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Full Length Research Paper

Tn5 tagging biomonitoring of *Rhizobium* inoculants

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***Sinorhizobium freddi* R0132, which forms nitrogen fixing nodules in association with *Vigna radatia* L. and fix nitrogen symbiotically, was the bioinoculant used in this study. Drug resistant *S. freddi* R0132::*Tn*5 remained constant at 40°C higher levels temperature for about 35 days (5 weeks) from the date of inoculation in solarzied and autoclaved biomanure VC-MA. The *Tn*5 mutagenesis was carried by the random transposon mutagenesis. The population of drug resistant *S. freddi* R0132::*Tn*5 in these treatments was significantly higher as compared to control. The present study attempted to evaluate the role of the said bio-manure VC-MA as carrier for survival of improved drug resistant *S. freddi* R0132::*Tn*5 in all the carrier material tested as compared to sample at higher (37 - 40°C) temperature, and higher population of drug resistant *S. freddi* R0132::*Tn*5 were observed when stored at relatively lower temperature (28°C). It is therefore not recommended to store the mass culture of biofertilizers particularly at higher temperature.**

Key words: *Rhizobium*, transposon Tn*5* mutagenesis, biomanure.

INTRODUCTION

Indiscriminate and excessive use of chemical fertilizers have badly affected the soil properties with growing concern over food security and sustainable agriculture, the use of ecofriendly technologies for agriculture production has become imperative. Biofertilizers may play important role in reducing environmental hazards by supplementing organic / biological nitrogen means and thereby reducing the inputs of chemical fertilizers. Over use of chemical fertilizers have seriously deteriorated the soil properties. The procedure use for random transposon mutagenesis was that given by Selvaraj and Iyeri (1983) and was modified by Khanuja (1987) because it is one of the most powerful tools for initial localization of gene or gene cluster and for preliminary analysis of the organization of genome, efficiently in many gram negative bacteria including Pseudomonas. *Sinorhizobium freddi* R0132::*Tn*5, which forms nitrogen fixing nodules in association with *Vigna radatia.* L. and fix nitrogen symbiotically, was the bioinoculant used in this study. The over all objective of the present study was to develop a low cost as well as growth supporter carrier for *S. freddi* R0132::*Tn*5. NTG mutagenesis was carried out for development of marker drug resistant of *S. freddi* R0132::*Tn*5 and was used to tag *Rhizobium* cells by insertion of transposon in to genome which was used as a marker gene for monitoring the population of *S. freddi* R0132::*Tn*5. This biomanure could not efficiently support the bioinoculant *S. freddi* R0132::*Tn*5 as such, probably because of high microbial load existing in the biomanure. Biomanure VC-MA has been proved to be the most efficient carrier material compared with other carrier material tested viz; charcoal, vermiculite and FYM (Tomer et al., 1998). Further studies are needed to standardize the inoculums load, moisture content and temperature 40°C for improving the shelf life of biofertilizers. Transposable (Tn) elements conferring drug resistance, e.g. Kanamycin, nornycin etc can be simple transposons and also more complex ones. Simple transposons, or insertion sequences (IS), which carry only the genes necessary for their own transposition that is the genes encoding for the transposes protein and the inhibitor protein. They can be detected in two ways: first, the insertion sequences interrupt and inactivate gene into which they insert, and the second, they may contain

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promoters that allows RNA polymerase to transcribe and thus turn on adjacent and even distal genes. Complex transposons, on the other hand, contain one to several genes in addition to those encoded by the insertion sequences which are essential to the process of transposition; typically they carry genetic markers for antibiotics resistance (Watsons et al., 1992).

Transposition

The process of transposition is the movement of a transposable element from one locus (donor site) to another locus on a DNA molecule (target site) which typically has little or no homology with the transposable element. Clearly, transposition gives rise to rearrangement of the host genome, including deletions and inversions. Transposition is a rare recombination event since excessive rearrangement of the genomic DNA is lethal to the host. Transposition occurs at frequencies which are comparable to spontaneous mutation rates. For example, the frequency of transposition is about 1/100,000 per generation for Tn5 in *E. coli* compared to spontaneous mutation rates of about 1/1 million per generation. The low frequency can be attributed to tight regulation, inefficient transcription signals, inefficient translation signals, and or inefficiencies which are intrinsic to the transposition process itself. Generally the frequency of transposition depends on the nature of the transposon and especially the relative amounts of transposase protein to inhibit protein which are present in the host cell. The DNA encoding for these two proteins constitutes the simplest transposon structure. The mechanism of transposition may involve cut-and-paste mechanism in which the transposon is transferred to a different locus, leaving a gap, which is potentially lethal, at the donor site on a DNA molecule or by a conservative two-step replicative mechanism in which a co integrate (fused replicon) structure is formed as an intermediate.

Transposon Tn*5*

The transposon Tn5 is a complex transposon of about 5818 base pair. There are two nearly identical insertion sequences (IS50L and IS50R, both 1533 bp in length) which are inverted with respect to a central region of DNA encoding three antibiotics resistance genes: kanamycin (kan), streptomycin (str) and bleomycin (bleo) resistance. On the other hand, the IS50R is a fully functional transposable element, encoding two proteins essential for transposition. The transposase protein (Tnp) and an inhibitor of transposase (Inh). The transposase consists of 476 ammo acids while the inhibitor is lacking the first 55-amino acids on the N-terminal end of the transposase such that the inhibitor has only 421-amino acids in length; both polypeptides are read from the same reading frame and share the same carboxyl-terminal. The IS50L differs from the IS50R at a single base pair such that it contains an ochre codon that results in synthesis of inactive proteins which are counterparts to the transposase and the inhibitor proteins (Wiegand et al., 1992). These are thought to have some inhibitory effect on the transposition effect, perhaps by binding to the Tnp-Inh heterooligomers.

In *Rhizobium*, Tn5 confer resistance to kanamycin. Two 1533 bp inverted repeats at the end of Tn5 (IS50) contain the genes necessary for transposition of Tn5 and its regulation.

Transposon mediated mutagenesis or tagging

Tn5 mutagenesis can greatly be divided in two major categories: "random Tn5 mutagenesis" and "site directed mutagenesis". The first category involves the introduction of Tn5 in to *Rhizobium* of interest via transformation, transduction, or conjugation with plasmid or phage vectors carrying Tn5. These Tn5 containing vectors are called "suicidal vectors" because of their instability (or inability to be suitably maintained) in the recipient bacteria.

The second category involves the Tn5 mutagenesis of DNA segments cloned in to (multicopy) plasmids in *Escherichia coli*. This is followed by the physical mapping of the Tn5 insertion and whenever possible determination of the "phenotype" of the Tn5 recombinant plasmid to construct a correlated physical and genetic map. The method is often coupled to the reintroduction of specific Tn5 mutated segments into their original bacterial background followed by replacement of the wild type gene or region with its Tn5 mutated homolog via forced double reciprocal recombination (gene replacement or homogenotization). In the present study random Tn5 insertion was carried out in the genome of *Rhizobium leguminosarum*, to serve as marker tagging of the strain.

MATERIALS AND METHODS

Preparation of packets containing different carriers

Different samples were taken out from different sources for biomanure VC-MA, vermiculite, charcoal (from market), and farm yard manure. These samples were weighed and mixed to give treatments as follow:

T_I. Biomanure VC-MA.
T_2. Farm yard manure (FYM)
T_3. Vermiculite
T_4. Charcoal
T_5. Biomanure VC-MA and Vermiculite (1:1)
T_6. FYM and vermiculite (1:1)
T_7. Biomanure VC-MA and Charcoal (1:1)
T_8. FYM and T Charcoal (1: 1)

Tn*5* marked drug resistant *S. freddi* was cultured in Yeast Extract

Table 1. Antibiotic sensitivity of mutant *of Sinorhizobium freddi* R0132::*Tn*5 (str^R) developed.

Antibiotics	Antibiotic concentration (µg/ml)							
	200.0	100.0	50.0	25.0	12.5	6.25	3.125	1.56
Gentamycin	-	-	-	-	-	-	-	-
Kanamycin	-	-	-	-	-	-	-	-
Tetracycline	-	-	-	-	-	-	-	-
Streptomycin	+	+	+	+	+	+	+	+
Nalidixic acid	-	-	-	-	-	-	-	+

Table 2. Optimization of incubation period for patch mating to transfer pGS9 plasmid into *Sinorhizobium freddi* R0132::*Tn*5.

S/No.	Incubation time (h)	Average titre of cells per ml (TY + str + kan)
1.	0	Nil
2.	4	Nil
3.	8	1.2×10^2
4.	16	6.2×10^3

Mannitol broth with 100 µg/ml streptomycin and 25 µg/ml kanamycin. After 36 h, it was incubated at 28°C on a rotatory shaker, the bacterial culture at ODA_{600}= 0.4 was centrifuged at 70000 rpm for 20 min and resuspended in sterile water. The 5 ml of the culture was then mixed with 25 g carrier uniformly. Each inoculated carrier material stored in polythene bag was incubated at 28 and 37^{0}C for 50 to 60 days, respectively. In these experiments the carrier materials was not sterilized. One gram of each carrier sample was taken from the polythene pack and was suspended in 10 ml of sterile water in 30 ml screw cap tubes and vigorously vortexes to mix. The suspension was allowed to stand for 10 min. The 10^2 to 10^6 dilutions were made from suspension and 0.1 ml solution was spread plated on YEMA containing 200 µg/ml of streptomycin and 50 µg/ml kanamycin. The colonies appearing after four days were counted in each plate.

RESULTS

Isolation of antibiotic resistant *Sinorhizobium freddi* R0132::*Tn*5

The drug resistance efficiency of *S. freddi* R0132::*Tn*5 was characterized by broth assay and Poison agar method. It is clear that the mutant was resistant to streptomycin as it could grow in media supplemented with 200 µg/ml of streptomycin. This strain was designated as R0132::*Tn*5 and was further used for transposon tagging (Table 1).

Tn5 tagging of rhizobial inoculants

The *Tn*5 mutagenesis was carried out by the random transposon mutagenesis procedure. The period of mating on agar had been found to significantly affect the vector transfer and recovery of *Tn*5 mutants. Therefore to standardize the mating period between WA803 (pGS9) and *S. freddi* R0132 ::*Tn*5, the patch from the mixture of their cells on TY agar (str + kan) surface were allowed to grow for 4, 8 and 16 h at 28°C.

Viability of *Tn*5 tagged drug resistant *Rhizobium* in biomanure VC-MA was subjected to different treatments. The population of drug resistant *S. freddi* R0132::*Tn*5 in untreated control could not be detected after 25 days when kept at 37°C. However, appreciable number of colonies could be observed in autoclaved biomanure VC-MA even after 50 days (Figure 1 and Table 2). The population of drug resistant *S. freddi* R0132::*Tn*5 in general, remained higher in various carriers when kept at 28°C. No significant change in population of drug resistant *S. freddi* R0132:Tn5 was detected in autoclaved biomanure VC-MA (Figure 2 and Table 3).

DISCUSSION

Low cost inert materials like charcoal are quite common earners for bioinoculants. Attempt were made to search and identify new carrier material which apart from being cheap, should also provide a good substrate for enhancing a survival and growth of bioinoculants. One of biomanure produced from agro-wastes generated at farm containing all major nutrients, was tested for suitability as a carrier for drug resistant *S. freddi* R0132::*Tn*5. Treatment of isolated strain of *S. freddi* R0132::*Tn*5 with sensitivity to common antibiotic streptomycin was tested; the major reason is being lack of suitable carrier which

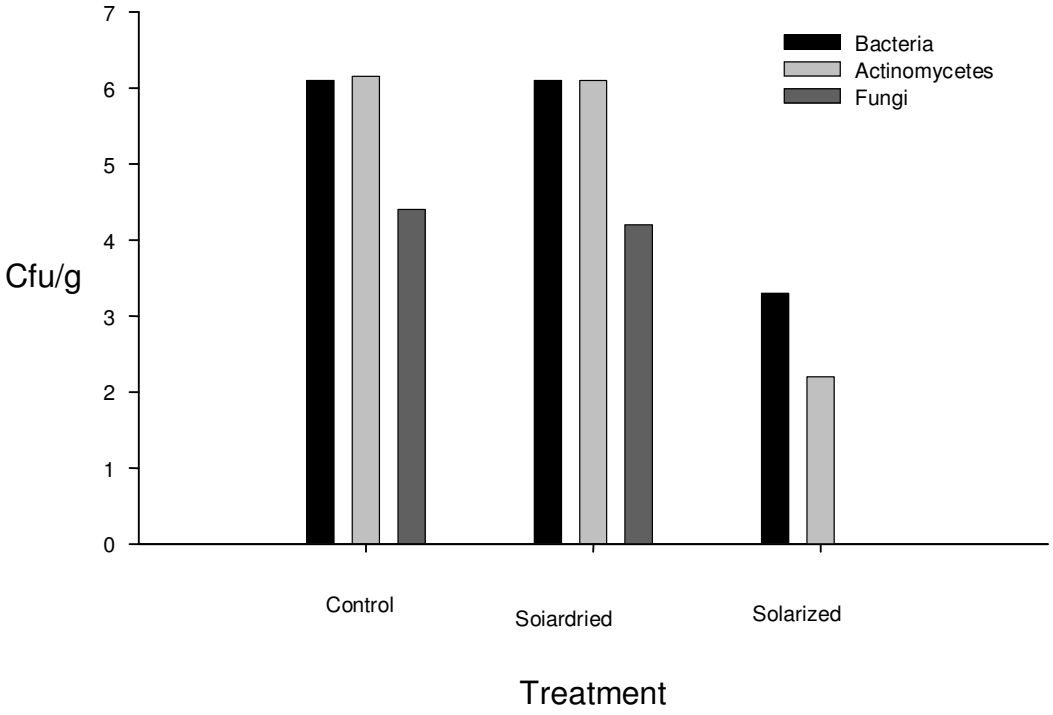

Figure 1. Microbial population of bacteria, actinomycetes and fungi biomanure VC-MA.

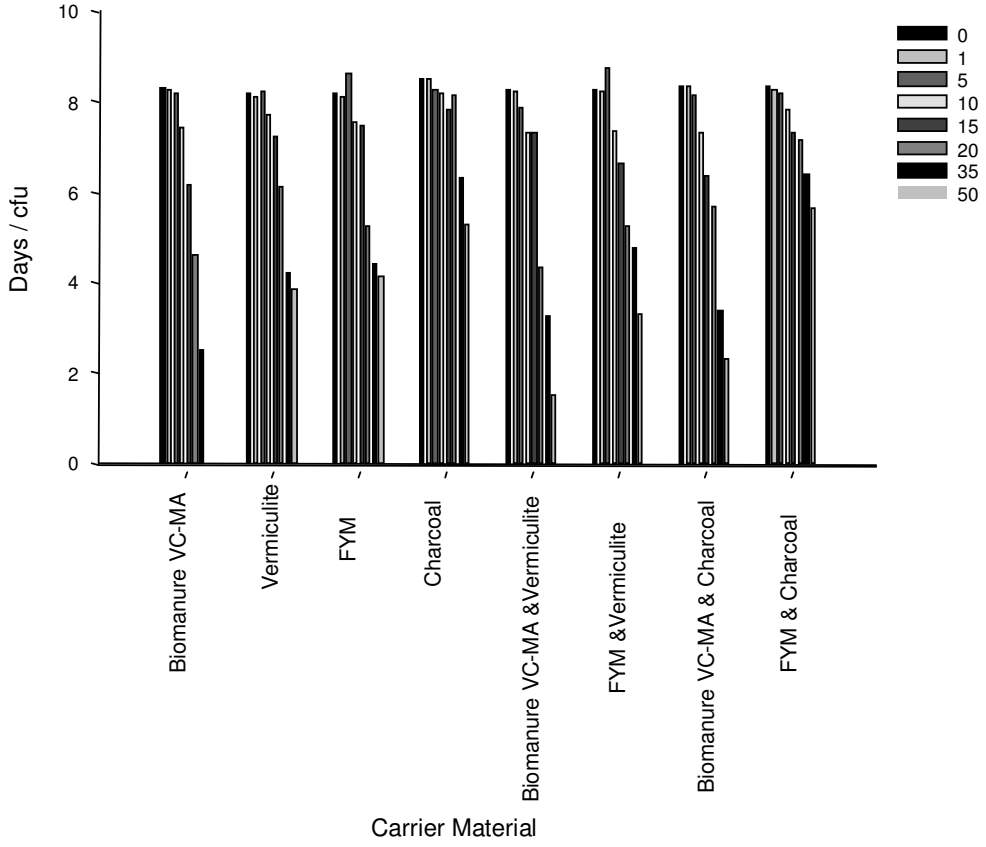

Figure 2. Population of drug resistant *Sinoshizodium freddi* in various carrier materials at 28°C.

Table 3. Microbial population of bacteria, actinomycetes and fungi in biomanure VC-MA.

S/No.	Treatment	Bacteria (cfu/g)	Actinomycetes (cfu/g)	Fungi (cfu/g)
1.	Control	1.07×10^6	1.52×10^6	4.0×10^4
2.	Solardried	1.0×10^6	1.0×10^6	$2 \times l0^4$
3.	Solarized	3.0×10^3	2.0×10^2	Not detected
4.	Autoclaved	Not detected	Not detected	Not detected

could not only increase the viability of bacteria but also support its growth and multiplication after application in the fields. So far, charcoal powder has been most commonly and extensively used as carrier for most of the *rhizobial* inoculants, though vermiculite (Sparrow and Ham, 1983), FYM (Chakrapani and Tilak, 1974), compost (Iswaran et al., 1972) has also been tested. The present study indicated that the biomanure VC-MA as such, could not support the growth of drug resistant *S. freddi* R0132::*Tn*5 and sharp decline in population with passage of time was observed. On the other hand high microbial density biomanure *S. freddi* R0132::*Tn*5 VC-MA probably did not allow drug resistant *S. freddi* R0132::*Tn*5 to establish itself. Some of the microorganisms present in biomanure *S. freddi* R0132::*Tn*5 VC-MA antagonistic or their metabolites produced in this biomanure might beto-xic to the test bioinoculant (drug resistant *Sinorhizobium freddi* R0132::*Tn*5. Temperature plays an important role in the survival viability and growth of any microorganism. Our results indicated that considerable decline in the population of drug resistant *S. freddi* R0132::*Tn*5 occurs when kept at higher (37 - 40°C) temperature. To improve its sustainability it should be kept in mind that the sample should be kept at cooler (28°C) places.

The major problem is being with the poor survival of bioinoculant in carrier materials. Some material like bio-manure VC-MA tested during the present investigations could be a better substitute. However, the post application population dynamics of the test organism *S. freddi* R0132::Tn5 need to be further studied.

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