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# DEVELOPMENT OF ANTIBIOTIC RESISTANT MUTANTS SIM *AZOSPIRILLUM BRASILENSE* TARRAND *et al.*

*Azospirillum*, the nitrogen fixing bacterium is gaining importance in Indian agriculture as a potential bio-fertilizer (Subba Rao, 1988). It hss been well documented that in addition to fixing nitrogen from the atmosphere, *Azospirillum* also synthesises quite a number oi plant growth regulating substances which help in augmenting crop growth (Tien *et al.*, 1979 and Govindan and Purushothaman, 1984). Of the many methods available to increase th3 nitrogen fixing capacity of micro-organisms, development of mutant strains with improved efficiency is the most tangible one. We have made an attempt to improve the nitrogen fixation in *Azospirillum* through mutation in this study.

The parent (wild) strain of *Azospirillum brasilense* (Pt, 85) used in this experiment was isolated from the root tissues of pearl millet (*Pennisetum americanum).* The culture was maintained on yeast extract glucose sgsr slants. The cells for mutain experiment were obtained by growing the isolate in NbF liquid medium (Dobereiner and Day, 1976). The intrinsic level of antibiotic resistance of the wild strain towards streptomycin (Str) and chloramphenicol (CAM) was determined by streak plate assay. The method followed for mutation was essentially the same as detailed by Clowes and Hayes (1968). The chemical mutagen ethyl methane sulfonate (FMS) was used at 100 $\mu$g per ml. Following the standard dilution plating technique, the cell populations were determined at frequent intervals of mutagen treatment for obtaining information on the percent survival of cells and frequency of mutation etc. As we wanted to have a high level of antibiotic resistance mutated cells were plated on glucose yeast extract medium containing 500 $\mu$g of tho antibiotic per ml. The mutant clones showing resistance were stabilized for five generations on antibiotic containing medium. The highest level of antibiotic resistance exhibited by the mutants was determined by streaking the mutants on media containing increasing concentrations of Str and CAM. The nitrogen fixing capacity of the parent strain and the mutants was determined by estimating the total nitrogen in the broth (Humphries, 1956) and by assaying nitrogenase (Hardy *et al.*, 1968).

The results have indicated that the parent strain had a very high level of intrinsic antibiotic resistance to Str and CAM viz., 250 $\mu$g per ml. This appears to be alarmingly very high. This might be probably due to the secretion of antibotics soil streptomycetes in the vicinity of pearl millet roots from where these isolate was obtained. The view of Baldani and Dobereiner (1980) on the high level of natural resistance towards antibiotics in *Azospirillum* lends support for the present observation.

The results have further indicated that 20 minutes of exposure to the mutagen resulted in 50 per cent mortolity of the cells (Fig. 1). The frequency of

Table 1

Frequency of mutation for antibiotic resistance in *Azospirillum brasilense*

| EMS exposure time (min) | Streptamycin resistance* | | Chloramphenicol resistance | |
|---|---|---|---|---|
| | Frequency of mutation among the survivors | Frequency of mutation among the total population | Frequency of mutation among the servivers | Frequency of mutation among the total population |
| 10 | $0.97\times10^{-5}$ | $0.77\times10^{-5}$ | $0.52\times10^{-5}$ | $0.41\times10^{-5}$ |
| 20 | $3.10\times10^{-5}$ | $1.19\times10^{-5}$ | $2.96\times10^{-5}$ | $1.82\times10^{-5}$ |
| 30 | $5.48\times10^{-5}$ | $2.17\times10^{-5}$ | $5.27\times10^{-5}$ | $2.08\times10^{-5}$ |

* 500 $\mu$g per ml of the respective antibiotic substance

Table 2

Nitrogen fixing efficiency of *Azospirillum* wild strains and drug resistant mutants

| Strains | N fixed, mg per g of malate | ARA (nmol of ethylene formed per mg protein per hour) |
|---|---|---|
| Wild type Pt 85 | 20.56 | 355.80 |
| Mutant $Str^r$ 2 | 11.38 | 257.14 |
| ,, $Str^r$ 3 | 22.31 | 375.00 |
| ,, $Str^r$ 4 | 7.44 | 137.00 |
| ,, $Str^r$ 5 | 13.44 | 261.03 |
| ,, $CAM^r$ 1 | 13.13 | 319.49 |
| ,, $CAM^r$ 2 | 10.90 | 200.00 |
| ,, $CAM^r$ 3 | 12.70 | 254.29 |
| ,, $CAM^r$ 4 | 9.19 | 169.70 |

(Data represent mean of three determinations)

ARA: Acetylene reduction activity.

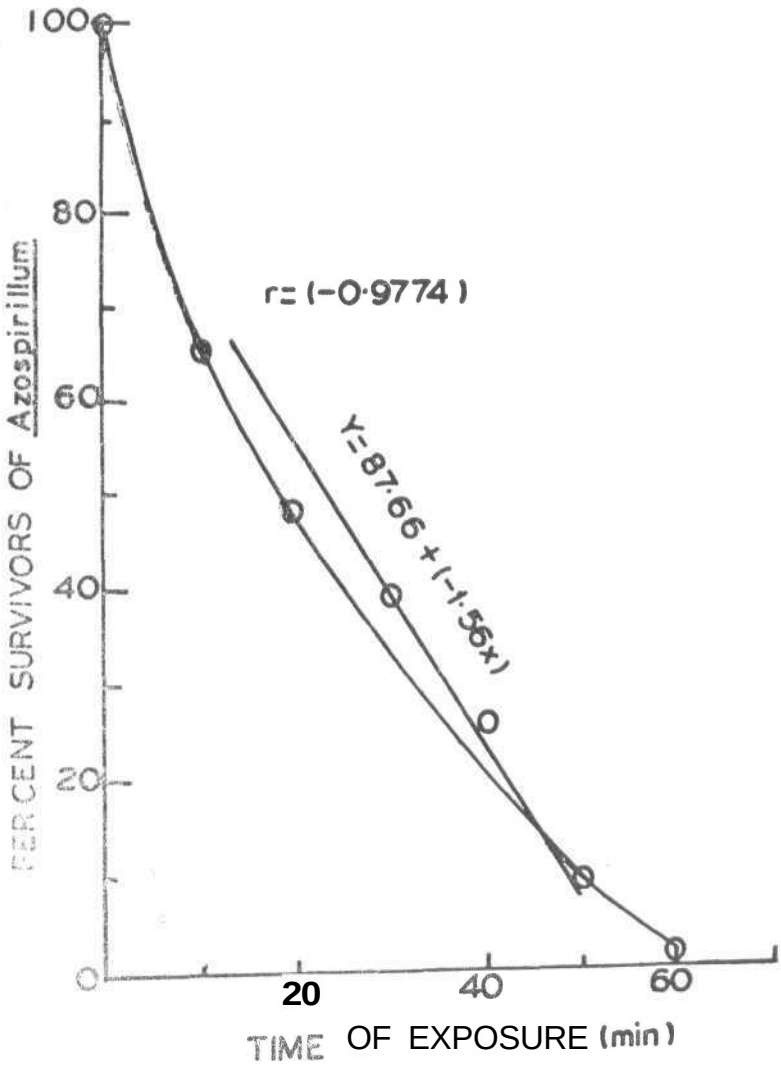


**Fig. 1** Effect of EMS on the survival of *Azospirillum* sp.

mutation for Str appeared to be higher than that for CAM (Table 1). This may probably be due to the loci determining resistance to these antibiotics are occurring far apart. The mutants CAM$^r$ 1, CAM$^r$ 2, CAM$^r$ 3 and CAM$^r$ 4 were exhibiting resistance up to 500 $\mu$g per ml of CAM. Similarly the mutants, Str$^r$ 2, Str$^r$ 3, Str$^r$ 4 and Str$^r$ 5 have registered up to 500 $\mu$g of streptomycin per ml. There was no evidence of antibiotic dependency of the mutants for growth.

The nitrogen fixing capacity of the mutants indicated a slight decrease; however the mutant Str$^r$ 3 has recorded higher fixation rate than the parent strain.

In respect of the antibiotic resistant character, the mutants were stable when examined up to ten generations. These mutants would be ideally suitable for studies on the population dynamics and rhizosphere-competition for survival etc.

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## References

Baldani, V. L. D., and Dobereiner, D. 1980. Host plant specificity in the infection of cereals with *Azospirillum* spp. *Soil Biol. Biochem.* 12: 433-439.

Clowes. R. C. and Hayes, W. 1968. *Experiments in Microbial Genetics.* Blackwell Scientific Publ. Ltd., Edinburgh, p. 244.

Dobereiner, J. and Day, J. M. 1976. Associative symbiosis in tropical grasses. characterization of microorganisms and dinitrogen fixation sites- In: Newton, W. E. and Nyman C. J. (ed). *Proceedings of the International Symposium on Nitrogen Fixation,* Washington State University Press, Pullman, p. 518.

Govindan, M. and Purushothaman, D. 1984. Production of phytohormones by the nitrogen fixing bacterium, *Azospirillum. Agri. Res. J. Kerala,* **22:** 133-138

Humphries, E. C. 1956. Mineral components and ash analysis. In: Paech, K. and Tracey, M. V. (ed.) *Modern Methods of Plant Analysis.* Vol. I Springer Verlag, Berlin, pp 468-502.

Hardy, G. W. F., Holsten, R. D., Jackson E. K. and Burns, R. C. 1968. The acetylene. ethylene assay for nitrogen fixation laboratory and field evaluation: *Plant Physiol.* **43:** 1185-1207.

Subba Rao, N. S. 1988. *Biological Nitrogen Fixation—Recent Developments.* Oxford & IBH Publ. Co. India, pp 1-16.

Tien, T. M., Gaskins, M. H. and Hubbell. D. H. 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl, Environ. Microbiol.* 37: 1016-1024.

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