JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4743-4747

Assessing the Establishment of *Lac Z* Marked Strains of Pentachlorophenol Degrading Rhizobacteria and their Effects on Plant Growth of Maize (*Zea mays*)

K.M. Avitha[1], K.S. Jagadeesh[1], P.U. Krishnaraj[2] and Sarita Sinha[3]

[1]Department of Agricultural Microbiology,
University of Agricultural Sciences, Dharwad - 580 005, India.
[2]Department of Biotechnology, University of Agricultural Sciences, Dharwad - 580 005, India.
[3]Ecotoxicology and Bioremediation Group, National Botanical
Research Institute, Lucknow - 226 001, India.

(Received: 06 April 2014; accepted: 19 August 2014)

Rhizobacteria isolated from the rhizosphere of PCP tolerant maize plant were investigated for establishment of *lac Z* marked strains of PCP degrading bacteria in the rhizosphere of maize (*Zea mays*) under green house condition. The identities of the isolates were determined by 16S rRNA gene sequencing as *Enterobacter asburiae* (MAZ1) and *Pseudomonas* (MAZ2). Both the *lac Z* marked strains were able to survive in rhizosphere of maize under greenhouse conditions and maintained a population of about 10^4 g"1 of rhizosphere soil up to 60 days study period. Positive effect of inoculation with PCP degrading bacteria on plant length and biomass were observed. Out of two, PCP degrading *Pseudomonas sp.* MAZ 2 was found to possess higher root colonization ability and plant growth promoting potentials.

Key words: PCP degrading, rhizobacteria, *lac Z* marked strains, Maize.

Pentachlorophenol is primarily used as a pesticide and also as a wood preservative. It is present in tannery effluents and is formed unintentionally in paper and pulp industries. PCP is highly toxic, and a recalcitrant organic compound because of its stable aromatic ring system and high chloride content, thereby persisting in the environment. Thus, there is a need for its removal from the environment. Different researchers have reported PCP degrading microorganisms from the natural environment. Several bacteria such as *Arthrobacter*, *Pseudomonas*, *Sphingobium chlorophenolicum*, *S. marcescens* capable of PCP degradation have been reported[1, 2].

* To whom all correspondence should be addressed.

Rhizodegradation is very attractive as a method for environmental remediation because it does not require the addition of nutrients to the soil, which is necessary for bioremediation. There have been a number of recent reports concerning rhizodegradation[3,4]. The prospects of manipulating crop rhizosphere microbial populations by inoculation of biodegradation bacteria to increase bioremediation have shown considerable promise in laboratory but responses have been variable under natural conditions. The performance of microbial inoculants depends largely on survival, successful establishment and root colonization under *in situ* conditions. Colonization of roots by inoculated microorganisms is a very complex and crucial process, which is not fully understood. To develop an insight of the process, the introduced strains need to be monitored to assess their fate. Thus, the aim of the present investigation was to monitor establishment of *lac Z* tagged PCP

degrading strains in maize rhizoshpere to study survival of these inoculated strains and their effect on plant growth parameters.

MATERIALS AND METHODS

Plasmids and cultural conditions

The transposon vector B20 *lac Z::*Tn 5 was kindly provided by Dr. Krishnaraj.P.U, Institute of Agricultural Biotechnology, UAS., Dharwad. Luria – Bertani solidified with 1.5 % bacto agar and were appropriately supplemented with kanamycin at final concentration of 25 µg/ml.

Characterization of PCP degrading isolates

PCP degrading isolates, MAZ1 and MAZ 2 were previously isolated from rhizosphere soil of maize plant after screening the plants to tolerate PCP at different concentrations and their PCP degrading potentials determined[5]. The selected bacterial isolates were characterized as per Bergey's Manual of Determinative Bacteriology[6]. The molecular characterization of isolates was done by 16S rDNA sequence analysis at National Center for Cell Science (NCCS), Pune (India). The 16S rDNA sequence was also deposited to GenBank database to get the accession number, and to identify most similar sequence alignment using www.ncbi.nlm.nih.gov/BLAST.

Construction of *lac Z*:: Tn5 by Patch mating

In order to assess the fate of the introduced organisms in the rhizosphere, *lac Z* tagging is generally done[7]. In the present study, The isolates MAZ 1 and MAZ 2 were used for the colonization studies on maize roots. Plasmid was transferred from *E. coli* to MAZ 1 and MAZ 2 by patch mating. Recipient strains were grown in Luria broth for 24 hours at 28 – 30 °C. donor for B 20 *lac Z*, *E. coli* was grown in LB (Luria Bertini) broth containing 25 µg/ml Kanamycin (Km) at 37 °C for 24 hours. Screening of blue coloured mutants of *lac* Z^+ by plating on luria agar supplemented with 25 µg/ml Kanamycin and X-gal and Isopropylthio-^{2}- galactoside (IPTG) (160 µl/100ml)(Plate 2). The transconjugants, MAZ 1:: *lac Z* and MAZ 2 :: *lac Z* were selected and maintained on the appropriate medium.

Colonization studies

The pots were filled with sieved black soil (10kg/pot). The experiment contained inoculation of MAZ 1:: *lac Z* and MAZ 2:: *lac Z* with seven replications. An uninoculated control was also maintained for comparison. Since observations were taken at four intervals i.e., 10, 30, 45 and 60 days of plant growth, each treatment was maintained in four sets. Thus, the treatments were as follows i) T_1 - Uninoculated, ii) T_2 - MAZ 1:: *lac Z*, iii)T_3 - MAZ 2:: *lac Z*

Seed bacterization and sowing

Maize seeds were bacterized, following the method of Weller and Cook (1983). The strains MAZ 1::*lac Z* and MAZ 2::*lac Z* used for seed treatment were grown for two days at 27°C in 10 ml Luria broth. Culture broth was then suspended with sterile carboxy methyl cellulose (1%). The surface sterilized seeds were treated with the respective tagged CMC based culture suspension (10^9 cfu/ml) and air dried for 2 to 3 h. Seeds in clumps were separated prior to sowing. MAZ 1::*lac Z* and MAZ 2::*lac Z* inoculated and uninoculated seeds were sown @ four seeds per pot. After germination, (7 days) they were thinned so as to maintain one plant per pot and watering done regularly to maintain the required moisture content in soil.

Observations

The plants were maintained for 60 days. The plant growth parameters such as root length, shoot length, root biomass, shoot biomass and total biomass were monitored and recorded.

Monitoring the introduced strains on the roots of Maize

The standard plate count method was used to assess the colonization of roots by the PCP degrading bacteria at 10, 30, 45 and 60 days of plant growth. The plants were depotted at above mentioned intervals and the roots thoroughly washed with tap water to remove all loosely adhering soil particles. The washed roots (1g wet wt. each) were suspended in 99 ml of sterile water and shaken for an hour to release bacteria from the root surfaces. The root samples were diluted serially and 100µl aliquot was plated on Luria agar supplemented with antibiotics and the chromogenic substrate (X-gal and IPTG) and incubated at 30±2 °C for 48 h. The bacterial populations growing on the media were recorded. Clones giving blue color of *lac Z* were counted and the viable counts per gram roots were recorded.

RESULTS AND DISCUSSION

Identification of the selected bacterial isolates

The selected potential bacterial isolates resistant to high levels of PCP were subjected to identification by determining their morphological and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology[6]. The identification of isolates MAZ1 and MAZ2 was further authenticated by 16S rDNA sequence analysis. The isolates were identified as *Enterobacter asburiae* (MAZ1) and *Pseudomonas sp* (MAZ2). Using forward and reverse primers, 16S rDNA sequence of the isolates MAZ1 and MAZ2 was obtained which was deposited at GenBank; with accession numbers HQ407230 and HQ641258 respectively (Table 1). Using BLAST search (www.ncbi.nlm.nih.gov/BLAST) of the obtained sequence, the culture exhibited maximum 99% similarity. The 16S rDNA gene is the most widely accepted gene employed for bacterial classification and identification. Signature nucleotide of 16S rDNA gene allows classification even if a particular sequence has no match in the database. PCP degradation by *Pseudomonas* sp. and *Enterobacter* sp. isolated from industrial dump sites, have been earlier reported[8, 9].

Rhizoplane population

The rhizoplane population of MAZ 1 and MAZ 2 were estimated on Luria agar (LA)

Table 1. Percent homology 16S rDNA sequence of PCP degrading bacterial strains

Strain	Organism with most similar sequence	Similarity (%)	Acc No*
MAZ 1	*Enterobacter asburiae*	99 %	HQ407230
MAZ 2	*Pseudomonas sp*	99 %	HQ641258

*Acc. Nos. obtained from the GenBank National Center for Biotechnology Information (NCBI).

Table 2. Population of *lac Z* gene tagged PCP degrading bacteria on roots of maize

Treatments		Bacterial population (CFUX10^4 / g roots)							
		Total population (DAS)				*lac Z* population (DAS)			
		10	30	45	60	10	30	45	60
T1	Uninoculated	12.71	68.14	53.86	87.57	0.00	0.00	0	0
T2	MAZ1::*lac Z*	19.29	150.71	82.29	105.57	15.57	16.00	16.86	16.29
T3	MAZ 2::*lac Z*	10.57	87.57	92.29	111.14	16.14	17.00	31.71	19.00
SEM±		2.44	15.97	7.56	25.28	3.77	1.79	2.35	1.91
CD	1%	NS	65.01	30.79	NS	15.37	7.32	9.59	7.80

Table 3. Root and shoot length of maize plants as influenced by inoculation with lac Z gene tagged PCP degrading bacteria

Treatments		Root Length (cm per plant)				Shoot length (cm per plant)			
		10 DAS	30 DAS	45 DAS	60 DAS	10 DAS	30 DAS	45 DAS	60 DAS
T1	Uninoculated	21.79	25.80	27.61	33.53	11.73	21.43	41.94	45.40
T2	MAZ 1::lac Z	20.63	22.71	30.43	38.93	12.40	20.64	36.57	45.00
T3	MAZ 2 :: lac Z	15.64	24.04	31.36	40.20	11.64	21.29	42.31	51.79
	SD±	1.12	1.24	0.69	1.41	0.77	0.21	1.24	1.45
	CD 1%	4.57	NS	2.80	5.73	NS	NS	5.04	5.89

Table 4. Total plant biomass of maize plants as influenced by inoculation with *lac Z* gene tagged PCP degrading bacteria

Treatments		Fresh Root biomass (g per plant)				Fresh Shoot biomass (g per plant)				Fresh Total plant biomass (g per plant)			
		10DAS	30DAS	45DAS	60DAS	10DAS	30DAS	45DAS	60DAS	10DAS	30DAS	45DAS	60DAS
T1	Uninoculated	0.80	1.74	2.39	2.89	1.87	7.47	22.90	26.47	2.67	9.21	25.29	29.36
T2	MAZ1 :: *lac Z*	0.87	1.84	2.39	2.53	1.84	10.33	20.26	29.03	2.73	12.17	22.65	31.56
T3	MAZ2 :: *lac Z*	0.53	1.64	2.76	3.31	1.54	10.73	26.31	32.74	2.07	12.37	29.07	36.05
SD±		0.07	0.19	0.31	0.13	0.07	0.62	4.80	1.23	0.14	1.50	1.05	1.20
CD 1%		0.30	NS	NS	0.52	0.30	2.53	NS	5.00	0.57	6.09	4.29	4.88

containing X-gal and IPTG (Table 2). Since the introduced strain was tagged with *lac Z* gene, it appeared blue on the medium and the natural bacterial colonies were creamish white.

In general, it was observed that the total bacterial population was significantly higher in the treatments inoculated with the tagged organisms (Table 2). Between T2 and T3, T3 (inoculated with MAZ 2::*lac Z),* resulted in higher total population at 45 and 60 DAS. The population of MAZ 2:: *lac Z* on maize roots remained higher than MAZ 1:: *lac Z,* on all days of sampling, indicating its superiority in terms of root colonization ability. Especially at 45 DAS, the population of MAZ 2:: *lac* Z accounted for about one-third of the total population (31.71×10^4 as against 92.29×10^4 per g root) .

Plant growth parameters

All the growth parameters namely, root length, shoot length; fresh root and shoot biomass and total biomass were significantly higher in the plants inoculated with MAZ 2::*lac Z* (Tables 3 and 4). Inoculation with MAZ 2::*lac Z* produced the highest plant height (51.79 cm per plant) and biomass (36.05 g per plant) at 60 DAS, thus, again showing the superiority of the strain. MAZ 2:: *lac Z* showed higher root colonization ability, which has reflected on higher growth and biomass. MAZ 1 and MAZ 2 strains have already been shown to produce IAA and thus characterized as PGPR strains[10].

CONCLUSION

The PCP degrading *Enterobacter asburiae* MAZ 1::*lac Z* and *Pseudomonas* sp. MAZ 2::*lac Z* promoted growth and biomass of maize. However, *Pseudomonas sp.* MAZ 2 was found to be superior with higher root colonization ability and plant growth promoting potentials as assessed by *lac* Z gene tagging experiments.

REFERENCES

1. Dams RI, Paton GI, Killham K., Rhizomediation of pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. *Chemosphere*, 2007; **68**: 864-870
2. Singh S, Chandra R, Patel DK, Rai V., Isolation and characterization of novel *Serratia marcescens* (AY927692) for pentachlorophenol

degradation from pulp and paper mill waste. *World J Microbiol Biotechnol*., 2007; **23**:1747–1754.

3. Siciliano, S.D., Germida, J.J., Enhanced phytoremediation of chlorobenzoates in rhizosphere soil. *Soil Biol Biochem* 1999; **31**: 299–305
4. Kuiper, I., Bloemberg, G.V., Lugtenberg, B.J.J., Selection of a plant– bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. *Mol. Plant–Microbe Inter.*, 2001; **14**: 1197–1205.
5. Avita K. Marihal, Jagadeesh K.S. and Sarita Sinha., Biodegradation of PCP by the rhizobacteria isolated from Pentachlorophenol - tolerant crop species. *Int. J. Environ. Sci. Engg.*, 2009; **1**(4): 189 – 193.
6. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST., *Bergey's Manual of Determinative Bacteriology*, 9th edn. Williams & Wilkins, Baltimore.
7. Rajkumar Shalini, Anil Kumar, Saxena and Kripa Shankar Jauhri. Influence of fertilizer level on establishment of *lac Z* tagged mutants of *Pseudomonas striata* in soybean rhizosphere. *World J. Agric. Sci*., 2008; **4**: 874-878.
8. Karn SK, Chakrabarty SK, Reddy MS., Pentachlorophenol degradation by *Pseudomonas stutzeri* CL7 in the secondary sludge of pulp and paper mill. *J Environ Sci*., 2010; **22**:1608-1612.
9. Karn, SK, Geetanjali and Xiangliang Pan., Pentachlorophenol remediation by *Enterobacter* sp. SG1 isolated from industrial dump site. *Pakistan J. Biol. Sci.*, 2011; **14**: 22-26.
10. Marihal, Avita K., S. M. Pradeep, K.S. Jagadeesh and Sarita Sinha, Rhizoremediation mediated by Plant Growth Promoting Rhizobacteria (PGPR) by In: Bioremediation of Pollutants, edited by DK Maheswari and RC Dubey, Published by IK Intl. Publishing House Pvt. Ltd., New Delhi, 2012.