

## Symbiotic Characteristics of Azide-resistant Mutants of Moth Bean (*Vigna aconitifolia*) *Rhizobium*

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**Abstract:** Spontaneously arising azide-resistant mutants were isolated from a hypermotile mutant strain of *Rhizobium* MR 125  $s_2$ - $sm^r$  8- $hyp5$  that nodulates moth bean (*Vigna aconitifolia* Jacq. Marechal). The mutants generally produced more nodules (30% more) than the parent. One of the mutants MR 125  $s_2$ - $sm^r$  8- $hyp5$ - $az^r$ 57 produced 3-fold more nodules than parent. Eleven mutants accumulated significantly more nitrogen than the parent in the plants grown in nitrogen-free Arnon's nutrient medium. Results indicate that incorporation of azide resistance frequently leads to improved effectiveness.

**Key words:** *Rhizobium*, azide resistance, symbiotic effectiveness,  $N_2$  fixation.

Rhizobia belonging to the genera *Rhizobium* and *Bradyrhizobium*, induce formation of  $N_2$  fixing root nodules. In many instances the rhizobial strains are known to be less effective in  $N_2$  fixation. Inoculation of seeds with effective strain of *Rhizobium* has, therefore, been practiced to harvest maximum nitrogen of biological origin. There are few indirect methods for isolation of more effective strains (Ishizuka, 1992). One of the methods is incorporation of spontaneous resistance to azide, which is frequently associated with increased effectiveness (Ram *et al.*, 1978; Vashisht *et al.*, 1986; Pancholy, 1996; Sharma *et al.*, 1997). However, the utilization of azide resistance as a generalized technique for improving symbiotic effectiveness awaits more species of rhizobia examined and elucidation at molecular level of the mechanism of resistance. The present study is concerned with symbiotic evaluation of 40 azide-resistant mutants of moth bean *Rhizobium*.

### Materials and Methods

The bacterial isolate used in the present investigation as parent is a hypermotile mutant derivative of moth bean *Rhizobium* strain MR 125  $s_2$ . This mutant, designated MR 125  $s_2$ - $sm^r$  8- $hyp5$ , has resistance to streptomycin @ 500  $\mu\text{g ml}^{-1}$  and forms a larger swarm than either MR 125  $s_2$  or its mutant derivative MR 125  $s_2$ - $sm^r$  8. While resistance to streptomycin was a spontaneous selection, the hypermotility was induced through UV mutagenesis and both the mutations were tested for their stability (Chakraborty, 1999).

Spontaneously arising azide-resistant mutants were isolated by enrichment of bacterial cells surviving the exposure of different doses of azide. A late exponential phase culture (0.2 ml) of isolate MR 125  $s_2$ - $sm^r$  8- $hyp5$  was inoculated to YM broth tube containing graded concentration of sodium azide, ranging from 0.05 mM to 1.0 mM. After 15 days of inoculation,

regrowth was observed in tubes upto 0.2 mM of sodium azide. From regrown culture clonal isolates were made and subjected to 3 passages of sub-culturing without azide and then tested for growth in the presence of azide (0.1 mM) and streptomycin (@ 200  $\mu\text{g ml}^{-1}$ ). The mutant cultures showing dual resistance were maintained on agar slants for further study.

For aseptic culture of plants, glass beakers of 400 ml capacity were used. Coarse pure sand was taken and filled upto 75% of the height of the beakers and sterilized in an oven at 200°C for 6 h. Healthy seeds of moth bean (cv. Jwala) were surface sterilized by suspending the seeds in 0.2%  $\text{HgCl}_2$  for 4 minutes and then in 70% alcohol for 15 s, followed by a washing through several changes of sterile distilled water. Washed seeds were sown 1 cm deep in beakers containing sand. Three plants were maintained in each beaker in a cage fitted with fluorescent tube lights emitting 5000 lux at a distance of 12 cm from the surface. Rhizobial inoculations were performed on 3<sup>rd</sup> day of seedling emergence. Five ml of the late exponential phase bacterial culture, having attained 0.8 to 1.0 OD was carried through 20 ml of N-free sterile Arnons' medium (1938) into each pot. The cell titer in the inoculums was made approximately uniform by making protein content equal, because if the number of cells in the different culture samples were equal their protein content determined colorimetrically would be equal, provided the samples were centrifuge-washed to remove exopolysaccharide and mucilage. The cell number in the aliquot was estimated to be  $5 \times 10^8$  CFU  $\text{ml}^{-1}$ . The pots were irrigated with N-free Arnons' medium as

and when required. The temperature within the cage was  $30 \pm 2^\circ\text{C}$ . The plants were harvested 40 days after inoculation and total N content was determined by micro-Kjeldahl method (Peach and Tracey, 1956). The symbiotic effectiveness in respect of an inoculation was measured as increase in nitrogen content over the uninoculated control. However, for comparing the superiority or inferiority of a mutant strain in respect of its symbiotic effectiveness, its N content was statistically compared with that of the parents' N content, i.e., MR 125  $s_2\text{-sm}^r$  8-*hyp5*. Statistical analysis was done as per CRD. The variances were analyzed for between and within inoculations.

## Results and Discussion

The azide-resistant mutants generally produced higher number of nodules than the parent (Table 1). Inoculations with 15 mutants resulted in significant increase in nodules/plant whereas 9 other mutants showed significantly lower number of nodules/plant as compared to the parent MR 125  $s_2\text{-sm}^r$  8-*hyp5*. The majority of the nodules were, however, small in size (~1-2 mm). The mutant MR 125  $s_2\text{-sm}^r$  8-*hyp5-az*<sup>r</sup>57 produced approximately three-fold more (123.66) nodules than the parent (44.66). A similar increase in nodule number, induced by azide-resistant mutants of *R. meliloti*, was reported by Sharma *et al.* (1997). It may be plausible to expect these results because there is evidence that genes conferring azide resistance and nodulation are borne on a DNA segment of 11.5 kb on a symplasmid of *R. leguminosarum* (Singh and Kumar, 1989) and hence linked to each other.

Table 1. Symbiotic characteristics of different azide-resistant mutants of moth bean *Rhizobium* isolate MR on *Vigna aconitifolia* cv. Jwala (measurements indicate mean of three plants)

Isolate number	Nodule number per plant	Nodule fresh weight per plant (mg)	Nodule dry weight per plant (mg)	Plant fresh weight (g)	Plant dry weight (mg)	N-content per plant (mg)
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5	44.6	38.39	7.19	1.153	143.0	10.73
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 31	61.6	36.90	6.00	1.585	206.33	11.53
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 32	75.6	42.89	7.85	1.078	127.33	8.02
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 33	61.0	35.82	7.05	1.465	172.00	8.72
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 35	26.3	20.88	4.17	0.980	117.33	8.21
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 38	46.0	33.67	5.76	1.443	168.66	11.93
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 40	56.6	33.27	5.93	2.204	267.00	13.37
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 42	97.3	45.24	8.88	2.367	252.33	12.74
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 44	35.3	32.71	6.56	1.204	160.00	8.68
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 46	77.6	45.28	8.88	1.198	145.33	9.82
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 48	76.3	54.70	10.78	2.239	297.33	14.63
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 50	62.0	36.62	7.28	1.356	158.66	9.75
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 53	85.3	48.62	9.48	1.659	199.33	11.60
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 55	35.6	29.39	5.68	1.381	167.33	11.48
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 57	123.6	53.80	10.77	1.165	210.00	13.82
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 59	79.3	40.19	7.86	1.487	167.00	11.55
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 61	50.6	40.35	7.43	1.187	150.66	10.69
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 63	46.3	33.01	5.82	0.967	104.00	9.28
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 64	75.6	42.93	8.26	1.722	202.66	14.21
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 66	43.0	34.74	6.00	1.017	102.00	8.07
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 67	72.6	42.26	7.63	1.366	149.00	8.77
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 68	26.3	24.64	5.27	1.191	120.33	7.26
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 69	97.6	46.08	8.94	1.583	193.33	11.62
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 70	45.0	40.98	8.01	1.513	204.00	11.52
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 71	48.0	44.45	8.69	1.938	217.00	15.71
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 73	58.3	38.64	6.95	1.415	172.00	13.73
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 75	66.0	40.97	7.78	1.462	187.33	9.83
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 76	55.6	35.58	6.73	1.185	160.66	9.73
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 79	99.0	47.47	9.21	1.879	218.00	13.48
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 80	32.3	27.68	5.57	1.410	167.60	8.69
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 81	29.0	24.05	4.66	1.066	108.33	8.90
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>F</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>F</sup> 82	34.3	27.75	5.75	1.277	122.66	11.00

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Table 1. *Contd.....*

Isolate number	Nodule number per plant	Nodule fresh weight per plant (mg)	Nodule dry weight per plant (mg)	Plant fresh weight (g)	Plant dry weight (mg)	N-content per plant (mg)
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 82	34.3	27.75	5.75	1.277	122.66	11.00
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 84	56.6	36.23	6.41	1.319	131.33	9.83
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 85	81.3	46.61	9.21	2.282	254.00	12.53
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 86	76.0	43.14	8.73	1.943	207.33	10.75
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 88	51.6	39.39	7.30	1.215	151.66	9.76
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 89	54.6	40.69	7.75	1.528	166.00	13.64
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 90	39.3	31.57	6.10	1.083	125.66	7.75
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 93	74.6	41.34	8.25	1.416	142.33	10.63
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 94	25.6	21.06	4.14	1.077	112.00	9.01
MR 125 <i>s</i> <sub>2</sub> - <i>sm</i> <sup>r</sup> 8- <i>hyp</i> 5- <i>az</i> <sup>r</sup> 95	76.0	43.72	9.17	1.585	201.33	13.95
Mean	60.0	38.14	7.31	1.453	171.47	10.93
CD at 5%	12.03	4.585	1.033	0.295	31.496	1.063

Mutants serialized from 2 to 16, 17 to 31 and 32 to 41 were isolated from 0.2, 0.15 and 0.10 mM

The dry matter of plants revealed that in 15 cases of inoculations the dry mass was significantly greater than the parent MR 125 *s*<sub>2</sub>-*sm*<sup>r</sup> 8-*hyp*5. Inoculations of three mutants resulted in significantly lower dry mass of plant. On the basis of N content of plant, eleven mutants accumulated significantly more nitrogen than the parent MR 125 *s*<sub>2</sub>-*sm*<sup>r</sup> 8-*hyp*5. The mutant MR 125 *s*<sub>2</sub>-*sm*<sup>r</sup> 8-*hyp*5-*az*<sup>r</sup>71 was one and half times more effective (15.00 mg N) than the parent (10.73 mg N). However, with the inoculation of 10 other mutants the N accumulation was significantly lower. These results indicated that the azide-resistant mutants were generally more effective, although inferior mutants in terms of nodule numbers and N content per plant do also result. A comparison of mutants with respect to the level of azide resistance used for their isolation revealed that both

superior and inferior mutants in terms of nodules per plant or N content per plant were equally common to the three groups (0.10 mM, 0.15 mM and 0.20 mM).

Since enhanced symbiotic effectiveness is of frequent occurrence among the individuals having spontaneous resistance to azide, a molecular mechanism underlying azide resistance is important. Since azide is an alternate substrate of nitrogenase, a depressed nitrogenase was shown to account for azide detoxification in the cyanobacteria (Singh and Singh, 1978). In *Rhizobium, ex planta* induction of the oxygen labile nitrogenase is restricted to only few slow growing species, that too under strict environmental conditions (Postgate, 1989). Accordingly in *Bradyrhizobium* spp. (*Cajanus*), the wild type and azide-resistant mutants, which tolerated 90 fg ml<sup>-1</sup> and

110 to 180  $\text{fg ml}^{-1}$  of azide, were also found to exhibit low and high *ex planta* nitrogenase activity, respectively (Bala and Gaur, 1996). Obviously, during aerobic growth the nitrogenase-mediated resistance to azide is not possible. In *R. meliloti*, Kashyap and Narula (1990) demonstrated that azide resistance was dependent on *fix ABC* gene products. This finding was further strengthened by the finding of Sharma *et al.* (1997) that one of the *az<sup>r</sup>* mutant of *R. loti* had higher symbiotic effectiveness and higher TMPD oxidase activity, which was indicative of improved micro-aerobic respiration. Since azide has an affinity to bind to *cyto3* the azide-resistant mutants might have prevented this binding, which in turn, results in improved electron transport (Ramkrishna and Charan, 1994). It seems, therefore, plausible that an efficient electron transport system is responsible for both azide resistance during the aerobic growth and enhanced symbiotic effectiveness observed by others (Ram *et al.*, 1978; Pancholy, 1996; Sharma *et al.*, 1997). There is evidence that *fix ABCX* gene products might be involved in electron transport to nitrogenase (Fischer, 1994).

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