CONFIRMING LOCATION OF NITROGEN FIXING GENES ON PLASMIDS IN *RHIZOBIUM* ISOLATED FROM *PISUM SATIVUM*

Balaji Hajare and Avinash Ade[1]

Department of Botany, Dr. B. A. M. University, Aurangabad, 431004 (MS) India
[1]Department of Botany, University of Pune, Pune 411007 (MS) India.

ABSTRACT

To confirm the location of the nitrogen fixing genes whether on the plasmids or on the genomic DNA the Rhizobial isolates isolated from pea (*Pisum sativum*) were treated with acridine orange with various concentrations and fixed nitrogen was estimated from the media in which these rhizobia were grown. There was no significant difference in between the cured and non cured strains of the *Rhizobium* which proved that the nitrogen fixing genes are not plasmid borne but these are located on the genomic DNA.

Key words: *Rhizobium*, plasmids, curing, nitrogen fixation

INTRODUCTION

Biological Nitrogen Fixation refers to the process of micro-organisms fixing atmospheric nitrogen, mostly within subsoil plant nodules, and making it available for assimilation by plants. Nitrogen supply is a key limiting factor in crop production. *Rhizobium* is the most studied and important genus of nitrogen fixing bacteria. It is able to fix atmospheric nitrogen in symbiosis with some types of leguminous plants. Biofertilizers have the potential of increasing yields of legumes as well as reducing the use and cost of chemical nitrogen fertilizers. The *Rhizobium* involved in the nitrogen fixation with leguminous plants carries an extra chromosomal DNA in the form of plasmid. The present study deals with the effect of removal of such plasmids on the total nitrogen fixation by Rhizobial species with in-vitro conditions.

Plasmids are self-replicating, extra chromosomal DNA molecules found in virtually all bacterial species (Birnboim *et al*., 1979). In nature, plasmids occur in exuberant profusion, varying in structure, size, mode of replication, number of copies per bacterial cell, ability to propagate in different bacteria, transferability between bacterial species.

Most prokaryotic plasmids are double stranded circular DNA molecules; however linear plasmids have been identified in both gram positive and gram negative bacteria. The smaller plasmids make use of the host cell's own replicative enzymes in order to make copies of them, whereas some of the larger ones carry genes for special enzymes that code for special enzymes that are specific for plasmid replication. A few plasmids are also able to replicate by inserting themselves into the bacterial chromosome. These integrative plasmids or episomes may be stably maintained in this form through numerous cell divisions (Hung *et al*., 1999)

Theoretically curing is the way to eliminate the plasmid from given microbial cell. This approach is encouraged by several *in vitro* and *in vivo* studies that show that plasmid associated characteristics of the organisms can be reversed by various ways since four decades (Molnar *et al*., 1977) by the ways such as use of detergents like SDS (Tomoeda *et al*., 1968), mutagenic agents like Acridine dyes (Hirota, 1960), Ethidium bromide (Watanabe *et al*., 1964) and Phenothiazines of which except last all are used in in-vitro studies.

Acridine dyes acts as a DNA intercalating agents, as they consist of three membered aromatic ring they can occupy the position of two base pairs connecting two strands of DNA in DNA double helix. Thus the intercalation of these dyes in place of base pair mutates the DNA during its replication. If the repair of such damaged DNA is not carried out during the preceding generation these mutations remains permanent and keeps inheriting them. It was observed that these dyes mutate both chromosomal and extra-chromosomal DNA equally but as the size of extra-chromosomal DNA is small in comparison with chromosomal DNA it gets mutated early.

Plasmid curing experiments shows very wide range of applications like study of extra chromosomal inheritance in bacteria, understanding the mechanism of bacterial recombination's, improvements of bacterial strains by the use of genetic engineering. In the present research we focused our efforts of plasmid curing towards genetic improvement of rhizobial strains selectively infecting *Psium sativum.* Upon the successful curing of plasmid the authors will be continuing the improvements in selected rhizobial strains through various ways like transformation, genetic recombination, random mutagenesis etc.

In the present work we have studied the effect of plasmid removal by using mutagenic agent acridine orange. To analyze it the *Rhizobium* was cultivated in nitrogen containing medium Yeast Extract Mannitol medium (YEM), where we have estimated the total nitrogen in the media before rhizobial inoculation by kjeldahl method (Sreenivasan and Sadasivan, 1939). There after the strain was subjected for plasmid curing using said mutagen and the cured strains were checked for plasmid curing using plasmid isolation followed by agarose gel electrophoresis. The plasmid cured strains were studied for their wild type characters, re-cultivated in YEMA medium and were checked for their effectivity of nitrogen fixation after plasmid curing.

MATERIAL AND METHODS

Isolation of *Rhizobium* strains

The experiment of plasmid curing was started with the isolation of rhizobial strains from *Psium sativum* by serial dilution method. The roots from pea plants were collected, surface sterilized, squeezed in sterile distilled water aseptically and finally the extract was serially diluted by tenfold dilution method. Each dilutions was then spreaded on CRYEMA (Congo Red Yeast Extract Manitol Agar) with the final volume of 100 micro liter and the plates were incubated for 40-72 hours at 37 ^{0} C. Upon incubation each plate was observed for the characteristic growth and features of rhizobial strains. Out of the various isolates of *Rhizobium* obtained by this method two of the morphologically dissimilar strains with unique colony characteristics were selected for further experiment. Further the isolates were studied for their GPA (Glucose Peptone Agar) test and salt tolerance test also.

After confirming the isolated strains, as the members of rhizobial genus, the strains were studied for their rate of nitrogen fixation by inoculating them in YEM media using kjeldahl method. After studying the rate of nitrogen fixation for 96 hours the strains were further taken for plasmid curing.

Plasmid curing

To start with the plasmid curing experiment both the selected strains were actively grown in the synthetic *Rhizobium* medium without the source of nitrogen. This actively grown culture was then equally divided into two subsets. Of these two subsets one set was directly taken for plasmid isolation experiment by using mini prep method whereas one set was subjected for plasmid curing by using acridine orange in increasing concentration with the difference of 100 ug/ml as the agent of plasmid curing incorporated in *Rhizobium* media without source of nitrogen. The *Rhizobium* plated thus prepared with the varying concentrations of acridine orange were spreaded with 100 micro liter of both the Rhizobial isolates and the plates were incubated for 72-96 hours at 37 ^{0} C in inverted position.

Upon incubation the plates were observed for the formation of colonies. Each colony was then selected and enriched in *Rhizobium* media and was screened for the presence of plasmid DNA by using mini prep method **(**Birnboim, 1983; Carlson *et al*., 1995 and Murphy *et al*., 1999**)**. Upon confirmation that few of the isolates have lost their plasmid at certain concentrations of used mutagen these strains were selected and were re-cultivated in YEM media and were analyzed for the rate of nitrogen fixation.

RESULTS AND DISCUSSION

The different rhizobial strains were isolated on YEMA (Yeast Extract Mannitol Agar) plate. The five isolates were observed randomly. Out of all, some showed circular whitish gummy colonies and some showed rhizoid, sticky colonies. On CRYEMA plates, white as well as reddish colored colonies were observed after 4 to 5 days of incubation period at 28-30^{o} C. From GPA test, it was observed that no colonies appeared on GPA medium. No growth was observed on YEMA medium in salt tolerance test.

A colony was formed after 3-4 days of incubation on YEMA plates. Of these two strains based on their fast growing feature were selected for future study**.**

Out of the five isolates three isolates have shown the higher nitrogen fixation and were selected for further study. The total rate of nitrogen fixation by each selected strain was as shown in table 2**.** After checking of rate of nitrogen fixation the strains were subjected for plasmid isolation (Fig 1) followed by plasmid curing, each stain showed the plasmid curing at differential concentration of mutagen as strain A 800 ppm, Strain B 300 ppm and Strain C 500 ppm respectively (Fig 2,3,4). Once the plasmid curing was confirmed by agarose electrophoresis the comparison was made in reference to rate of nitrogen fixation before and after plasmid curing (Table 2).

Rhizobia are the most popular kind of biofertilizers used in common practice today for enhancing the fertility of farming soil. The growing needs of high level of nitrogen fixation require some genetic improvements in rhizobial strains towards high efficiency nitrogen fixation. As a one step towards the said statement we have initiated our efforts with the designing of experiment for removal of pre-existing plasmids in the selected *Rhizobium* strains. By this exercise the confirmation of the location of nitrogen fixing genes whether on the plasmids or on the genomic DNA. The transfer of the efficient plasmids will lead to the improvement in the nitrogen fixing ability of other *Rhizobium* strains. In the present study the efforts were taken to check the effect of plasmid removal on the rate of nitrogen fixation by rhizobial strains.

Table 1: **Colony characteristics of isolates after the treatment of acridine orange.**

Sr. No.	Colony character	Isolates 1	Isolates 2	Isolates 3	Isolates 4	Isolats 5
1	Size	1-2 mm	2mm	2-4mm	1-2mm	2-4mm
2	Shape	Circular	Circular	Rhizoid type	Circular	Rhizoid type
3	Colour	Colourless	Colourless	Colourless	Colourless	Colourless
4	margin	Entire	Entire	Entire	Irrgular	Irregular
5	Elevation	Raised	Flat	Raised	Flat	Raised
6	Surface	Smooth	Smooth	Smooth	Smooth	Smooth
7	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
8	Consistancy	Non Sticky	Sticky	Sticky	Sticky	Sticky
9	Emulsifialbilty	Emulsifiable	Non- emul	Non- emul	Non-emul	Non-emul
10	Motility	Motile	Nonmotile	Motile	Motile	Motile
11	Odour	Musky	-	Musky	-	Musky
12	Grams nature	Gram –ve	Gram -ve	Gram -ve	Gram -ve	Gram -ve

Table 2: comparative analysis of amount of nitrogen fixed in mg/lit before and after plasmid curing

Sr. No.	Strains	Total nitrogen in media mg/lit	Amount of nitrogen fixed (after hrs) in mg/lit							
			Before plasmid curing				After plasmid curing			
			24 Hrs	48 Hrs	72 Hrs	96 Hrs	24 Hrs	48 Hrs	72 Hrs	96 Hrs
1	Strain A	1913	45	153	223	278	38	148	217	265
2	Strain B	2042	58	179	217	286	49	165	209	282
3	Strain C	1848	48	182	233	267	44	174	224	259

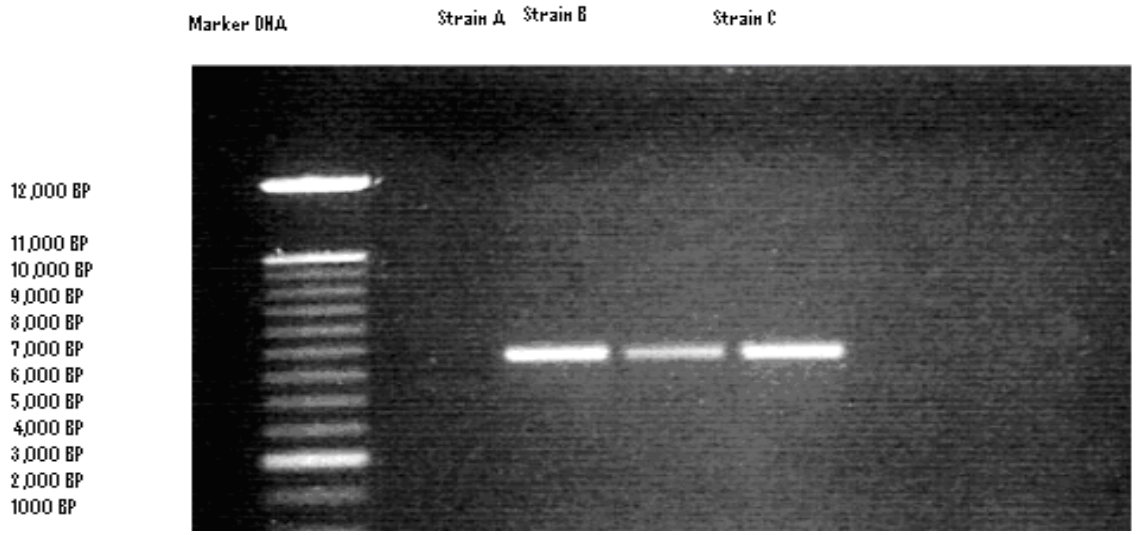

Fig.1 Plasmid isolation by mini-prep method

Fig: 2.1= DNA Marker, 2= Plasmid DNA without RNase treatment, 3= Plasmid DNA with RNase Treatment, 4, 5, 6, 7 = Plasmid Cured DNA. Final Concentration of Acridine Orange 800 ppm. strain A

Fig: 3.1= DNA Marker, 2= Plasmid DNA without RNase treatment, 3= Plasmid DNA with RNase Treatment, 4, 5, 6, 7 = Plasmid Cured DNA. Final Concentration of Acridine Orange 300 ppm Strain B

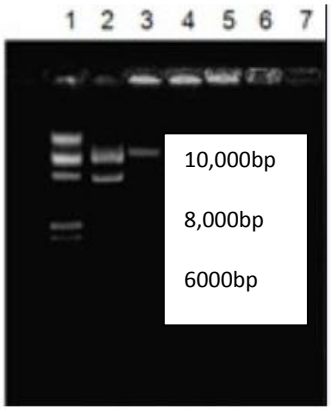

Fig: 4. 1= DNA Marker, 2= Plasmid DNA without RNase treatment, 3= Plasmid DNA with RNase Treatment, 4,5,6,7= Plasmid Cured DNA. Final Concentration of Acridine orange 500 ppm Strain C.

Several chemical curing agents, including acridine dyes, novobiocin, acriflavin, ethidium bromide, and SDS, have been successfully used in the curing of bacterial plasmids (Caro *et al*., 1984). Of these compounds we have selected acridine orange where it was noted that the final concentrations of this dye required for the curing of plasmid for each strain was 800 ppm strain A, Strain B 300 ppm, Strain C 500ppm respectively.

The present study shows that though the Rhizobial species carries the extra chromosomal DNA in the form of plasmid DNA which is expected to play certain role in nitrogen fixation, our study reveals that the removal of plasmid from Rhizobial species does not have drastic effect on the rate of total nitrogen fixation by Rhizobial strains in-vitro condition hence genes for nitrogen fixation in the Rhizobium are present in the genomic DNA.

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