Int.J.Curr.Microbiol.App.Sci (2018) *7*(7): 2800-2806

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 07 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.707.327

Molecular Diversity of *Azotobacter* sp. Isolates from Rhizosphere of Tropical Tasar Silkworm Host Plants

Manjappa*, I.G. Prabhu, Niranjan Kumar and A.K. Sinha

Central Tasar Research and Training Institute, Ranchi, Jharkhand, India

**Corresponding author*

Keywords

Molecular diversity, *Azotobacter* sp., Rhizosphere, Tasar Silkworm

Article Info

Accepted:
20 June 2018
Available Online:
10 July 2018

A B S T R A C T

Azotobacter is an aerobic, free living bacteria fixes atmospheric nitrogen in soil besides producing plant growth promoting substance. Eight Azotobacter isolates were isolated and purified from different tasar rearing regions of West Singhbhoom, Jharkhand on Jenson media by using serial dilution technique. Molecular diversity among these isolates was analysed using RAPD primers. Of the 20 decamer primers screened, 10 primers amplified clear and distinguishable fragments and yielded a total of 103 fragments, of which 101 fragments are polymorphic (98%). Unweighted neighbour joining dendrogram based on Dice indices divides eight Azotobacter isolates in two major clusters. Dissimilarity matrix showed a maximum difference of 76% between the isolate from Hatghamaria (Azo 21-1) and Talaburu (Azo 29-1) and minimum difference of 39% between the isolates derived from the same region, Kharswan (Azo 14 and Azo 15-1). Thus RAPD marker analysis was found to be easy and rapid in assessing diversity among Azotobacter isolates.

Introduction

India is the only country producing all five known commercial silks *viz.*, Mulberry, Tropical Tasar, Oak Tasar, Eri and Muga. India is producing 28,472 MTs of raw silk that includes 20,434 MTs of mulberry silk, 2,818 MTs of Tasar silk, 5,054 MTs of Eri silk and 166 MTs of Muga silk (CSB, 2015-2016). Among five types of silks tropical tasar silk is unique in its sheen, durability and thermal properties (Gangwar *et al.*, 2007) and has been an integral part of traditional culture since centuries. In tropical tasar culture, silkworm cocoons are collected from forest plants (*Shorea robusta* or *Terminalia tomentosa*) or silkworm is reared on forest/systematic plantation (*T. tomentosa* and/or *T. arjuna*). As tasar culture is forest based ecofriendly cottage industry, it forms an integral part of tribal economy, employing 1.25 lakhs of tribal families residing in forest associated rural areas of Jharkhand, Bihar, Chhattisgarh, Orissa, Andhra Pradesh, Maharashtra, West Bengal and Uttar Pradesh (Reddy *et al*., 2015). In recent decades tasar culture is shifting from forest based cocoon collection (subsistence culture) to intensive silkworm rearing under systematic/natural plantation (commercial culture).To facilitate tribal tasar farmers, systematic plantations (*T. arjuna*) have been raised in forest land, government waste land,

marginal land and non-agricultural landsby various government projects *viz*., Cluster development and cluster promotion programmes (CDP, CPP), Mahila Krishi Shashktikaran Pariyojna (MKSP) and Swarnjayanti Gramin Swarojgar Yojna (SGSY) (Gargi *et al*., 2015). Hence the area under systematic plantation is being increased. Conversely, under commercial rearing, the quality of cocoon is being decreased as compared to forest based cocoons. Poor leaf quality under commercial rearing could be the major factor influencing cocoon quality, which is associated with decreasing soil nutrient contents through continuous silkworm rearing. Decreasing soil nutrient status is primary cause of poor leaf quality (Subbaswamy *et al*., 2004). To maintain soil nutrient status, external application of fertilizers/manure is not a feasible approach due to two reasons. First, tribal farmers are not affordable to manures/fertilizers due to their poor economic status and it increases input cost. Second, majority of tasar plantations are located in forest area, where application of chemical fertilizer is not advisable. Under this circumstance, application of biofertilizer is an ecofriendly, sustainable and feasible approach, which is affordable to farmers in low cost.

Among plant growth promoting rhizosphere microorganisms (PGPMs), *Azotobacter* is a free living bacteria known to fix atmospheric Nitrogen and produces plant growth promoting substances *viz*., hormones, vitamins and amino acids. Besides, they act as biocontrol agent against soil borne pathogens by secreting antifungal substances, hydrogen cyanide and siderophores (Gurikar *et al*., 2015). In the selection of bacterial inoculum to crop plants, indigenous isolates are more preferred as they can adapt to local ecosystem and more competitive than non-indigenous of inoculum (Bhattarai and Hess, 1993).Therefore the objectives of present study were to isolate *Azotobacter* strains from tasar host plant rhizosphere of different tasar rearing regions and to compare diversity among indigenous strains.

Materials and Methods

Collection of soil samples

For the isolation of *Azotobacter* bacterial isolates rhizosphere soil samples were collected from different tasar rearing regions of West Singhbhoom region of Jharkhand state (Table 1). Soil samples were collected from both forest as well as block plantations covering *T. tomentosa* and *T. arjuna* host plants. In each sampling location three rhizosphere soil samples were collected by following three-point sampling method (Malik *et al*., 1982) at soil surface layer, one feet depth and one meter apart from the trunk of host plant. Three rhizosphere soil samples were then mixed and stored immediately in polythene cover at 4°C, which were later transported to lab. Soil samples were processed after removing debris and pebbles. The soil pH and electrical conductivity was also been recorded using pH meter and conductivity meter respectively.

Isolation and purification of strains

Azotobacter sp. strains were isolated from rhizosphere soil samples by serial dilution technique, where 100 µL of10^{-4} and 10^{-5}inoculum was inoculated on Jenson agar (Jensen, 1951) (sucrose 20.0g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4$ 0.1 g, Na_2MoO_4 0.005 g, $CaCO_3$ 2.0 g, Agar 15.0 g, distilled water 1 L) by pour plate method. Petri dishes were then incubated at 30°C for 48 hr. Based on colony morphology different isolates were purified by streaking and single colonies were stored in slants for further use. Among several isolates, one isolate from each sampling location (8 isolates) was selected for molecular diversity.

Genomic DNA isolation

For the genomic DNA extraction Eight *Azotobacter* bacterial isolates were inoculated in Luria broth and incubated overnight at 30°C. About 2 ml of liquid culture was centrifuged at 5000 rpm for 10 minutes and supernatant was removed. To wash pellet,500 µl of Millipore water was added and centrifuged at 10,000 rpm for 10 min, this step was repeated twice. Bacterial genomic DNA was isolated by using DNA isolation kit (Bioline, USA) as per the given protocol. The DNA was quantified on 0.8% Agarose gel using a known quantity of λDNA (10 ng/µl) as a standard before use in subsequent PCRs.

PCR amplification of DNA using RAPD markers

Eight isolates were screened for their diversity using RAPD markers. Initially 20 RAPD primers were screened to test the amplification, of which 10 (Table 2) were shown clear and distinguishable bands. PCR reactions were performed in a final volume of 25 µL containing 30 ng of template DNA, 200 µM dNTP, 1.5 mM $MgCl_2$, 2µL of 10X reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.05% (v/v) NP40 and 0.05% (v/v) Triton X-100), 2.5 µL of 5 pmole primer and 1 unit of Taq DNA polymerase (Sigma Aldrich Chemicals, Bangalore, India). Polymerase chain reactions (PCR) were performed in Techne TC-512 thermocycler (Techne, TC-512, USA) with the program consisting of initial denaturation at 94°C for 5 min followed by 40 cycles each consisting of denaturation at 94°C for 1 min, primer annealing at 36°C for 1 min, primer extension at 72°C for 2 min and a final extension of 72°C for 10 min. The amplified PCR products were size fractioned by electrophoresis on 1.5% Agarose gel (UAB, Affymetrix, Inc.) in 1x in 1 × Tris borate EDTA buffer and gels were stained with Ethidium Bromide (0.5 µg/ml).A 1kb standard molecular marker (MassRuler DNA Ladder Mix, Thermo Fisher Scientific, USA) was used in every electrophoretic run and gel was visualized in UV-Trans illuminator (*genetix*, Biotech Asia Pvt. Ltd.).

Molecular data analysis

Each RAPD markers across eight *Azotobacter* isolates were scored by comparing standard size marker run along with the gel and scoring was recorded in binary system ('1' for presence and '0' for absence). Binary data was used in the computation of Dice indices (Perrier *et al*., 2003), which was calculated using following formula.

$$d_{ij} = \frac{b + c}{2a + (b + c)}$$

Notations:

dij: dissimilarity between units i and j
xi, xj: variable values for units i and j
a: number of variables where xi = presence and xj = presence
b: number of variables where xi = presence and xj = absence
c: number of variables where xi = absence and xj = presence

Dissimilarity matrix was developed by using Dice indices with a bootstrap value of 10,000.Dissimilarity matrix was then used to construct a dendrogram using Unweighted Neighbour Joining method of clustering using DarWin v.6 software (Perrier *et al.*, 2003). To evaluate the robustness of dendrogram and its confidence limits, bootstrapping with 10000 replication was performed.

Results and Discussion

In the preparation of biofertilizer for any crop, indigenous bacterial isolates are preferred due to better adaptation to their crop ecosystem and good agronomic performance. Hence, in present study *Azotobacter* isolates were isolated from rhizosphere soil samples of tasar

host plants (*T. arjuna* and *T. tomentosa*). The rhizosphere soil samples were collected from different tasar rearing areas of West Singhbhoom, Jharkhand state. About 18 Azatobacter isolates were isolated from eight locations. One isolate from each location was analysed to understand diversity among the isolates.

Initially twenty RAPD primers of arbitrary sequences were screened on two isolates DNA, out of which 10 primers were selected based on their distinct and clear amplification profiles (Table 2). Polymorphic gel profiles of RAPD primer PWD6 is shown in figure 1. Screening of primer is essential to save time and cost, and to reject primers which are not informative for the analysis (Prakash *et al*. 2002.). A total of 103 clear and readable RAPD fragments were produced from 10 RAPD primers (Table 2), of which 101 (98.05%) fragments were polymorphic. The number of RAPD fragments per primer ranged from 5 (OPAJ4 and OPAJ07) to 21 (OPA21) with an average of 10.3 fragments per primer.

Table.1 Details of *Azotobacter* isolates used in the molecular diversity

Isolate Code	Place of collection	Host plant	Plantation type
Azo 14-1	Karsawan(Farmers field),	Arjun	Block plantation
Azo 15	Karsawan (Farmers field)	Asan	Block plantation
Azo 21-1	REC, Hatghamaria	Arjun	Block plantation
Azo 23	REC, Hatghamaria	Asan	Forest
Azo 26-1	PPC, Debrasai	Arjun	Forest
Azo 26-2	PPC, Debrasai	Arjun	Forest
Azo 29-1	Thalaburu (Farmers field)	Arjun	Block plantation
Azo 31	CTR&TI, Ranchi	Arjun	Block plantation

Table.2 The sequence and level of polymorphism of selected polymorphic primers in *Azotobacter*

Sl. No.	Primers	Sequence (5'-3')	No. of amplified fragments	No. of monomorphic bands	No. of polymorphic bands
1	OPW 6	AGGCCCGATG	14	0	14
2	OPW 9	GTGACCGAGT	9	0	9
3	OPW 16	CAGCCTACCA	15	0	15
4	OPK 10	GTGCAACGTG	7	0	7
5	OPKO4	CCGCCCAAAC	10	0	10
6	OPA 21	GAGGACTAGG	21	1	20
7	OPA 11	CAATCGCCGT	6	1	5
8	OPAJ 4	GAATGCGACA	5	0	5
9	OPAJ 07	CCCTCCCTAA	5	0	5
10	OPAJ 15	GAATCCGGCA	11	0	11
	Total		103	2	101

Table.3 Genetic dissimilarity matrix of 8 *Azotobacter* isolates based on polymorphism of 10 RAPD markers

14-1	**0.000**							
15	0.394	0.000						
21-1	0.538	0.647	0.000					
23	0.500	0.528	0.610	0.000				
26-1	0.643	0.521	0.561	0.619	0.000			
26-2	0.686	0.508	0.500	0.544	0.571	0.000		
29-1	0.688	0.590	0.760	0.718	0.652	0.647	0.000	
31	0.507	0.518	0.708	0.627	0.541	0.618	0.550	0.000

Figure.1 RAPD profiles of Eight *Azotobacter* isolates for OPW6 primer. Lane 1-8 contains amplification profiles of eight isolates. Lane M: 1kbstandard DNA marker

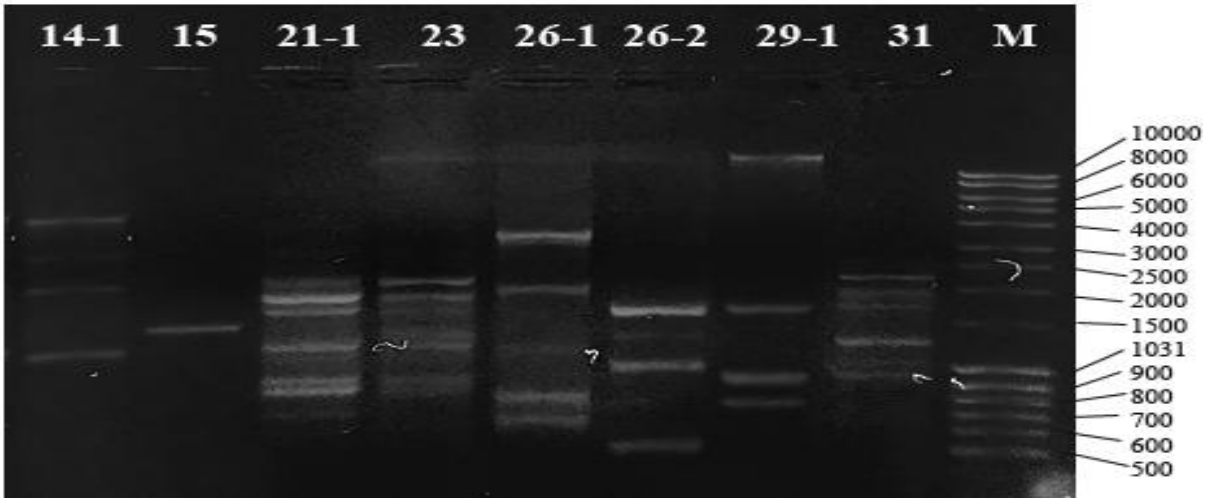
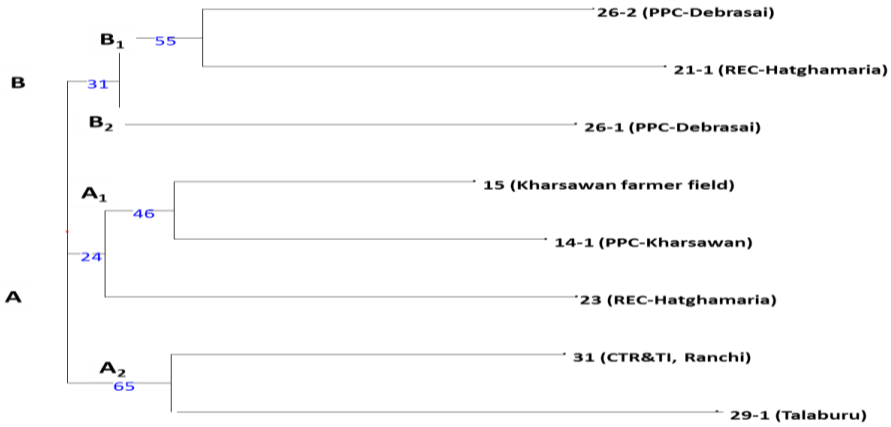


Figure.2 Cluster analysis. Dendrogram showing RAPD-marker-based genetic relationships among 8 *Azatobacter* isolates and grouping them into two clusters 'A' and 'B' with five and three isolates, respectively



Similarly Anatha Naik *et al.*, (2008) also obtained high polymorphic RAPD fragments (84.4%) among ten *Azotobacter crococcum* isolates isolated from different agro-climatic zones of Karnataka. The polymorphic RAPD profiles of eight *Azotobacter* isolates were used in computation of dissimilar matrix using Dice indices (Perrier *et al*., 2003). The dissimilarity among eight *Azotobacter* isolates, as revealed from dissimilarity matrix varies from 0.394 to 0.760 (Table 3). Isolates from Talaburu (Azo 29-1) and REC, Hatghamaria (Azo 21-1) were found to be more diverse, whereas isolates from PPC Kharswan (Azo 14) and Farmer field, Kharswan (Azo 15-1) are less diverse (Table 3).

Unweighted neighbour joining dendrogram constructed from dissimilarity matrix has clustered eight *Azotobacter* isolates into two major clusters i.e. cluster A and cluster B and four sub clusters i.e. A_1, A_2, A_3 and A_4 (Fig. 2). It is noticed that, isolates derived from closer geographical regions have clustered together in cluster B(Azo 21-1, Azo 26-1 and Azo 26-2) and in sub cluster A_1 (Azo 14-1 and Azo 15).

All the *Azotobacter* isolates in the present study were collected from different geographical locations with diverse agro-climatic conditions and hence showed relatively high polymorphism as revealed by dissimilarity matrix and dendrogram. Hence, RAPD–PCR based DNA fingerprinting of the genomic DNA with arbitrary primers is a rapid and sensitive method for the detection of genetic variation among different isolates of *Azotobacter*.

Acknowledgement

The financial support (Project No. PPA-4717) of the Central Silk Board (CSB), Ministry of Textiles, Govt. of India is gratefully acknowledged. The Silkworm breeding and Genetics and Silkworm pathology departments are acknowledged for laboratory support.

References

Anantha Naik, T.,N. Earanna,P. Narayanaswamy and Luke, S. 2008. Molecular characterization of *Azotobacter chroococcum* strains isolated from different agro-climatic zones of Karnataka, India. Dynamic Soil, Dynamic Plant. 2(2): 73-76.

Bhattarai, T. and Hess D., 1993. Yield responses of Nepalese spring wheat (*T. aestivum* L.) cultivars to inoculation with *Azospirillum* spp. of Nepalese origin. Plant and Soil. 151: 67-76.

CSB (Central Silk Board), 2016. Note on the performance of Indian silk industry & functioning of central silk board.

Gangwar, S. K., R. Kumar and Srivastava, S. K., 2007. Tasar culture- Principles and practices: Tasar silkworm biology, Vol-II. Central Tasar Research and Training Institute, Central Silk Board, Ministry of Textiles, Piska Nagri, Ranchi.

Gargi, R.Kumar, M.Deka, H.Yadav and Alok Sahay, 2015. Crossability studies in *Terminaliaarjuna* and *T. tomemtosa*. *International Journal of Applied Biology and Pharmaceutical Technology*.6(3): 211-216.

Gurikar C., M.K. Naik and Sreenivasa, M.Y., 2016. *Azotobacter*: PGPR Activities with special reference to effect of pesticides and biodegradation. D.P. Singh *et al.*, (eds.), Microbial inoculants in sustainable agricultural productivity. 229-244. doi: 10.1007/978-81-322-2647-5_13

Jensen, V., 1951. Notes on the biology of *Azotobacter*. *Proc. Soc. Appl.*

Bacteriol., 74: 89–93.

Malik, K.A., Zafar, Y., Hussain A., *et al*., 1982. Associative dinitrogen fixation in *Diplachne fusca* (Kallar grass). Biological nitrogen fixation technology for tropical agriculture. 503-507.

Perrier, X., A. Flori,F. Bonnot, 2003. Data analysis methods. In: Hamon, P., M.Seguin, X. Perrier, Glaszmann, J. C. Ed., Genetic diversity of cultivated tropical plants. Enfield, Science Publishers. Montpellier. 43 - 76.

Reddy, P.M. Muniswamy, V.P. Gupta and Lokesh, G., 2015. Impact of trainers training programmes of integrated skill development scheme (ISDS) in tasar technologies. Global Journal of BioScience and Biotechnology. 4(1): 17-20.

Subbaswamy, M.R., N.R.Singhvi, B. V. Naidu, M. M. Reddy, H. Jayaram, and Suryanarayan, N. 2004. Effect of source of nitrogen on phosphorus uptake and arginine content in mulberry. Indian J. Sericulture. 40: 182–184.

How to cite this article:

Manjappa, I.G. Prabhu, Niranjan Kumar and Sinha, A.K. 2018. Molecular Diversity of *Azotobacter* sp. Isolates from *Rhizosphere* of Tropical Tasar Silkworm Host Plants. *Int.J.Curr.Microbiol.App.Sci.* 7(07): 2800-2806. doi: https://doi.org/10.20546/ijcmas.2018.707.327