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# Preliminary Characterization of Root Nodule Bacteria from *Vigna aconitifolia*

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**Abstract:** Indigenous rhizobia nodulating *Vigna aconitifolia* from three agroclimatically different zones of semi-arid region of Rajasthan state of India were investigated. The rhizobia consisting of 146 isolates were characterized on the basis of intrinsic resistance to different antibiotics, UV and azide and colony properties. Fifty three per cent of the isolates produced waxy mucilage. The isolates representing most arid region were both antibiotic and UV sensitive whereas those from least arid zone were relatively tolerant to both of these. Of these rhizobia, two isolates which showed pink and big nodules on the host plants, MR125s2 was not only antibiotic and UV sensitive but also susceptible to phage. These two isolates also produced waxy mucilage. It is inferred that waxy mucilage and susceptibility to both biotic and abiotic factors may confer the bacteria an increased adaptedness to both plant and soil.

**Key words:** Intrinsic antibiotic resistance, legume, rhizobia, symbiotic $N_2$ fixation, *Vigna aconitifolia.*

Soil borne symbiotic bacteria, belonging to the genera *Rhizobium* and *Bradyrhizobium*, form dinitrogen fixing nodules on the legume roots. These bacteria may exist in (i) bulk soil, (ii) rhizospheric soil and (iii) host nodule tissue (Bauer and Caetano-Anolles, 1990). The last mode of existence is important, being responsible for generation of new variants resulting from spontaneous mutation and establishment of a base line population of native rhizobia for nodulation of crop in season. The nitrogen fixation by native rhizobial nodulation is inefficient in common bean, berseem clover and lentil (Moawad *et al.*, 1998; Moawad and Bohlool, 1992). For realization of maximum benefits of the symbioses, an effective strain of inoculant *Rhizobium* must be able to compete with native rhizobia for nodule occupancy (Das, 1991). Brockwell *et al.* (1982) have listed 10 attributes which an inoculant strain should possess. While such an ideal strain may not be available in nature, it is, nevertheless, feasible to improve a strain in a stepwise manner. The present study is concerned with the possible selection of a strain that is well adapted to the environment and plant, from the native rhizobia that nodulates moth bean (*Vigna aconitifolia*).

## Materials and Methods

To isolate rhizobia of *Vigna aconitifolia*, nodules were collected from moth bean fields from three districts Churu, Bikaner and Jobner where moth bean is cultivated. Nodules were collected from sites where there was no previous history of inoculation and preserved under fused $CaCl_2$ tubes. For recovery of the bacteria, desiccated nodules were imbibed in sterile distilled

water for 6 h and then surface sterilized by immersing in 0.2% $HgCl_2$ for 3 min, then in 70% alcohol for 15 s, followed by washing through several changes of sterile distilled water. The exudate of each nodule was streaked on Yeast Extract Mannitol (YEM) agar plates containing 0.02% Congo red stain. The plates were incubated at 30±2°C for 3 to 7 or 10 to 12 days in a growth chamber. One non-stained colony further purified through 3 passages of single colony isolation was maintained on YEM agar slants in respect of each nodule plate (Vincent, 1970). Two hundred fifty isolates, thus developed, were tested for nodulation on the host plants as described by Vincent (1970). Seeds of *Vigna aconitifolia* (cv. 'Jwala') were surface sterilized by suspending them in 0.2% $HgCl_2$ for 4 min and then in 70% alcohol for 20 s followed by washing through several changes of sterile distilled water. Seeds were then incubated for germination in the Petriplate containing sterile wet filter paper. After germination the seed coat was gently removed and the seedlings transferred on agar slants (in 200 x 25 mm glass tubes) prepared in Arnon's medium (Arnon, 1938). The seedlings were incubated in light for 15 to 20 days. An isolate capable of inducing root nodules was authenticated as *Rhizobium*.

The sensitivity of the rhizobia to different physical and chemical agents was determined. The physical agents included pH (5 to 10 with increments of 0.5), temperature (28°C, 35°C and 42°C) and ultraviolet (UV) exposure of 10, 20, 30 and 40 s at a distance of 55 cm from the source (30 W germicidal lamp Phillips, Holland, principally emitting at 256 nm). Similarly chemical treatments included polyethylene glycol (1, 2, 3, 4 and 5%), NaCl (4 to 10% with increments of 1%), sodium azide (0.05 mM to 0.40 mM with increments of 0.05 mM) and antibiotics tetracyclin (Tc)– 1, 2, 3, 5 and 10 µg/ml; streptomycin (Sm)– 10, 15, 20 and 30 µg/ml; neomycin (Neo)– 15, 20, 25, 30 and 40 µg/ml; ampicillin (Amp)– 20, 30, 40 and 50 µg/ml ; trimethoprime (Trim)– 10, 20, 30, 40 and 50 µg/ml; chloramphenicol (Cam)– 10, 20, 30, 40 and 50 µg/ml and erythromycin (Ery)– 30, 40, 50, 60 and 100 µg/ml. A multi-inoculator carrying 49 inoculations was used and inoculated on 2% YEM agar plates containing the chemical wherever required for screening. The index score in respect of an isolate was developed as follows: An isolate was assigned the weighing of '1' when resistant and '0' (zero) when susceptible to an agent. The weighings gained by an isolate in respect of various agents was summed up and termed as "index score". Results of agents like NaCl, temperature, pH and PEG were not used because isolates did not show any variation among each other. In this way index score varied from zero to eight for an individual isolate. The dose of an agent at which nearly 50% isolates were either resistant or susceptible was chosen for the purpose of developing index (Sm: 10 µg/ml, Tc: 5 µg/ml, Neo:20 µg/ml, Amp: 20 µg/ml, Trim:10 µg/ml, Cam: 20 µg/ml, Ery: 50 µg/ml and azide: 0.10 mM). A scatter diagram was developed by plotting index score against the UV sensitivity of 146 individual isolates

on the host plants as described by Vincent (1970). Seeds of *Vigna aconitifolia* (cv.

'Jwala') were surface sterilized by suspending them in 0.2% $HgCl_2$ for 4 min and then in 70% alcohol for 20 s followed by washing through several changes of sterile distilled water. Seeds were then incubated for germination in the Petriplate containing sterile wet filter paper. After germination the seed coat was gently removed and the seedlings transferred on agar slants (in 200 x 25 mm glass tubes) prepared in Arnon's medium (Arnon, 1938). The seedlings were incubated in light for 15 to 20 days. An isolate capable of inducing root nodules was authenticated as *Rhizobium.*

For the detection of phages infectious to the bacterial isolates, soil samples were collected from around the harvested crop. The soil lysates were prepared for phage plaque detection as described by Dhar and Ramkrishna (1987). The existence of phage in non-enriched soil sample against a bacterial host was taken to mean that the host genotype or related genotypes are of frequent occurrence in that habitat.

## Results

The nodulation test under controlled conditions revealed that only 146 isolates, out of 250 tested, induced root nodules. The colony characteristics of these nodulating cultures were studied. On the basis of mucilage, these isolates could be distinctly placed in 3 groups (Table 1). The waxy mucilage producing colonies were most frequent (53%). Very small (less than 1 mm) compact colonies were also represented (about 20%) and visually detectable only between 10-12 days of incubation (these may be tentatively considered as bradyrhizobia, although it was not confirmed through any biochemical test).

These isolates were screened for tolerance to different stress agents. There was a marked variation among the isolates when screened against different antibiotics, UV irradiation and azide. Based on resistance shown by isolates towards azide, ampicillin, streptomycin, tetracyclin and UV irradiation the rhizobia could be placed in 28 groups. Although using 5 different stress agents, theoretically 32 groupings are possible. The observed grouping is quite close to this figure indicating heterogeneity of the rhizobia studied.

The resistance pattern of the isolates was expressed as index scores using results obtained with seven antibiotics and azide. Their scattering on the basis of UV resistance revealed that Jobner isolates were generally more tolerant to UV and antibiotics, whereas, Bikaner isolates were frequently sensitive to both UV and antibiotics. On the other hand, isolates of Churu had both higher and lower tolerance to these stress agents.

During nodulation test with $MR125s_2$ and MR110, nodule formation could be detected on $6^{th}$ day after inoculation. With both of these isolates the nodules were maximum (~20), pink and big. Both of these isolates belonged to Churu district and formed waxy mucilage. Isolate $MR125s_2$ was sensitive to both UV rays and antibiotics, whereas isolate MR110 was moderately resistant (index score 5) to both UV and antibiotics.

These two isolates were examined for their susceptibility to the bacteriophages of the soil. In case of $MR125s_2$ even

*Table 1. Origin and characteristics of 146 rhizobial isolates of Vigna aconitifolia*

| | Locations | | | |
|---|---|---|---|---|
| | Jobner | Churu | Bikaner | |
| Soil type | Alluvial soil with a texture of sandy loam | Shallow soil having stony surface, with saline and/or sodic patches | Desert soil with a texture varying from loamy sand to coarse sand | |
| Rainfall | 500 to 600 | 300 to 500 | 100 to 300 | |
| No. of cultures taken | 62 | 64 | 20 | |
| Colony morphology | | | | |
| (1) Producing watery mucilage, colonies visible 4 days after incubation | | | | |
| (a) Big (8 to 10 mm) | 11 | 12 | – | (23)[a] |
| (b) Small (4 to 6 mm) | 6 | 9 | 4 | (19) |
| (2) Producing waxy mucilage, colonies visible 6 days after incubation | | | | |
| (a) Big (4 to 6 mm) | 8 | 9 | 9 | (26) |
| (b) Small (2 to 4 mm) | 34 | 11[b] | 7 | (52) |
| (3) Non-mucilage, small/compact colonies (less than 1 mm), visible 10-12 days after incubation | 3 | 23 | – | (26) |

a = Figures in parenthesis indicate the total number of isolates under this category.
b = The isolates selected as most adapted belong to this group.

non-enriched soil lysates of Churu and Jobner revealed plaques, whereas on MR110 several attempts of plating, with enriched soil lysates did not reveal any plaque (Table 2).

## Discussion

The rhizobia of moth bean (*V. aconitifolia*) were investigated with a view to characterize their adaptation to both plant (nodulation) and environment and identify an isolate which can become a base for further genetic improvement (Chakraborty *et al.*, 2003). The rhizobia were collected from areas with no previous history of inoculation, and hence, considered as indigenous to their respective geographies. Further, since moth bean is traditionally cultivated in these areas, we expected an adequate heterogeneity among the rhizobial isolates. Results of intrinsic resistance to antibiotics, azide and UV revealed that the rhizobia were heterogeneous. In a number of studies the pattern of intrinsic antibiotic resistance have been effectively used in chacterizing the diversity of native rhizobia (Brockman and Bezdicek, 1989; Josey *et al.*, 1979; Kremer and Peterson, 1982; Moawad *et al.*, 1998; Teaumroong and Boonkerd, 1998; Vasquez-Arroyo, 1998).

*Table 2. Detection of phage plaques on two effective isolates of moth bean Rhizobium*

| Place | Soil sample | PFU/g dry soil | | | |
|---|---|---|---|---|---|
| | | Isolate MR125s2 | | Isolate MR110 | |
| | | Without enrichment | With enrichment | Without enrichment | With enrichment |
| Churu | I | 5 | 375 | – | – |
| | II | 10 | 340 | – | – |
| | III | 6 | 345 | – | – |
| | IV | 3 | 315 | – | – |
| | V | 2 | 316 | – | – |
| Jobner | I | 2 | 320 | – | – |
| | II | – | 188 | – | – |
| | III | – | 175 | – | – |
| | IV | 3 | 315 | – | – |
| | V | 7 | 388 | – | – |

The rhizobia also showed considerable degree of location specificity. The strains isolated from Bikaner zone were generally sensitive to antibiotics. Since this area is relatively arid, the present findings contradict the earlier report of Odee *et al.* (1997) that arid soils favor the growth of antibiotic producing organisms, which, in turn, impose a selection pressure on evolution of antibiotic resistant types of other microflora of the soil. Our results indicate absence of such a selection pressure at these extreme arid environments. The isolates of Churu, however, ranged from relatively most sensitive to most resistant. Climatically also, Churu lies between Jobner and Bikaner (Table 1). The rhizobia from Jobner were generally resistant to antibiotics. These facts may tempt one to speculate that the moisture content of the soil may contribute to the evolution of resistant genotypes of the rhizobia through affecting the antibiotic producing organisms, particularly in arid and semi arid regions.

The UV tolerance of the isolates had a similar trend to that observed for antibiotics. The present results may not explain the location specific variation in UV tolerance among the isolates. Since, UV irradiation causes dimmer formation between adjacent cytosines or thymidines, the relative sensitivity to UV may be attributed to relative abundence of adjacent pyrimidines in the bacterial genome (Freifelder, 1990). Alternatively, an efficient genetic repair system may be responsible for observed tolerance.

On the basis of colony characteristics majority (53%) of the isolates were found to produce waxy mucilage. Possibly waxy mucilage is associated with better survival in the arid soils.

The results of nodulation when combined with index scores led to interesting conclusions. Initially it was thought that isolates with higher tolerance (higher index scores) to antibiotics and UV could have better representation in the rhizobial

collection, but two of the isolates, MR125s2 and MR110 which developed maximum pink and big nodules were found to belong to most sensitive groups, i.e., with index score of '0' and '5', respectively, indicating their adaptation to the plant. The frequent occurrence of MR125s2 was substantiated with demonstration of phage plaques. Both Jobner and Churu soils showed the plaques without enrichment indicating the presence of related bacterial host genotypes.

From these results it may be inferred that the possibility of selecting a rhizobial strain adapted to both rhizospheric environment and plant may be based on extreme sensitivity to antagonistic soil factors of both biotic and abiotic nature and waxy mucilage production.

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