years of evolution (Devine and Kuykendall 1996) as different adaptive solutions evolved in response to challenge of survival in highly divergent complexes of symbionts. Provorov (1996) suggested that synchronization of the rates of bacterial and plant genome evolution was the condition necessary for co-evolution of root nodule bacteria and legumes.

Legume symbioses are thought to have evolved from loose associations between free-living bacteria (Rhizobium's ancestors) and the legumnioseae family (Dilworth and Parker, 1969). Kennedy and Tchan (1992) seem to agree with this, that nodulation is an advanced character compared to a situation where relatives of rhizobium (*Azorhizobium* and *Bradyrhizobium*) could fix N_2 ex-planta. The nature of *Rhizobium* to hide from O_2 for N_2 fixation would, however, suggest that it is at a lower evolutionary level compared to others, who can live and fix N_2 in the presence of O_2.

DNA sequence molecular data indicates a single origin of the predisposition for root nodule symbiosis within this more restricted group. These findings indicate that only one small group of angiosperms in a single clade possesses the ability to host nitrogen fixing microsymbionts. The initiation of N_2 fixation was not an absolutely unique event. Possibly, some plant species and their ancestors may have periodically lost and regained a symbiotic state in response to altered selection pressures. The complexity of the molecular dialogue now recognized as controlling the establishment of many legume-*Rhizobium* symbioses presumably represents a refinement of simpler relationships existing formerly. However, no mechanism can readily be proposed, that would allow a sequential development of such a complex interactive process without initial advantages such as mutually beneficial nitrogen fixation. Thus we may assume that even legume symbiosis involved a prototype stage of development involving fewer genes but of lower stability. Alternatively, advantages such as improved mineral nutrition, stimulation of plant root growth or mild parasitism may have provided initial mutual benefits favouring acceptance of microbes by the plant host, with insertion of nitrogen fixation at a later stage.

Is legume-bacterium association a symbiotic relationship: In true sense of the meaning, the relationship is of symbiotic nature i.e., both the partners benefit from each other in one way or the other. The bacterium gets C and energy source from the legume, while the later meets most of its N demands through the courtesy of the former. The question, however, arises that to what extent this symbiotic co-existence is based on mutual understanding between the two partners, and whether or not any mutual damage is also involved. It would probably be more appropriate to term the association "parasymbiosis" since some sort of damage is certainly involved. Nodulation starts with the initial damage done to the legume by bacterium, while the later renders the former impotent to get the benefits in terms of fixed N. All signs are that, the association is more like a compulsion than choice. The entire terminology used in the literature to define/discuss the legume-bacterium association e.g., host, bacterial invasion, infection, infection thread, resistance to infection, and legume defense mechanism etc. suggests something else than a symbiosis; mutual benefit not withstanding. According to Sharifi (1984), the pre-infection events, the process of infection and nodulation in the colonization of the legumes by the *Rhizobium* are similar to those of other parasitic associations. Likewise, the host responses to the *Rhizobium* entry, infection thread synthesis and bacteroid formation are comparable to those of other plants when they encounter phytopathogens. Evolutionary processes acted in the selection of biotrophy. The fine control and regulation of the extracellular enzymes of the necrotrophic *Rhizobium* converted the association into biotrophy. The nutritional dependence of the *Rhizobium* on the legume, the requirement of the plant for combined nitrogen and the *Rhizobium* potential to meet this requirement drove the biotrophic association into mutualism. This became possible when regulation of the nitrogen-fixing system of the *Rhizobium* was modified and the oxygen carrying protein leghemoglobin was acquired or evolved by the legume to enhance nitrogen fixation. Broughton and Perret (1999) used the term invasion to describe bacterium legume interaction. According to these authors, once the contact has been initiated by flavonoids and NodD proteins, constant signal exchange fine-tunes these symbiotic demands, especially to overcome defense reactions. However, it is not known how rhizobia benefit from nodulating legume hosts because they fix nitrogen only after becoming bacteroids, which are terminally differentiated cells that cannot reproduce (the un differentiated ones in and around the nodule can reproduce). In some hosts, their kin may persist in the nodule as viable, undifferentiated bacteria. In other hosts, no viable rhizobia survive to reproduce after nodule senescence. By getting transformed into bacteroids and sacrificing their potency, the N_2 fixers make a last ditch effort to arrange some food for survival of their kin, although the plant is basically providing a kind of subsistence food to the bacteroids to make them continue the donkey work (transport of photosynthates actually gets enhanced following infection), but a part of it may benefit the kin as well. They do this by synthesizing "rhizopine" in the nodules and smuggling them out for their kin that are the only ones to catabolize these (Simms and Bever, 1998). The host, essentially unaware of this, goes on supplying necessary substrate for the synthesis of rhizopine (the rhizobia that can synthesize rhizopine should be considered at a higher evolutionary level than the ones unable to do so). However, rhizopine genotypes are relatively rare.

Sprent and de Faira (1989) emphasized that many of the widely accepted dogmas for "normal" symbioses, e.g., root hair infection, formation of infection thread and the necessity of the bacteria to be released from infection threads before they differentiate into N_2 fixing forms, are not universal. Infection through wounds is now well established as a normal part of nodule initiation, so that the idea of a fixed, obligatory procedure cannot be sustained any further. Infection threads develop subsequent damage of cells, and the damage could be caused in any manner. Despite genetical studies lasting more than a decade, no evidence has been found that the legume-rhizobium symbiosis involves unique plant gene products that are necessarily restricted to plants that are reported to successfully nodulate and fix the N_2. Neither do any of the proposed steps in this symbiosis offer a uniqueness that would absolutely exclude different plant species from also allowing establishment of persistent symbioses with diazotrophs. In fact, enzymatic treatment of the root hair of white clover has been shown to remove the barrier of host specificity and increase the range of rhizobia able to nodulate this legume (Al-Mallah *et al.*, 1989).

Indeed nodulation or nodule-like structures may result not necessarily from bacterial invasion alone. Also, the structures formed after bacterial invasion or due to physical root damage may differ. In fact, different structures are formed as a result of differences in the agent e.g., types of rhizobia. Different rhizobia could secrete different types of chemicals similar to 2,4-D in function. These chemicals may be different for different organisms leading to variation in the degree of damage caused to the root system and plant responses expressed in the form variable types of nodular structures. Genetic diversity of the host is also an important factor in the maintenance of polymorphism within the symbiont population. That formation of nodules is not necessarily the result of symbiotic relationship is supported by reports on paranodules. Glagoleva *et al.* (1997) claimed for the first time the formation of paranodules in the roots of rape (*Brassica napus*) due to 2,4-D and bacterial inoculation. The nodules formed on 2,4-D treated roots in the presence of bacteria were structurally different than those formed without inoculum. Thus nodules or nodule-like structures can be formed by chemical treatment of roots, presence of bacterium only extends this occurrence to a sort of effective nodulation commensurate with N_2 fixation. Increase in bacterial number and nitrogenase activity was demonstrated in chemically treated roots. In non-nodulating soybean, physical damage of roots by 2,4-D was found to cause nodulation (Akao 1991). Thus non-nodulating soybean seems to have evolved the mechanism to prevent cell wall damage by the bacteria i.e., the first step towards nodulation.

Physical wounding and rhizobial inoculation results in the triggering of similar genes e.g., chalcone synthase gene which codes for the first enzyme of the flavanoid pathway (Rolfe *et al.*, 1997). Differences in cell wall chemistry of non-nodulating and nodulating legumes may have an important bearing on the subsequent nodulation. The bacteria that do well once in the nodule but are unable to cause nodulation will be lacking cell wall degrading chemicals. This would also mean that legume is not attracting the bacteria, but it is all along a coincidence that the two partners get together somehow, and then who uses whom is the question to be answered after thorough investigations. Even the expression of haemoglobin gene may not necessarily involve rhizobial interaction sicne expression of these genes in root and seed tissues of barley has been reported under anaerobic conditions (Taylor *et al.*, 1994). Similarly, *Casuarina glauca* has a gene encoding haemoglobin that is expressed in a number of plant tissues (Jacobsen *et al.*, 1995).Thus expression of these genes may not necessarily result from microbial involvement but may be considered an adaptation to physical rather than biological conditions. Nevertheless, bacteria with a 2,4-D like effect must succeed in causing nodulation. Some of these diazotrophs have indeed been used in an attempt to establish an N_2 fixing association with non-legumes treated with 2,4-D (Kennedy and Tchan, 1992, Kennedy *et al.*, 1997). This approach has also been used to induce nodulation in cereals or at least effective N_2 fixation through the agency of endophytic bacteria other than rhizobia (Tchan and Kennedy, 1989; Kennedy and Tchan, 1992). The most well known successes in this regard being *Rhizobium-Parasponia* symbiosis (Trinick, 1988).

Nodulation a relationship of invasion by bacterium and its imprisonment by legume:

The legume nodule is a unique plant organ. It has been considered homologous with lateral root modifications of actinorrhizal plants and *Parasponia*. However, legume nodules differ from these in the developmental origin, anatomy and the patterns of gene expression (Hirsch and LaRue, 1997). Iannetta *et al.* (1997) used both terms like host and invader for legume and *Rhizobium*, respectively. Basically it must be treated as damage-repair mechanism i.e., a damage caused by any physical or biological means has to be repaired by legume like any other living-being that responds to such a challenge. The question arises that how do compatible rhizobia manage to breach the host cell wall and evade a host plant defense response and what enzymes are responsible for host cell wall damage. These questions still await convincing answers. Iannetta *et al.* (1997) suggested that species of rhizobia have evolved towards symbiotic status by development of cell-wall degrading enzymes on 2 levels, firstly to stop or limit secretion and/or synthesis of wall-degrading enzymes capable of eliciting HPDR (host plant defense reaction), and secondly to express high levels of enzymes stimulated by the presence of compatible host tissue polysaccharides or polysaccharide-containing moieties. They also suggested that rhizobia might have evolved from a pathogenic ancestor. Indeed, the so-called plant-microbe specificity will depend on specific compounds released into the rhizosphere and the resultant threshold level of the particular microbial population to achieve effective plant-microbe association.

In fact, if damaged using 2,4-D, non-nodulating lines of soybean have been shown to nodulate even in the absence of bacteria, while when bacteria are also inoculated simultaneously, effective nodulation could be established (Kennedy and Tchan, 1992). Such nodules are inducible on non-nodulating plants as well, meaning thereby that a sufficiently damaging substance is required for malformation or so-called nodulation, followed by bacterial entry into the wound, where it may or may not work depending upon the preferences and capabilities of the plants. One of the 2,4-D effects is to induce many lateral root initials simultaneously, possibly stressing the plant by causing excessive demand for photosynthates. In addition, curtailing root elongation may also lead to new roots being formed at places not initially destined for this purpose. However, the roots become normal after the hormone is relieved.

What actually happens before or after the two partners come in contact with each other. Examples from other systems suggest that as an instinct, the plant will not let the bacterium enter into its tissues. In fact, the entry seems to be circumstantial rather than intentional/planned. Quite certainly, the plants that nodulate do not have the means to resist the entry of Rhizobia into their roots, while the ones which do not nodulate have such abilities. Even once in the root, N_2 fixing types have some mechanism to make the bacteria stay there and fix N_2, while the others cannot provide those conditions required for this system to work. Thus there may not be a genetic basis for the interaction to take place, although sustainability of the relationship does have a genetic basis. There is ample evidence to suggest that most common mode of infection is through fissures (where lateral roots emerge) other than via root hairs. Same (crack entry) is indeed true for endophytic root colonization of other plants. It will appear therefore that the Rhizobia become part of the nodule only after the plant has started reaction to some foreign agent (exogenous chemicals like 2,4-D or biochemicals produced by microbes including those destined to become microsymbiont). In rape plants e.g., the rhizobia may enter the roots but are killed, so survival/activity and not merely the entry is what matters. Once in the plant tissue what situation they have to face, whether they are accommodated (like in legumes) or destroyed (like in brassicas e.g., rape) will depend on the host characteristics. In fact it is not the matter of accommodation either, the so-called compatible legume does not have the necessary infrastructure to destroy the invader but it does have means to restrict its spread into the other tissues. Thus the legume does not welcome the bacterium but has no way to get rid of it either. In fact, as soon as the plant is aware of the enemy in its vicinity that has incurred initial damage to the young root or root hair, the plant immediately reacts and builds walls against the invading bacterium. The bacterium, seeing initial success in damaging the root cells (caused by the bacterium itself or by some other agent) and getting some food in return, is encouraged. It becomes greedy and in search of yet better meals enters into the enclave, to find itself confined within the strong walls of the nodule, just to render services to the legume - a kind of forced labour. The bacterium may not be even an invader but just looking for some food. Sensing/smelling the possible source of carbon and energy, it attacks the legume with wall-degrading chemicals (if it is compatible or host specific) without knowing the cost involved i.e., life long imprisonment in return for some food material, but with no surety of survival or continuity of its race. Legumes in fact develop a containment facility for their security and would not let the invaders have free access to other plant parts.

The presence of enemy means activation of possible defense mechanisms. Being under stress, the plant would need to transport more energy materials to the site of combat. However, sort of unknowingly, the extra carbon transported to the roots is happily welcome by the bacterium. Further, the supply of extra amounts of photosynthates also means providing bait to the invaders and thus to engage them at a specific site. Nodulation is a stressful process from initiation to sustenance that induces the transfer of more photosynthates at the site of infection to enable the plant withstand the stress as is true for other physical stresses. Once, the bacterium gets to work, a source to sink relationship automatically develops for the photosynthates.

The supply of carbonaceous materials to the site of nodulation may also be considered as "bait" for the bacterium to get engaged thus restricting it to a certain place where the damage has been done. The plant can at the same time, initiate roots or root hair at some other site to compensate for the loss. For example, increase in the root proliferation following *Agrobacterium* infection may be considered as one such manifestation of the plant. In addition, the work of Hawes (1991) clearly demonstrates that the advanced plants e.g., monocots, has acquired a mechanism whereby they are able to defy the possible pathogens/invaders/parasites. According to these workers, the root cap cells (true replica of the living cell in all their characteristics) are regularly released during the process of root elongation. What this means is that the actively growing root sheds off living cells at regular intervals. These living cells engage the potent invaders (parasites) at the point of production/release and the root continues growing further. Although an expensive business, it could not have been avoided to ensure the survival of the plant that depends on healthy and well-established root system in the wake of possible invasion/damage by microbes. In general as well, greater amounts of C are transferred to the rhizosphere as rhizodeposition in the presence than the absence of microbes (Graham *et al.*, 1981, Reid *et al.*, 1983). Upon being accommodated within the nodule, the bacterium becomes a prisoner. The so-called compatible host plant must have the ability to feed the prisoner and get the desired work out of it till needed (till maturation), and then let it free half-dead. Why actually the plant should apply "terminator technology" (by rendering it unable to reproduce) on the bacterium that does a good job for it. Because, it was all along an accident for the plant and not a welcome gesture for the bacterium (which has always been considered as an invader and danger). This means that plant foresees the loss of at least something when nodulated compared to when not nodulated. Thus by rendering the rhizobium incapable of further reproduction the plant at least makes an attempt to break the continuity of rhizobial race -albeit not that successful. The plant sees the bacterium as an unfriendly, unfamiliar entity and starts reacting almost immediately by way of supplying more energy materials (photosynthates) at the site of invasion. An example from *Alnus rubra* and *Frankia* interaction will help understand the contention. Markham and Chanway (1999) observed that plants with familiar *Frankia* strains were half the size and derived less fixed N from their symbiont compared with those inoculated with unfamiliar *Frankia*. Familiar means that plant knows the worth of that strain and the consequences of interaction and need not react aggressively i.e., in terms of photosynthate supply. At the same time, however, it suffers from N limitation because of reduced activity of the microsymbiont. In case of the unfamiliar strain, the plant sends down higher amounts of photosynthates to combat the danger. It will do this without knowing that the entire energy is being diverted to the invader. However, this way the invader gets engaged in its activities one of which is N_2 fixation. Thus in a way the plant unknowingly supports the bacterium and gets some benefit in terms of increased N supply and improvement in growth. This implies that N_2 fixation could possibly be enhanced by changing the microsymbiont i.e., an unfamiliar strain may result in increased N_2 fixation compared to the familiar one. It may also be possible to change the microsymbiont and use a consortium of microbes. The unfamiliar strain will probably lack appropriate enzymes to cause initial cell damage, and once this is facilitated somehow, further process of infection may not be a big problem.

Thus being less well-evolved compared to monocots, legumes have adopted a different and defensive than an aggressive strategy to contain the rhizobial enemy. In this way they get the benefit in terms of improved supplies of N. But even then it does not give the microbes complete freedom and builds a strong fort around the bacterium, thus banning its further spread into the interior (in fact, the cell wall chemistry of nodules will be very different compared to that of root hairs i.e., in terms of resistance to damage by the bacterium). Indeed this may become a so-called rigorous imprisonment for the bacterium that is made to work for the plant. That the plant was actually not willing to have the bacterium and thus captured it while tress passing is evident from the fact that the plant renders the invaders impotent, probably better and heavy-duty workers as "mules".

Concluding remarks: Legume-bacterium association has all along been considered as resulting from exchange of genetic information between the two partners. It is becoming increasingly apparent, however, this partnership although of a symbiotic nature, may not be so specific as generally considered. Rather, it will depend upon the ability of a particular bacterium to cause initial root damage and subsequently on the way the legume responds in terms of C supplies and cellular modifications at the site of interaction.

Success in sustained N_2 fixation by the bacterium will also depend on the microenvironment created by the legume including the measures taken to prevent interference by oxygen supply within the nodules. Breaking the resistance of legume to cell wall damage (by modified bacteria or using some chemicals like 2,4-D) may help find more efficient N_2 fixing bacteria and a wider range of host plants. So far, the failure to achieve this goal could mainly be the result of the enigma of so-called host specificity rather than its absolute existence.

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