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Symbiotic Effectiveness of Different Antibiotic Resistant Mutants of Moth bean (*Vigna aconitifolia*) *Rhizobium* MR 67

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Abstract: Spontaneous mutants of moth bean, *Rhizobium* (*Bradyrhizobium* sp.) MR 67, resistant to streptomycin, kanamycin, neomycin and ampicilin were isolated at the graded levels of antibiotics. The mutation frequency was 2.54 x 10^{-8}, 2.66 x 10^{-8} and 3.54 x 10^{-8} with streptomycin, neomycin and kanamycin, respectively. Strain MR 67 was completely resistant to ampicilin. All the twenty mutants isolated and tested for each antibiotic, were stable in their resistance and sensitive to lysis by lytic phage MRV 67. Studies on the performance of the mutants with moth bean variety "Jwala", under controlled conditions revealed, that the strains were symbiotically effective. The mutants produced lesser nodules, but their symbiotic efficiency was comparable with the parents. Mutants superior in effectiveness were frequently associated with kanamycin resistance than streptomycin or neomycin resistance.

Key words: Moth bean *Rhizobium*, symbiotic efficiency and antibiotic resistance.

Moth bean (*Vigna aconitifolia*) is a drought tolerant pulse grown in the arid and semi-arid regions of Rajasthan. Though the crop benefits from symbiotic nitrogen fixation under field conditions, it is generally known that use of an appropriate strain of *Rhizobium* improves the symbiotic N_2 fixing in many grain legumes, including moth bean (Dixon and Wheeler, 1986; Ladha *et al.*, 1992 and Buttery *et al.*, 1992). There is an evidence that the strains of moth bean *Rhizobium* differ in their effectiveness under field conditions (Rao and Venkateshwarulu, 1983). Several reports indicate that selection of spontaneously occurring antibiotic resistant mutant strains is useful approach to improve symbiotic effectiveness over parent strains (Cole and Elkan, 1979; Srivastava *et al.*, 1980; Ramkrishna, 1985). However, in *Rhizobium*, *Lequminosarum* and *Rhizobium trifolii* strains, the symbiotic effectiveness was shown to be lost due to acquisition of antibiotic resistance (Schwinghamer, 1967). Based on these findings, Kuykendall (1981) pointed out that genetic background of the strain employed for isolation of the mutants and the antibiotic used have strong influence on symbiotic behaviour of the mutant strain. The present investigation was, therefore, taken up to characterise spontaneously isolated antibiotic resistant mutants of an effective *Rhizobium* strain under controlled conditions.

Materials and Methods

The moth bean *Rhizobium* strain MR 67 employed in the present study is a laboratory isolate, at the place of investigation.

Table 1. Spontaneous mutation frequency estimated at graded levels of different antibiotics for moth bean Rhizobium strain MR 67

Antibiotics	Mutation frequency at different doses of antibiotics ($\mu g\ ml^{-1} \times 10^{-8}$)					
	20	50	100	200	300	500
Kanamycin	2.50	2.80	3.87	2.50	2.75	3.54
Neomycin	2.87	3.29	3.79	3.37	3.62	2.66
Streptomycin	2.66	3.08	2.62	2.12	2.58	2.54
Ampicilin	R	R	R	R	R	R

R = Stands for complete resistance as profuse bacterial lawn was observed.

The rhizobiophage MRV 67 is also a laboratory isolate lytic to bacterial strain MR 67. The isolation, purification and preparation of phage lytic was done as described by Dhar and Ramkrishna (1987). Spontaneously arising antibiotic resistant mutants (kanamycin, neomycin, streptomycin and ampicilin were isolated at different concentrations of antibiotics as described by Dhar *et al.* (1979). Twenty colonies surviving at 500 $\mu g\ ml^{-1}$ of the drugs were picked up and subjected to five subculturing in antibiotic free YEM broth at 28±2°C.Each isolate thus subcultured was tested for resistance to antibiotic and sensitivity to the phage MRV 67 lysate as described by Dhar *et al.* (1979). Fifteen mutants in respect of streptomycin, kanamycin and neomycin, which exhibited resistance to the antibiotic and lysis by the phage, were studied further for their symbiotic effectiveness on host variety "Jwala" under controlled conditions. For asceptic culture of plants, five surface sterilized seeds of moth bean variety "Jwala" were sown 1 cm deep in sterile pure sand filled in glass beaker (400 ml). After 3 days of seedling emergence, each beaker received 5 ml (mid log phase) of the bacterial suspension (Parent/Mutant) as inoculum. After two days of inoculation, only three seedlings in a beaker were maintained and grown under fluorescent light system (5000 lux) for 16 hours per day at 28±2°C and irrigated as and when required with Arnon's nitrogen free nutrient solution (Vincent, 1970). Plants in uninoculated beakers served as control. The treatments were replicated thrice. After 37 days plant growth, observations on various symbiotic traits were recorded and means were used for analysis of variance. Total nitrogen content was determined by the Standard Laboratory methods.

Results and Discussion

Spontaneously arising mutants resistant to antibiotics were sought at 5 different levels (20 to 500 $\mu g\ ml^{-1}$). Colonies of putative mutants appeared on kanamycin, neomycin and streptomycin containing plates (Table 1). In the case of ampicilin, thick bacterial lawn was developed at all the concentrations indicating that the parent is resistant to this antibiotic. This is likely to be due to the presence of a genetic determinant that confers resistance to ampicilin. In case of other three antibiotics, the mutation frequency at all the concentrations was comparable. As the maximum tolerance to antibiotic by the mutants at lower grades such as 20 to 50 $\mu g\ ml^{-1}$ of antibiotic was not determined, therefore, it was difficult to draw any plausible con-

Table 2. Symbiotic effectiveness of various spontaneously arising mutants of moth bean Rhizobium MR 67 resistant to three different antibiotics on moth bean variety "Jwala" grown under controlled conditions

Characters	Parent's mean	Mean of Mutants	C.D. (5%)	Mutants* superior to parents
Streptomycin				
Nodules/plant	31.33	18.33	6.03	None
Nodule fresh wt./plant (mg)	14.16	14.12	6.01	Sm′ 20(20.9)
Nodule dry wt./plant (mg)	3.06	2.03	1.32	None
Plant fresh wt. (mg)	819.50	896.02	205.98	Sm′ 7(1067.93), Sm′ 9(1279.83) Sm′ 14(1035.26), Sm′ 16(1100.10) Sm′ 18(1067.73)
Plant dry wt. (mg)	116.60	104.88	35.42	Sm′ 9(156.1)
N-content/plant (mg)	11.58	13.2	3.66	Sm′ 7(18.75), Sm′ 9(19.87) Sm′ 20(15.28)
Kanamycin				
Nodules/plant	22.41	7.79		None
Nodule fresh wt./plant (mg)	19.49	8.96		Kan′ 11(37.06)
Nodule dry wt./plant (mg)	3.16	1.68		Kan′ 11(4.96)
Plant fresh wt. (mg)	978.70	312.03		Kan′ 6(1384.33), Kan′ 11(1538.76) Kan′ 14(1225.23), Kan′ 18(1160.63)
Plant dry wt. (mg)	118.89	36.21		Kan′ 11(153.83), Kan′ 14(164.96)
N-content/plant (mg)	13.58	2.88		Kan′ 4(14.92), Kan′ 6(17.04) Kan′ 11(19.21), Kan′ 14(17.14) Kan′ 18(16.16), Kan′ 19(15.36)
Neomycin				
Nodules/plant	18.54	5.81		None
Nodule fresh wt./plant (mg)	14.02	5.41		Neo′ 11(21.16), Neo′ 20(25.36)
Nodule dry wt./plant (mg)	2.43	1.04		None
Plant fresh wt. (mg)	857.12	280.56		Neo′ 9(1286.08), Neo′ 11(1121.53) Neo′ 20(1213.36)
Plant dry wt. (mg)	101.23	36.22		None
N-content/plant (mg)	11.72	2.55		Neo′ 17(17.37)

* Mutants statistically superior to the parents are shown in the parenthesis.

clusion from these observations. These three antibiotics belong to aminoglycosidic group of antibacterial agents. The bacteriostatic action of streptomycin is different from that of neomycin and kanamycin, and hence, different genetic locus/loci would be mutated to confer the resistance. Further, unlike streptomycin (which binds to 30 s subunit of ribosome), both for neomycin and kanamycin, there are two binding sites, atleast one on 50 s subunit. It may, therefore, be inferred that the selection pressure caused by employed antibiotics renders to pickup mutants affected at the deferent loci. Since mutants were able to tolerate the respective antibiotics (500 $\mu g\ ml^{-1}$)after five subculturing in absence of the drug, the mutations can be considered stable. Further, the sensitivity of the mutants to lysis by phage MRV 67 indicates that the mutants are true derivatives of parent strain MR 67 and not possible contaminants. The symbiotic effectiveness of the 15 mutants in respect of streptomycin, kanamycin and neomycin was studied. Variance analysis revealed that mutants of the three groups produced significant difference in respect of various symbiotic traits studied. Nodule number per plant was invariably and significantly reduced as compared to parent (Table 2). Although, in the present study, the selection was done for isolating spontaneous mutants resistant to antibiotic, the selected individuals also showed general reduction in nodule per plant, and occasionally exhibited relatively better symbiotic effectiveness. These may therefore be attributed to possible pleiotrophic effects of the point mutation that confers resistance against the antibiotics. Although other symbiotic traits were statistically at par for parent and mutants in respect of three antibiotics, occasionally the mutants exhibited superiority than the parent. Particularly for total nitrogen content per plant, six mutants for same traits were superior in case of kanamycin resistance as compared to one with neomycin and three with streptomycin resistance.

Since no loss of symbiotic effectiveness was noted, our observations were consistent with those of inference drawn by Kuykendall (1981). Under field conditions however, the performance of the superior mutants cannot be extrapolated as their competitiveness in the rhizosphere remains to be studied.

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