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Symbiotic Efficiency of Azide Resistant Mutants of *Prosopis cineraria* Rhizobia

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Abstract : Seven rhizobial mutants, with enhanced resistance to sodium azide (25 μg ml^{-1}), have been isolated following ultraviolet-irradiation of the native rhizobial strain, PC-1 (azide resistance 5 μg ml^{-1}), of *Prosopis cineraria*. Five out of the seven isolated mutants (azi-1, azi-2, azi-4, azi-6, azi-7) showed significant increases in per cent shoot nitrogen (33-45%) and plant dry weight (14-40%) over the parent strain, PC-1. However, two isolated mutants, azi-3 and azi-5, did not show any improvement in nitrogen-fixing efficiency, in terms of per cent shoot N and plant dry weight possibly due to high reversion frequencies. It can be inferred that increased rhizobial resistance to azide and effectiveness are associated with each other in legume-Rhizobium symbiosis system.

Key words : *Prosopis cineraria*, rhizobia, azide, mutants, symbiosis.

The importance of biological nitrogen fixation by *Rhizobium* spp. in symbiosis with leguminous plants and trees is well known. Seed inoculation with *Rhizobium* strains selected for high nitrogen fixing capacity helps obtain maximum benefit from the symbiosis. This envisages better prospects for arid lands of India where the principal constraint for low biomass production in 3.2 lakh sq. km of Indian arid zone is the poor soil nitrogen status (Dhir, 1977; Joshi *et al.*, 1989).

Introduction of sodium azide resistance in rhizobia through mutations is gaining importance in recent years for enhancing the effectivity of legume-*Rhizobium* symbiosis, but, there are contradictory reports about the performance of these mutants of rhizobia. Ram *et al.* (1978), in *R. leguminosarum* L4, and Vashishat *et al.* (1986), in slow-growing mung bean *Rhizobium*, reported that the rhizobial strains resistant to higher concentrations of azide, fixed nitrogen more effectively than strains resistant to lower concentrations in hosts *Pisum sativum* and mung bean, respectively. Therefore, they have opined that resistance to azide can be used as a potentially useful marker to select efficient rhizobial strains. On the contrary, Yadav *et al.* (1992) have observed in case of *Rhizobium* sp. (Vigna) that the development of azide resistant mutants may not always lead to an increase in symbiotic effectivity. This puts a question mark on the use of azide resistance as an easily selectable characteristic for the identification of efficient rhizobial strains. Hence, the main objective of the present study was to find out the likely reasons for the contradictory behaviour of the sodium azide resistant mutants of rhizobia with regard to symbiotic effectivity, using the symbiotic system of desert tree legume, *Prosopis cineraria*, and its associated rhizobia.

Materials and Methods

Rhizobial strains

The rhizobial strains, PC-1, PC-3 and PC-6 were isolated from root nodules of *Prosopis cineraria* from one-year-old seedlings following Vincent (1970). The cultures were maintained on GSY medium slants (Ram *et al.*, 1978) and stored at 4°C. Strain PC-1 was resistant

to the antibiotics novobiocin (Nv), erythromycin (Em) and ampicillin (A). Strain PC-1 also tolerated 5 μg ml^{-1} of sodium azide. The resistance to antibiotics was used as genetic marker in mutagenesis studies for strain identification.

Mutagenesis

Mutants with enhanced resistance to azide were isolated from strain PC-1 by exposing 10 ml of cell suspension having 10^8 cells ml^{-1} in 0.1 M phosphate buffer to 30 W Phillips germicidal tube at a distance of 45 cm for 50 sec. The irradiated cells were plated without any dilution on GSY medium containing 25 μg ml^{-1} of sodium azide under yellow light and incubated in dark for 4-5 days at 28°C. The colonies appearing on azide containing medium plates were picked up and treated as azide-resistant mutants, their cell suspension made in sterile, distilled water and further streaked on GSY plates containing sodium azide (25 μg ml^{-1}) for single colony isolation. Well separated colonies of mutants of PC-1, with enhanced azide resistance, were picked up and maintained on GSY slants stored at 4°C.

Symbiotic efficiency studies

The symbiotic efficiency studies of azide-resistant mutants were conducted under sterilized soil conditions. Polythene bags of 1 kg capacity were filled with sterilized soil. Chemically scarified *P. cineraria* seeds were grown on 1% agar plates. The seedlings were inoculated with the test strains by dipping for 15 minutes in a cell suspension having 10^6 cells ml^{-1}. The inoculated seedlings were transferred to the soil-filled polythene bags (3 replicates per strain) kept under natural light conditions and watered daily with quarter strength of Jensen's nitrogen free mineral salt solution. After 60 days of growth, the plants were uprooted, the nodules from each treatment were detached and kept separately for nodule reisolation studies. The whole plants were dried in an oven at 70°C till constant weight and plant dry weights were recorded. Per cent shoot nitrogen was estimated through micro-Kjeldahl method.

Nodule reisolation studies

Five nodules per plant were taken, surface sterilized and each nodule crushed separately in 2 ml of sterile, distilled water. The contents of each nodular suspension were streaked separately on GSY medium plates for single colony isolation. Five randomly isolated colonies were picked up and transferred separately on YEMA slants. From these slants, the isolates were tested for level of azide resistance and presence or absence of antibiotic resistance markers, using antibiotic susceptibility discs of Hi media.

Statistical analysis was done using analysis of variance (Snedecor and Cochran, 1967).

Results and Discussion

Ultraviolet irradiation of the parent rhizobial strain PC-1 of *P. cineraria* yielded seven mutants with enhanced azide resistance (25 μg ml^{-1}) to sodium azide (azi-1 to azi-7). These mutants were tested for their symbiotic performance and compared with that of parent strain PC-1 and two other native rhizobial strains PC-3 and PC-6 of *P. cineraria*, which could tolerate 10 μg ml^{-1} of azide.

The results show that the native strains PC-1, PC-3 and PC-6 do not vary significantly from each other in terms of per cent shoot N and plant dry weight of the host seedlings (Table 1). This indicates that increase in sodium azide resistance from 5 to 10 μg ml^{-1} has no beneficial effect on symbiotic nitrogen fixing efficiency.

When the azide resistance was raised to 25 μg ml^{-1} after ultraviolet irradiation of the strain PC-1, the resultant seven mutants (azi-1 to azi-7) behaved differently in symbiotic ef-

Table 1. Effectivity of sodium azide resistant mutants of Prosopis cineraria rhizobia

Strains	Sodium azide resistance (μg ml^{-1})	Shoot N (%)	Plant dry weight (g $plant^{-1}$)
Uninoculated control	–	1.70 (7.49)	1.64 (7.34)
Native strains :			
PC-1 (Parent strain)	5	2.65 (9.37)	2.70 (9.46)
PC-3	10	2.1 (8.33)	2.97 (9.98)
PC-6	10	2.20 (8.53)	2.79 (9.63)
Mutants			
azi-1	25	3.64 (11.01)	3.95 (11.46)
azi-2	25	3.83 (11.24)	3.63 (10.94)
azi-3	25	2.51 (9.10)	2.65 (9.46)
azi-4	25	3.93 (11.39)	3.27 (10.47)
azi-5	25	2.34 (8.82)	2.77 (9.63)
azi-6	25	3.44 (10.70)	3.92 (11.39)
azi-7	25	3.66 (11.09)	3.55 (10.86)
C.D. 1%		1.76	1.28
C.D. 5%		1.21	0.88

Values in parenthesis are angular transformed values.
C.D. = Critical difference.

ficiency. Five of the seven azide resistant mutants, upon inoculation of the host seedlings, resulted in significant increases in per cent shoot N ($P<0.05$ in azi-1, azi-6 and azi-7 and $P<0.01$ in azi-2 and azi-4) and plant dry weight ($P<0.05$ in azi-4 and $P<0.01$ in azi-1, azi-2, azi-6 and azi-7) (Table 1). Performances of azi-3 and azi-5 were not as expected. These behaved like parent strain PC-1 in terms of shoot N percentage and plant dry weight instead of showing enhancement in these parameters.

To find out the reason for low symbiotic performance of the mutants azi-3 and azi-5, the bacteria were reisolated from the nodules of the host plants inoculated with azi-3 and azi-5 and two others (azi-1 and azi-2) with enhanced effectivity for comparison (Table 2). It was found that in case of azi-1 and azi-2, all the 75 reisolated bacterial clones were resistant to 25 μg ml^{-1} of sodium azide. On the other hand, in case of azi-3 72% and in azi-5 76% of the reisolated bacterial clones were showing azide resistance only upto 5 μg ml^{-1}, but the pattern of antibiotic resistance was the same as that of the original mutant, thus ruling out the possibility of contamination. This indicated that the mutations leading to increased resistance to sodium azide in azi-3 and azi-5 had high frequencies of reversion to low azide resistance and that most of the nodules were formed by these revertants in case of azi-3 and azi-5. This explains the low symbiotic performance of the mutants azi-3 and azi-5. Had these reversions not taken place, azi-3 and azi-5 would also have behaved like other azi-resistant mutants and showen enhanced symbiotic effectivity.

From these results, it can be inferred that: (1) induced enhancement in the level of sodium azide resistance in the bacterial symbiont in

Table 2. Nodule reisolation studies with azide resistant mutants of Prosopis cineraria rhizobia

Mutant strains	No. of nodules tested	No. of clones tested	% clones with azide resistance (mg ml^{-1})		Resistance to Nv, Em, A
			25	5	
azi-1	15	75	100	Nil	+
azi-2	15	75	100	Nil	+
azi-3	15	75	28	72	+
azi-4	15	75	24	76	+

+ indicates presence of antibiotic resistance markers.

desert woody legume-*Rhizobium* system does lead to an increase in nitrogen-fixing effectivity of the system, (ii) the possible reason for the contradictory behaviour of the azide resistant rhizobial mutants, as reflected in the previous reports, is the reversion to low azide resistance in some of the isolated mutants.

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