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Research Article

ECOLOGICAL MONITORING OF *LACZ* MARKED STRAIN OF *AZOTOBACTER CHROOCOCCUM* IN THE RHIZOPLANE OF *TRITICUM AESTIVUM*

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ABSTRACT

The potential of *A. chroococcum* as a biofertilizer to improve the crop productivity is well recognized which has accelerated its use as bioinoculants. Unfortunately a huge amount of microbial inoculants are being dumped into the environment without understanding the ecological factors that control their fate and importance. The introduction of marker genes that can be easily identified using a chromogenic substrate offers an opportunity for the survival studies of the inoculants. The establishment of lacZ marked strain of *Azotobacter chroococcum* Mac 27 in the rhizosphere of *Triticum aestivum* under pothouse conditions was studied. The lacZ marked strain formed blue colonies on selective media and could be identified from soil on the basis of this character. The transconjugant was able to survive in the rhizosphere of *Triticum aestivum* under pothouse conditions and maintained a population of about 10^6 cfu/plant root after 90 days of sowing in plants treated with *A.chroococcum* Mac 27 L and the strain has been found to be reliable for ecological studies.

Key words: *lac* Z, β-galactosidase, *Azotobacter chroococcum*, inoculants

INTRODUCTION

Due to deliberate use of chemical fertilizers in the soil and the deteriorating soil quality, the bacterial inoculants have gained momentum in the last decades. Many bacterial species have been implicated as biofertilizers for improving soil nutrient status. Amongst many, *Azotobacter* is one that has multifarious effects on plant growth. It not only fixes atmospheric nitrogen and excretes a part of fixed nitrogen in soil but also produces antimicrobial substances, siderophores and growth regulators. Developing countries like India having large area under cultivation can exploit *Azotobacter* as a cheap alternate source of nitrogen, growth regulators and antimicrobial agents by the use of *Azotobacter* as biofertilizer for cereals, oilseed crops, cotton and vegetable crops.

To assess the potential of agriculturally important microorganisms including *Azotobacter chroococcum* in terms of increase in yield, it is essential to monitor the survival and persistence of inoculated strains of bacteria in soil. Strain identification has been done using different methods based on serological properties[1], bacteriophage susceptibility[2], antibiotic resistant markers[3], DNA homology[4] and plasmid profile[5,6]. Since such studies are very laborious, there is a need for simple and specific methods of strain identification. In recent years a major technical development in the detection of microorganisms in their natural environment has been the tagging of a particular strain with specific marker genes like lac Z, gus A, etc. Lac Z has been a convenient marker for ecological studies since the β galactosidase activity can be measured very easily and quickly using chromogenic substrates without the need of much sophisticated equipments. Moreover the assay is sensitive enough to monitor the β galactosidase activity. In this study, lac Z marked *A. chrroococcum* Mac 27 was used to monitor its survival in the rhizoplane of *Triticum aestivum.*

MATERIALS AND METHODS

Bacterial Cultures and growth media

Azotobacter chroococcum Mac 27 and lac Z marked transconjugant (*A.chroococcum* Mac 27 L) was procured from the culture collection of Department of Microbiology, CCS Haryana Agricultural University, Hisar, India and maintained on Burks medium. X-gal (5- Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to precooled sterilized medium.

Assay of β galactosidase activity

One ml actively growing bacterial broth culture was taken in Eppendorf tube and centrifuged at 8000g for 10 min. The cell pellet was washed twice with phosphate buffer and resuspended in 500 µl of phosphate buffer (pH 7.0). The cells were permeabilized by vortexing for 10 seconds with a solution containing 25 µl of toluene. To 100 µl of permeabilized cells, 10µl ONPG was added. The reaction mixture was incubated at 37 ^{0}C in water bath till the development of yellow colour. Reaction was stopped by adding 2 ml of 0.4 M Na_2CO_3 solutions. Colour intensity was determined at 420 nm using a spectrophotometer against a blank containing all ingredients except the cell culture. β-galactosidase activity is expressed as Millers unit calculated as per formula given below:

Millers unit = Absorbance 420 / Absorbance 600 x T x V X 1000

where T=Time of reaction (min), V=Volume of the cell culture used (ml)

Enumeration of *A. chroococcum* Mac 27 and Z from the rhizoplane of *Triticum aestivum*

Seeds of *Triticum aestivum* were surface sterilized and inoculated with about 10^8 cells of *Azotobacter chroococcum* Mac 27. *A.chroococcum* Mac 27 L. Seeds were sown in pots containing 7 Kg unsterilized soil. Each treatment was replicated six times. The plants were uprooted after 30 and 60 days of growth and the rhizospheric soil samples were analysed for total and lacZ marked populations. The appropriate serial dilutions were plated on Burks medium plates with and without X-gal. The plates were incubated for 48 h and the number of colonies that appeared on both types of media was counted.

RESULTS AND DISCUSSION

Identification of strains in field experimentation with strains of various biological nitrogen fixating bacteria is one of the major problem. Many studies have been conducted to monitor and identify the inoculated strains under field conditions using different techniques but information on the survival of inoculated strains is not reliable. So a better way of identifying a strain under natural conditions is the introduction of foreign genes that can be easily identified using a chromogenic substrate. In the present study, lac Z marked *A. chroococcum* was used to monitor its survival in the rhizoplane of *Triticum aestivum.* However, there are a variety of contaminants present in the environment which may interfere in the expression of β- galactosidase activity of lac Z marked *A. chroococcum.* To ascertain if the β-galactosidase activity of *A. chrooocoocum* Mac 27 lacZ+ strain is affected by the presence of contaminants such as *Bacillus* spp, *Rhizobium* spp., *Pseudomonas* spp., etc. in the soil, the indigenous β-galactosidase activity of some of the common soil bacteria were tested and were found to possess almost negligible β-galactosidase activity (Table-1).

Table-1: β-Galactosidase activity of some commonly occurring soil bacteria

Culture	β-Galactosidase Activity (M.U.)			
	5'	10'	15'	30'
Bacillus	42.8	42.6	2.84	1.64
Rhizobium spp p-9038	110	114	3.8	12.6
Rhizobium spp G-14	7.4	8.2	4.10	2.80
Rhizobium	106	112	3.7	12.4
Azotobacter	110	129	4.3	18
Pseudomonas-1	62.2	68.4	2.3	7.6
Pseudomonas-2	31.4	34.0	1.17	3.8

The presence of these bacteria had no apparent effect on the β-galactosidase activity of the lac Z marked strain. The β-galactosidase activity was found to be 11000-12000 MU in the presence or absence of other bacteria (Fig-1).

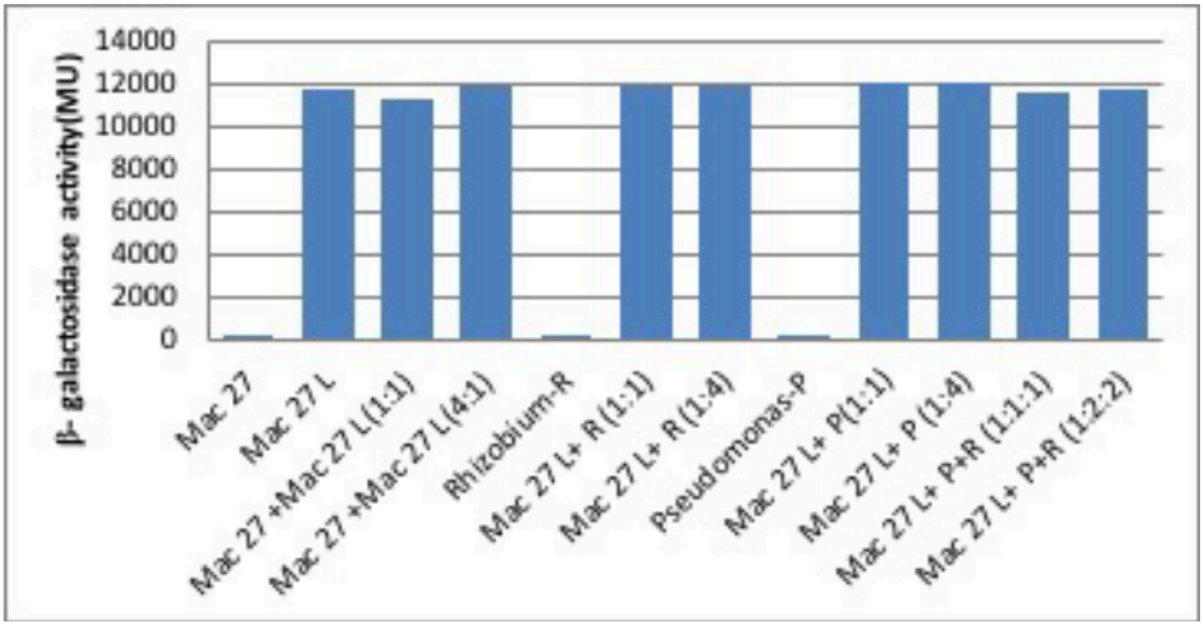

Figure-1: β-Galactosidase activity of *A. chroococcum* Mac 27 L as affected by other bacteria

Also, there was no effect on the β- galactosidase activity of the transconjugant by the presence of *Bacillus* spp. in concentration as high as 10 times that of *A. chroococcum* Mac 27 L (Table-2).

Table-2: β-Galactosidase activity of *A.chroococcum* Mac 27 L as affected by different concentrations of *Bacillus* spp.

Culture	Cell concentration (600 nm)			Colour Intensity (M.U.)
	Mixed Culture	Lac Z marked	Absorbance (420 nm)	
Bacillus	1.1	-	0.011	-
Mac 27 L	0.945	0.945	0.646	9660
Mac 27 L+ *Bacillus* (1:1)	0.964	0.482	0.317	9300
Mac 27 L+ *Bacillus* (1:2)	0.998	0.332	0.236	10000
Mac 27 L+ *Bacillus* (1:3)	1.000	0.250	0.176	9900
Mac 27 L+ *Bacillus* (1:4)	1.060	0.212	0.151	10060
Mac 27 L+ *Bacillus* (1:5)	1.032	0.172	0.120	9860
Mac 27 L+ *Bacillus* (1:6)	1.060	0.151	0.106	9920
Mac 27 L+ *Bacillus* (1:7)	1.030	0.128	0.087	9600
Mac 27 L+ *Bacillus* (1:8)	1.060	0.117	0.800	9660
Mac 27 L+ *Bacillus* (1:9)	1.026	0.103	0.072	9870

The transconjugant was used to monitor its survival in the rhizoplane of *Triticum aestivum.* The transconjugant was detected by direct plating on Burk's medium containing X-gal. It was observed that out of 2.5x106 cfu/plant root, 4.1x105 cfu/plant root were that of *A.chroococcum* Mac 27 L (Table-3) .

Table-3: Enumeration of different strains of *Azotobacter* from the rhizoplane of *Triticum aestivum*

Treatment	30 DAS		60 DAS		90 DAS	
	Total Count	Count of lacZ marked	Total Count	Count of lacZ marked	Total Count	Count of lacZ marked
A.chroococcum Mac27 L	$7.82X10^6$	$6.6X10^6$	$5.45X10^6$	$5.32x10^6$	$2.5X10^6$	$4.1X10^5$
A.chroococcum Mac27	$8.15X10^6$	-	$5.89X10^6$	-	$1.2X10^6$	-
Control	$8.56 X10^6$	-	$8.14X10^6$	-	$9.4X10^6$	-

The transconjugant could be detected in the presence of other bacteria capable of growing on Burks agar medium containing X-gal on the basis of lac Z marker even after 90 days of inoculation. The present study clearly indicates that the lac Z marker can be effectively used for monitoring the survival of the introduced strains in the soil.

Various Strains *of Pseudomonas, Rhizobium* and other plant growth promoting rhizobacteria with lacZ fusion have been constructed and used for ecological studies earlier also.Drahos et al[7] reported that the lacZ marker system enabled the detection of Lac^+ transformants at a sensitivity of <10 CFU/g soil(1). Hofte et al[8] marked the rhizopseudomonas strain 7NSK2 with a Mu d (lac) element for ecological studies. They concluded that MPB1 can reliably be used for ecological studies since no genes essential for growth, siderophore production, survival in sterile and nonsterile conditions, plant growth stimulation and root colonization had been damaged. They also reported that upper root parts were effectively colonized upon seed inoculation of Maize with MPB1, while soil inoculation resulted in a complete colonization of the root system. Krishnan and Pueppke[9] also reported that gene fusions can be used to greatly simplify the assessment of nodulation competitiveness in rhizobia, Kamboj et al[10] and Khokar et al[11] tagged *Rhizobium* and *Pseudomonas* respectively to monitor their persistence in soil. Pal et al[12] employed the Tn5::lacZ marker to monitor the rhizobacterium *Pseudomonas glumae* EM85, an effective biocontrol agent against *Rhizoctonia solani* from cotton. Solanki and Garg[13] have found lac Z marker to be reliable in detection of inoculated strains in soil as well as in charcoal based inoculants.

CONCLUSION

LacZ can be used as a stable, sensitive and selectable chromogenic marker to monitor the colonization of various rhizobacterial strains on plant roots. The tagged bacteria can be monitored effectively with high sensitivity in natural environment by direct plating of soil sample on selective media even in the presence of other soil bacteria.The use of marker genes has been demonstrated to work with a number of plant growth promoting strains on a variety of plants and the introduction of the marker gene per se does not show any impact on growth attributes.

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