OnLine Journal of Biological Sciences 1(12): 1134-1137, 2001
© Asian Network for Scientific Information 2001

Biodiversity and Performance of *Rhizobia* in Relation to Plasmid Genes and Their Translational Products

Gardezi, S.D. Ahmad
University College of Agriculture Rawalakot, Poonch, Azad Jammu and Kashmir, Pakistan

Abstract: The *Rhizobium* isolates collected from various ecological conditions of Azad Jammu and Kashmir were characterized using conventional and molecular techniques. The conventional techniques like the antibiotic resistance and *in vitro* and *in vivo* nodulation tests proved that there exists large diversity among the isolates. The isolates showed great potential for BNF under natural conditions, however, their potential could only be exploited if their genetic and biochemical markers are identified. The molecular techniques like plasmid profiles and the cellular protein profiles were investigated to identify some marker genes and their protein products for practical BNF and for the manipulation of the DNA based markers for better activity of the bacteria in plant nodules. The isolates differed in nodulation test also showed variability in their plasmid and protein profiles. The relationship between plasmid and protein profile depicts the presence of the variable genes on plasmids, which produce their influence in the form of protein products. These proteins may have their role against the environmental stresses in which the bacteria are adopted. The status of the genes once confirmed would be of great importance because plasmid are easy to handle, easy to manipulate and easy to transfer from one strain to another strain which may have otherwise desirability in BNF programme.

Key words: *Rhizobium,* plasmid profile, protein profile, nitrogen fixation

Introduction

The state of Azad Jammu and Kashmir extends from low subtropical plains to the south to high alpine slopes of 3,000 meters or more in elevation in the north. The most acute problem like low fertility, uneven distribution of rain, low temperature and water erosion hinder the farmers to improve their farm produce. Optimum use of industrially fixed nitrogen with physical means of soil conservation can help to increase the soil fertility and rehabilitation to some extent. However, the high cost of physical work, the fertilizers and other chemicals and their restricted availability poses serious and continuous threat for the farmers of this area. The use of synthetic chemicals like the nitrogen fertilizers even if made available will have some serious environmental reservation, when global awareness about the environment is increasing very rapidly. The use of synthetic chemicals is hazardous for the soil micro flora and fauna of great value. It has been reported that an hectare of soil contain about 1000 kg of earthworm, 1000 kg of arthropods, 150 kg of protozoan's, 150 kg of algae, 1700 kg of bacteria and 2700 kg of fungi, all of which are involved in recycling the nutrients needed by plants (Pimentel *et al*., 1995; Ahmad and Chaudhry, 1995).

The nitrogen cycle is obviously of considerable importance in the biosphere and the nitrogen fixation component demonstrates the tremendous potential for replacing artificial nitrogen fertilizers by biologically- fixed nitrogen, especially from the *Rhizobium*- legume symbiosis. The interaction between leguminous plants and bacteria of the genera *Rhizobium* and Bradyrhizobium is the single most important beneficial association between plants and bacteria in agriculture. As the two organisms behave differently under different environmental conditions, their behaviour under environmental stresses is not fully understood. Many investigations showed that the symbiotic effectiveness of the bacteria decreases with the increase of physical/environmental factors like soil pH, temperature and moisture (Saud, 1990; Ahmad and Hanif, 1994). It has been observed that strains of *Rhizobium* performing well in laboratory do not perform well in field conditions (Lindstrom and Myllyniemi, 1987).

By genetic evidence it has been found that the *Rhizobium* nodulate the legumes because of nod, sym and fix genes. All members of Rhizobaceae family harbour large plasmids (circular DNA pieces) with variable number and size. Representatives of nif, fix and nod genes have been located on the symbiotic plasmids of different *Rhizobium* species (Johnston *et al*., 1987).

The improvement to a strain's nitrogen fixation efficiency can be made by replacing its resident symbiotic (sym) plasmid with one, which is more effective. This is particularly attractive if the strain has useful characteristics other than those encoded by its sym plasmid. Chen *et al*. (1991) improved the symbiotic properties of an acid tolerant strain of *Rhizobium leguminosarum* bv. *trifolii* ANU 1173. The resulting strain gave a 17 % increase in nitrogen fixation under acid conditions compared with the original stains. The most encouraging attribute in this experiment was that the conjugated plasmid was maintained stably in the recipient strain. Skot *et al*. (1990) demonstrated to transfer toxin genes from *Bacillus thuringiensis* to *Rhizobium* species plasmids to control the pest attack and to maintain nodule productivity. Mutagenic techniques to *Rhizobium* itself or to its plasmids for better nitrogen fixation have been utilized (Paau, 1989). It is very difficult, most often to reconstitute the inoculated strains from the field, when indigenous strains are also prevalent in the field. Genetic markers especially if identified on plasmids could help significantly to investigate and identify the inoculated strains. These markers could be identified either by DNA analysis (plasmid profiles) or by the analysis of proteins (Protein profiles) using the techniques of electrophoresis. Before the identification of genetic markers the manipulation of *Rhizobial* strains will not help in the production of competitive strain(s) of the bacteria. The markers identified on either plasmid genes or gene products will also have greater significance in analyzing the biodiversity among the strains. The investigation was carried out: 1. To analyze genetically the local strains especially their plasmid profiles and their relationship with protein profiles in relation to their BNF efficiency. 2. To identify the plasmid based markers for later genetic manipulation and strain identification using molecular techniques.

Materials and Methods

The investigation was carried out at University College of Agriculture Rawalakot during the years 1993-1997. Nodules from white clover roots were collected from different representative ecological zones of Azad Jammu and Kashmir, cultured, authenticated and stored following the method given by Somasegaran (1985) and Jensen's (1942). The isolates were allotted 'UCR' number in series from 1-26. *In vitro* nodules formation was recorded just after initiation and then

its maturation. Photographs were taken for record and time taken to nodulate a single plant for individual isolate was also recorded. Intrinsic antibiotic resistance test were made as described earlier (Ahmad and Hanif, 1994). For field inoculation clover seeds were sown by hand in 10 plots, 5 each for white and red clover to test the efficiency of *Rhizobium* for nodulation and nitrogen fixation under field conditions. Once the seedlings started to emerge, each plot was sprayed with *Rhizobium* isolates (UCR 3, UCR 6, UCR 7, UCR 8 and UCR 12) performing better under laboratory conditions. The inoculum was prepared in liquid TY media using rotary shaker. One litre of a seven day old culture was applied to each plot in 20 litre of water using knap- sack sprayer.

Gel electrophoresis of DNA: Separating gel was prepared and used for plasmid profile about different strains of *Rhizobium* by electrophoresis techniques using similar procedure as described by Sambrook *et al.* (1989). Agarose gel concentration was 0.8% along with a continuous stripe for SDS- agarose just bellow the wells in order to lyse the bacterial cells within the gel. At the place of continuous stripe SDS- agarose was poured with few drops of bromophenol blue to monitor the movement of DNA. Freshly grown bacterial culture was used for *in situ* plasmid isolation. One ml culture taken in appendorf tube was centrifuged in microfuge at 13000 rpm for five minutes. The pellet was suspended in 30µl suspension buffer for electrophoresis. The samples were loaded into the gel slots and were electophoressed under submerged mode. The gel after electrophoresis was stained overnight using ethidium bromide. Photographs of the gel were taken after visualizing the stained gel on UV trains- illuminator using Polaroid DS- 34 direct screen instant camera.

Gel electrophoresis of proteins: The vertical gel apparatus was assembled in casting mode and a spacer was placed into it. Two combinations of the gel were prepared for separating and stacking gels. Separating gel was prepared by mixing 9.07 ml separating acrylamide stock solution, 4.24 ml separating gel buffer, 170 µl 10% SDS, Glycerol (99.9%) 1.70 ml and 1.75 ml st. distilled water in a flask by mixing over the magnetic stirrer. The solution was degassed for several minutes while mixing. 48 µl of ammonium per sulfate solution (10%) and 24 µl TEMED were added to the solution by gentle swirl to avoid the bubble formation. The mixture was carefully poured in to the casting tray to a level about 4 cm from the top using wide bore long needle attached with a syringe. The gel was allowed to polymerize for some time and was kept as such for several hours. The stacking gel was prepared by mixing 1.26 ml stacking acrylamide stock solution, 1.25 ml stacking gel buffer, 50 µl 10% SDS, Glycerol (99.9%) 0.50 ml and 1.91 ml dist. water in a flask. The mixture was stirred using magnetic stirrer and was deaerated to remove the air bubbles. 15 µl ammonium per sulfate (10 % solution) and 12 µl TEMED was added to the mixture.

Fresh cultures of the selected bacterial isolates were prepared and were mixed with equal volumes of 2 X treatment buffer in an appendorf tube. The tubes were kept in a boiling water bath for 90 seconds and were chilled on ice to lyse the cells. The wells in the gel were rinsed with distilled water, drained and the samples were loaded. Constant current of 60 mA was adjusted on power supply until the tracking dye moved to the bottom of the gel. After electrophoresis the gel was carefully removed from the assembly and was treated with the staining solution for 2-4 hours with gentle shaking. The gel was treated with destaining solution to remove the background. Photographs were taken for reference and the difference in banding pattern was compared either directly from the gel or from the photographs.

Results

Rhizobium isolates collected from different agro- climatic conditions of Azad Kashmir were cultured on growth medium, authenticated and investigated initially for preliminary information like growth performance on culture media without exposing them on different environmental stresses, like variable soil pH, temperature and moisture. As the isolates original habitat differed greatly in term of temperature, rainfall, acidity and height from the sea level, attempts were made to see the genetical differences among the isolates based on their original habitat. Preliminary investigations showed that there exists great genetical variation

Table 1: Field performance of *Rhizobium* isolates

Trifolium repens Isolate	Mean number of nodules per plant			
	Total	>2mm	1-2mm	>1mm
UCR8	20	3.3	7.9	8.8
UCR6	18	2.4	6.5	9.1
UCR12	15	1.8	5.2	8.0
UCR3	12	1.8	3.5	6.7
UCR7	11	0.7	3.6	6.5
Mean	15.2	2.0	5.34	7.82
Trifolium pratense				
UCR6	19	2.8	7.2	9.0
UCR3	18	2.6	7.0	8.4
UCR8	16	1.9	6.6	6.0
UCR7	13	1.8	4.2	7.0
UCR12	11	0.9	3.7	6.4
Mean	15.6	1.88	5.74	7.46

Figure based on 25 plants assessed in each 5 plots for both species of *Trifolium*.

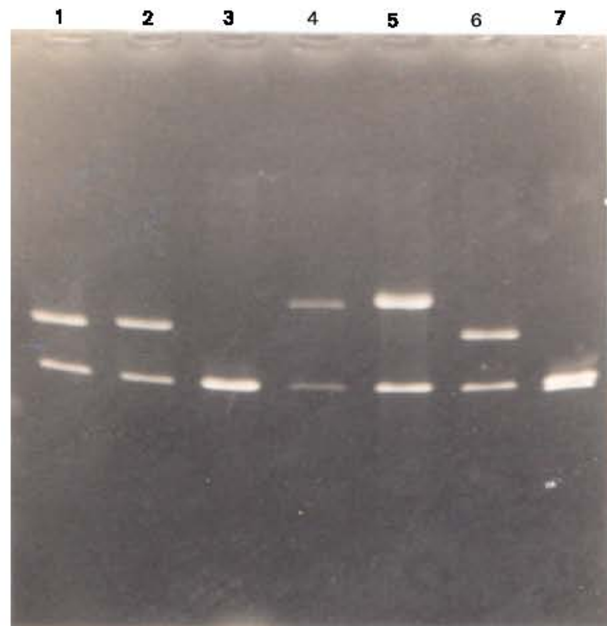

Fig. 1: Plasmid profiles of different isolates of *Rhizobia*. Lanes from 1-7 are UCR3, UCR6, UCR7, UCR8, UCR12, UCR13 and UCR18 respectively.

(biodiversity) among the strains (Ahmad and Hanif, 1994), however, it was not clear whether the variation is because of the chromosomal genes or of the criptic plasmids harboured by the bacteria.

In vitro nodulation tests were performed using the seedlings of

white clover (*Trifolium repens*) as well as red clover (*Trifolium pratense*). Young seedlings were placed on sterile Jensen's (1942) nitrogen free agar slants in test tubes or in conical flasks. Different isolates of *Rhizobium* were inoculated from freshly grown liquid (TY) culture. Most of the isolates initiated nodules after 6 weeks of inoculation but few isolates of *Rhizobium* initiated nodules at very early stage (4-5 weeks) after inoculation. The nodulation activity of UCR3 (collected from pH 6.00) was fast than any other strain followed by UCR12 UCR8 (Collected from pH values of 6.2 and 6.4 respectively). The nitrogen fixation efficiency of the isolates also differed as indicated by the volume of the nodules and their colour. Although the volume of nodules in all the plants inoculated with different isolates was under the range of 0.8 to 2 mm. But in some plants the average number of large sized nodule was higher compared to the others. The isolates forming nodules earlier were again in better position with larger number of nodules falling in the range of 1-2 mm in size. UCR3 was at first place followed by UCR8 and UCR12.

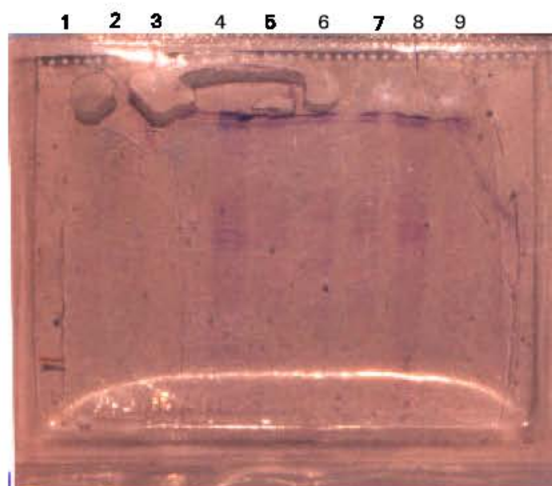

Fig. 2: Protein profiles of different isolates of *Rhizobium*. Lanes from 1-9 are UCR1, UCR2, UCR3, UCR4, UCR5, UCR6, UCR7, UCR8, UCR12, UCR13 and UCR18 respectively.

When same isolates were inoculated to the seedlings under field conditions the behaviour of *Rhizobium* isolates was different. It is to be noted that the field experiments were randomized and all the factors including sowing date, tillage practices, the inoculation date and time and the apparent soil conditions were kept constant for all the isolates under investigation. The promising strains giving better performance under laboratory conditions were included for the field experiments and comparisons were made for both species of *Trifolium* (white and red clover). Field data for nodule formation and size of the nodules for five best performing isolates is given in Table 1.

The isolates performing very well under laboratory conditions did not produce similar results under field conditions. UCR3 isolate showed very good performance under laboratory conditions but its field performance was not as good. UCR6 stands at fifth position under laboratory conditions but under field conditions it took second position after UCR8 which took first position at least in one experiment.

Plasmid profiles of the isolates: The plasmid profiles for different isolates of *Rhizobium* were investigated using agarose gel electrophoresis. The plasmid profiles for 26 different isolates from Azad Kashmir were almost similar, producing similar pattern of fractionation in agarose gel electrophoresis. In Fig. 1 the plasmid profile for 7 best performing isolates was compared. The profile was similar in UCR3, UCR6 and UCR13, whereas UCR8 and UCR12 were having similarity in their profiles. The two isolates UCR7 and UCR18 were altogether different in profiles showing one and two plasmids of about the same size respectively. Attempt was made to see the total cellular protein profiles of the isolates to see whether that can give some clear picture about the individual isolate and the information can be utilized for isolate identification.

Protein profiles of the isolates: Protein profiles of bacterial cells from different isolates of *Rhizobium* were analyzed using vertical slab -acrylamide gel electrophoresis. Protein profiles for 9 different isolates are shown in Fig. 2. From the figure it is clear that almost all isolates produced different protein profile as against the plasmid profiles, which were similar in many isolates. It indicates that the significance of the technique to differentiate the isolates for their performance and identification of the strain under field conditions could not be ignored. The protein bands in UCR1, UCR2 are not very much conspicuous because of their low intensity, but a single band of variable size was observed in the gel in a close view (Fig. 2). The isolates UCR3, UCR6, UCR8, UCR13 and UCR18 produced two clear bands, which again were of variable molecular weight. The two bands seen in UCR3, UCR6 and UCR13 seems to be of similar molecular weight but due to non availability of the molecular weight marker their similarity could not be ascertained. UCR8 was altogether different as it showed a band of very high molecular weight and another band of very low molecular weight. The single band seen in the lanes of UCR7 and UCR12 was at least comparable with one band of UCR3, UCR6 and UCR13. It is very much interesting to note that the protein profiles and the plasmid profiles of UCR3, UCR6 and UCR13 were similar. However, the protein profiles of UCR8 and UCR12 were different in contrast to their plasmid profiles as seen in agarose gel electrophoresis. The results about UCR7 and UCR18 were also different when compared with their plasmid profiles. The isolates UCR3 and UCR6 showed best performance for nodulation and nitrogen fixation trials both under laboratory and under field conditions and also showed some resemblance in their plasmid and protein profiles. The protein profile produced in the lane of UCR8 was also very much interesting because of its total variability from the other lanes. It indicates that the protein profile for UCR8 can very effectively be used for its identification and reconstitution from the field

Discussion

The *Rhizobium* isolates were collected from different ecological zones of Azad Jammu and Kashmir, which differ greatly in term of soil properties, daily temperature, height from the sea level and rainfall. Primary investigation was carried out to note whether any genetical diversity exists among the isolates and to what extent. All strains were compared using similar conditions to see their growth pattern on agar plates, broth culture and their response to different antibiotics. The results demonstrated that the isolates differ in their response to culture and their ability to grow against the stresses of the environment. These results were in conformity to the previous results, where *Rhizobium* response to environmental conditions has been found to be variable (Richerdson and Simpson, 1989; Saud, 1990).

When the nodulating efficiency of *Rhizobium* isolates was checked under laboratory and field conditions using white and red clover seedlings, the isolates showed variable performance. The application of inoculum by injection under

laboratory conditions and by spraying under field conditions provided an extra measure to ensure that *Rhizobium* population of the introduced isolates were efficiently high to nodulate the legumes. It was under laboratory conditions, as plants were raised on nitrogen free Jensens's agar medium and there was no chance of contamination of other *Rhizobium* or any bacteria. Whereas under field conditions one could speculate that the nodules appeared on clover plants were may be due to the presence of the indigenous strains of the *Rhizobium*. Generally the inoculum produced is either applied on to the seeds by mixing a slurry or it is mixed with the seeds at the time of sowing. However, the application of inoculum by spraying at least under experimental conditions has been found to be very much successful (Jones and Lewis, 1993). The success of the method has been proved by using the inoculum in sterile soils used in pot cultivation (Saud, 1990). Rogers *et al*., (1982) used a spray inoculant technique as a post-emergence remedial treatment when nodulation and establishment was deficient. It was observed that some isolates that did not show excellent performance under laboratory conditions were quite good under field conditions. UCR6 and UCR13 although perform well under laboratory conditions were after UCR3, UCR8 and UCR12 but attained second and third position under field conditions. Such controversies in the performance of *Rhizobium* have already been observed in many studies (Lindstrom and Myllyniem, 1987). Although it is certain that the nodules produced in *Trifolium* species under field tests at least partly were by the inoculated isolates, their actual performance could not be determined unless and until some markers are identified and utilized for such observations.

It is well known fact that most of the genes related to the performance of *Rhizobium* under different environmental stresses i.e., soil pH, soil temperature, soil moisture and antibiotic resistance are located on the plasmids (Nuti *et al*., 1979; Johnston *et al*., 1987; Saud, 1990; Chen *et al*., 1991). Improvement to a strain's nitrogen fixation efficiency can be made by replacing its resident symbiotic (sym) plasmid with one, which is more effective. This is particularly attractive if the strain has useful characteristics other than those encoded by its sym plasmid. Chen *et al*. (1991) improved the symbiotic properties of an acid tolerant strain of *Rhizobium leguminosarum* bv. *Trifolii* ANU ii73. Mutagenic techniques to *Rhizobium* itself or to its plasmids for better nitrogen fixation have also been utilized (Paau, 1989).

The results indicated that, although the isolates of *Rhizobium* differ in their performance under laboratory as well as field conditions, but the plasmid profiles were not altogether different. However, when the plasmid profiles for 7 best performing isolates were compared small differences were found in the number of plasmids and their size. These differences may help in identifying the isolates as a marker and also to see their varying ability to fix nitrogen under ecological stresses. The little differences found in the plasmid profiles can be strengthened if similar differences could be identified in protein profiles. The relationship of plasmids variability with the similar variability in proteins will provide accurate measure of the competitive ability of the *Rhizobium* under investigation. The genes present on the plasmid may be expressing certain proteins which make the strain better adapted against the natural stresses in the soil.

The protein profiles were almost different among the strains as against the plasmid profile, where similarity was greatly prevailing. It is very much important to know that the highly competitive strains identified during the investigation showed remarkable relationship of the protein profiles with the plasmid profiles. The plasmid profile for UCR8 and UCR12 were similar but the protein profile for UCR8 was distinct and may prove its worth in its identification and reconstitution from the field. The plasmid profiles of UCR3, UCR6 and UCR13 were similar and these isolates were highly competitive with very little difference among themselves. Likewise their protein profiles were also similar at least in the number of protein bands and their molecular weight. The results in this respect are highly commendable. Because it would confirm that the plasmid present in the cells of bacteria are very much important, as they express particular proteins, which may have an important role in their competitive ability to fix nitrogen under stresses of the environment.

Acknowledgment

This work was conducted under the Pakistan Science Foundation research grant No. PSF/Res./AJK/UCR/ Agr (126).

References

Ahmad, S. D. and M. B. Chaudhry, 1995. Development of grass-legume mixture-a potential source of sustainable development of agriculture in Hilly areas of Pakistan. Sci. Technol. Dev., 14: 15-22.

Ahmad, S. D. and T. Hanif, 1994. Biological nitrogen fixation (BNF) and the use of *Rhizobium* under low temperature and low pH in Azad Kashmir. Proceedings of the International Symposium on Biotechnology for Sustainable Development. Dec. 26-31, 1993, NIBGE Faisalabad, Pakistan

Chen, H., A. E. Richerdson and E. Gartner, 1991. Construction of an acid tolerent *Rhizobium leguminosarum* bv. *trifolii* strains with enhanced capacity for nitrogen fixation. Appl. Environ. Microbiol., 57: 2005-11.

Jensen, H. L., 1942. Nitrogen fixation in leguminous plants-1. General characters of root- nodule bacteria isolated from species of Medicago and Trifolium in Australis. Proceedings of the Linnean Society New South Wales, pp: 98-108.

Johnston, A. W. B., J. A. Dowinie, L. Rossen, C. A. Shearman, J. L. Firman, Borthakur, E. A. Wood, D. bradley and N. J. Brewin, 1987. Molecular analysis of the *Rhizobium* genes involved in the induction of nitrogen-fixing nodules on legumes. Phil. Trans. R. Soc. Lond., B3 17: 1993-207.

Jones, D. G. and M. Lewis, 1993. *Rhizobium* inoculation of crop plants. In: Exploitation of Microorganisms (Ed. D. G. Jones). Chapman and Hall, London.

Lindstrom, K. and H. Myllyniemi, 1987. Sensitivity of red clover rhizobia to soil acidity factors in pure culture and symbiosis. Plant and Soil, 98: 353-362.

Nuti, M. P., A. A. Lepidi, R. K. Prakash, R. A. Schilperoot and F. C. Cannon, 1979. Evidence for nitrogen fixation (nif) genes on indigenous *Rhizobium* plasmids. Nature, Lond., 282: 533-535.

Paau, A. S., 1989. Improvement of *Rhizobium* inoculants. Appl. Environ. Microbiol., 55: 862-865.

Pimentel, D., C. Harvey, P. Resosudarmo, K. Sinclair, D. Kurz, M. McNair, S. Crist, L. Shpritz, L. Fitton, R. Saffouri and R. Blair, 1995. Environmental and economic costs of soil erosion and conservation benefits. Science, 267: 1117-1123.

Richardson, A. E. and R. J. Simpson, 1988. Enumeration and distribution of *Rhizobium trifolii* under a subterranean clover-based pasture growing in an acid soil. Soil Biol. Biochem., 20: 431-438.

Rogers, D. D., R. D. Warrens and D. S. Chamblee, 1982. Remedial post emergence legume inoculation with *Rhizobium*. Agron. J., 74: 613-619.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989. Molecular cloning. A laboratory Manual (Second Ed.) Cold Spring Harbor Laboratory Press, USA.

Saud, H. M., 1990. Ph.D. Thesis, University of Wales, Aberystwyth, UK.

Somasegaran, P. and H. J. Hoben, 1985. In: Methods in Legume-Rhizobium Methodology. NifTAL, Hawaii, USA.

Skot, L., S. P. Harrison and A. Nath, 1990. Expression of insecticidal activity in *Rhizobium* containing the endotoxin gene cloned from *Bacillus thuringiensis* subsp. tenebrionis. Plant and Soil, 127: 285-295.