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Short Communication

Isolation of Auxotrophic Mutants of *Rhizobium* of *Prosopis cineraria*

Anjly Pancholy
Central Arid Zone Research Institute, Jodhpur 342 003, India

Legume *Rhizobium* symbiosis is a well known biological system which fixes atmospheric nitrogen efficiently. Scherrer and Denarie (1971) reported that glycine-dependent mutants of *R. meliloti* were more effective than the parent strain, and prototrophic revertants of some of the ineffective auxotrophic mutants were also found to be more effective than the wild-type strain. Studies were undertaken to explore the possibility of isolating auxotrophic mutants of rhizobia of *Prosopis cineraria*, an important multipurpose tree of the desert, and to develop a standard protocol for their isolation.

The rhizobial strain, PC-3, used in the present investigation, was isolated from the nodules. It was sensitive to ampicillin (Ap) (30 μg ml^{-1}) and resistant to trimethoprim (Tr) (350 μg ml^{-1}), with a mean generation time of 3.6 h. The complete medium (GSY) used for its cultivation was as per Ram *et al.* (1978) and minimal medium (MM) was as suggested by Fedorov and Zaretskaya (1978). When required, MM was supplemented with different amino acids (20 μg ml^{-1}).

Loopful of rhizobial cells, taken from GSY slants, was used to inoculate 30 ml of GSY liquid medium and grown under constant shaking for 3 days. Cells taken from this culture were mutagenised by 1.5% ethylmethane sulphonate (EMS). Mutagenesis, enrichment and isolation of auxotrophs were carried out by a modified method of Kohli and Vashishat (1985). The major modifications were (i) mutagenesis done with 1.5% EMS for 40 min. giving 4% survival, (ii) for isolation of auxotrophs, the Nutrient Broth (NB) supplement added to MM instead of GSY, (iii) various concentrations of NB (range 0.2 to 1.5%) tested for maximum appearance of small colonies in Auxotroph Isolation (AI) medium, and (iv) although both large and small colonies appeared on AI medium, only small colonies were tested for auxotrophy.

The mutagenised cells were phenotypically expressed in GSY medium for 40 h and starved in MM containing 0.05 M sorbitol and 0.01 M $MgSO_4$ for 20 h. During starvation, the auxotrophic mutants stopped growing, but the wild type cells continued the growth. Mutagenised cell suspension was treated with Ap (100 mg ml^{-1}) for 12 h, followed by lysozyme (100 mg ml^{-1}) for 3 h. The cells were centrifuged, washed and plated on Auxotroph Isolation medium. After 4-5 days, both small and large colonies appeared on AI medium, small colonies were tested for auxotrophy and large ones discarded.

Nodulation test was conducted by inoculating seedlings of *P. cineraria*, growing in polythene bags containing sterile sand, under natural light. The bags were watered daily with sterile tap water. Observations on nodulation were made 45 days after inoculation.

After mutagenesis and enrichment, MM supplemented with 0.6% NB gave maximum percentage (5.0) of small colonies, while further increase in NB resulted in a decrease in percentage of small colonies, with only 1% small colonies at 1.5% NB level. Hence, this combination (MM + 0.6% NB, v/v) was selected for isolation of auxotrophic mutants. Out of 403 small colonies tested, seven colonies (PC-3.1 to PC-3.7) failed to grow on MM and were regarded as presumptive auxotrophic (P.A.) mutants. Single colony isolates of all the seven PA mutants were tested for their growth requirements on MM

supplemented with individual amino acids. It was revealed that rhizobial strains, auxotrophic for adenine (PC-3.1 and 3.3), valine (PC-3.2 and 3.7), tryptophan (PC-3.4), histidine (PC-3.5) and arginine (PC-3.6) were all resistant to trimethoprim and produced nodules on the host like the parent strain, showing that these were derived from the parent strain PC-3 only and were not contaminants. The spontaneous reversion frequencies of various mutants obtained varied between 0.38×10^{-7} to 83.1×10^{-4} indicating that these were point mutations as expected from EMS and not deletions.

Determination of an optimum concentration of NB (0.6% , v/v) in the partially supplemented MM (Auxotroph Isolation medium) was a crucial step. It reduced the number of small clones to be tested for auxotrophy to the minimum. Out of 403 small colonies tested, seven proved to be auxotrophs. Thus, the percentage of auxotrophic cells in the final enriched medium was 1.2 which is quite high, compared to 0.35 reported by Kohli and Vashishat (1985). Replacement of GSY medium with MM, in the Auxotroph Isolation medium, also increased the frequency of small colonies in the enriched medium to a great extent.

With the possibility of mutants being more efficient in nodulation and N_2 fixation, temperature tolerance needs to be further investigated.

References

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