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Nodulation Efficiency in Term of Nitrogenase Activity of *Rhizobium* Mutants and their Wild Type

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Abstract: This study aimed to select suitable strains that can be used as inoculants to enhance legume production and simultaneously reduce the use of inorganic fertilizers. It benefited growth of V. radiata and it was more easily cultured on solid and liquid media than any of the other strains tested including R0132 (1112)::Tn5, R0132 (0097)::Tn5 and R0132 (1106)::Tn5. Mutant R0132 (1106)::Tn5 exhibited superior growth promoting ability under extreme environmental condition; therefore it has potential to be used in India. A slow growing nitrogen-fixing strain of *Vigna radiata* (mung bean), *Rhizobium fredii* which expressed nitrogenase activity in a synthetic medium was isolated from its native population. Mutants with decreased and increased nitrogenase activity were derived from this strain by Tn5 mutagenesis. These mutants were tested for in vivo symbiotic activity. The efficiency of mutants R0132 (1106)::Tn5 with increased nitrogenase activity in the culture medium was higher than the parent strain (R0132); This suggests that the plant is perhaps a limiting factor in the full expression of rhizobial nitrogenase in the nodules. Currently involved in researen aimed at improving symbiotic nitrogen fixation (SNF) and root nodule sustainability in *Vigna radiate*.L.

Key words: Nitrogenase activity, symbiotic activity, inoculation, mutant strains, nodulation and nitrogen fixation

INTRODUCTION

In legume-*Rhizobium* symbioses, variabilities in symbiotic effectiveness which are either due to variations in nitrogen fixing potential of *Rhizobium* strains or due to host genotypic compatibility (Brockwell and Katznelson, 1976), are often observed. Since the nitrogen-fixing genes are known to be confined to the bacterial genome (Gibson *et al.,* 1976), the host effects are mainly on symbiotic establishment and on controlling the induction and expression of nitrogenase in the nodules. The variability in the effectiveness of native *Rhizobium* isolates even on a single cultivar of a legume crop, gives the impression that the nitrogen- fixing ability of *Rhizobium* could be improved either by strain selection or by genetic manipulation.

Since the nitrogen-fixing ability is expressed only in symbiotic association, it is not possible to find out whether the restriction on the bacterial gene expression is due to the bacterial genome or the plant. Recently, methods for inducing the nitrogenase under culture conditions in some slow growing *Rhizobium* strains have been reported (Kennedy and Pankhurst, 1979). Having standardized the conditions for the expression of nitrogenase in a culture medium by a mung bean-*Rhizobium* strain R0132, we have derived mutants with increased and decreased nitrogenase activity as compared with the parent strain. In this paper, we report the symbiotic behavior of these mutants in relation to the parent strain.

MATERIALS AND METHODS

Percent mercuric chloride and acidified with 5 ml/L concentrated HCI for about 2 min. Seeds were washed again several times with sterile distilled water and sown at different location of C.C.S. University, Meerut. The field was irrigated whenever required. The nodulation usually started about 9–10 days after emerging of the seedling. For the measurement of activities of different enzymes the cells of different Rhizobium isolates were grown in the defined medium (containing gm L–1; K2HPO4 1.0, KH2PO4 1.0, MgSO4.7H2O 0.25, CaCl2.6H2O 0.1, KNO3 1.0, mannitol 3.0, arabinose 3.0 and amount of trace elements and vitamins were same as used in defined medium previously. The pH of medium adjusted to 7.2 and cultures incubated at 28˚C ± 1˚C on an incubator shaker at 200 rpm.

Rhizobium strain designated as R0132, isolated from the root nodules of locally grown *V. radiata* plants.Transposon (Tn5) mutagenesis (Mutagenesis of *Rhizobium* sp. R0132)

Culture of Escherichia coli WA 803 (pGS9) was obtained from IARI New Delhi. Antibiotic was procured form sigma corporation for U.S.A and other media and chemicals were procured from this media private Limited. Rhizobium sp R0132 was grown to log phase in LB at 30oC, rpm 200. To raise WA 803, Kan 50 and Clm10 was added (subscripts indicate concentration in µg/ml). One ml each culture spun at 5000 rpm for 5 min and re-suspended in 1.0 ml 10 MgSO4.

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Equal volumes (200 µl) of these two washed suspensions were mixed, centrifuged at 5000 rpm for 5 min and Resuspended in 100 µl, 10 mM, MgSO4 this was spotted for patch-mating on LA surface and incubated for 8 h at 30oC. The cells were re-suspended later in 2.5 ml of 10 m M MgSO4, plated on M9 medium with Kanamycin (50 µl/ml) and incubated at 30oC for 24 h. Individual R0132:Tn5 exconjugants streaked for analysis on HAM Agar.

Estimation of Nitrogenase activity: The measurement of nitrogenase activity was based on the reduction of acetylene to ethylene as quantities by gas chromatography. Acetylene reduction was performed by a protocol modified from (Silvester, 1983). Acetylene made directly from calcium carbide chips immersed in water was injected into each jar to give a final concentration of 10 % (v/v). Jars were incubated on the lab bench and analyzed for ethylene (C_2H_4) after approximately one hour. Ethylene was analyses by standard flame ionization gas chromatography (Shimadzu GC8A) standardized with pure ethylene and results expressed as pmol of C_2H_4 produced per jar per minute.

RESULTS AND DISCUSSION

Fig. 1 shows that nitrogenase activity of plant nodule inoculated with different mutants and their parent isolate varies from 1.96 to 10.36 in mutants and 0.46 to 3.21µmol/h/mg FW of nodule in wild type isolate. The maximum nitrogenase activity has been observed in isolates R0132 (1106)::*Tn*5, while minimum nitrogenase activity has been recorded in isolates R0132 (0072)::*Tn*5 and R0132 (0599)::*Tn*5 Middle range of activity of nitrogenase enzyme has been recorded from isolates R0132 (0056)::*Tn*5.

Regarding the nitrogenase activity it has been observed in the present investigation that different *rhizobial* isolates had variable activity of nitrogenase as induced in free living state. The free living forms of many *rhizobial* strains when cultured in an appropriate medium exhibit nitrogenase activity (Anupma, 1999). This activity can only appear on nitrogen free medium (Beegerson, 1978). Present observations as well as the observations of the other are clear indicator of the fact that genetic information for synthesis of nitrogenase enzyme resides only in the *rhizobia* as suggested by many workers like. This renders invalid the theory held by (Dilworth and Parker, 1969) that part of the nitrogenase in legume might be specified by plant DNA. Analysis of present data on induction of nitrogenase activity *in vitro indicate that* in presence of different sources of nitrogen *viz*, ammonium chloride, glutamine, glutamate, casein hydrolysate and potassium nitrate indicate that these nitrogen sources are derogatory with regard to induction of nitrogenase activity either in terms of turnover or in terms of molecular activity. It has also been observed in the present investigation that out of all nitrogen sources ammonium

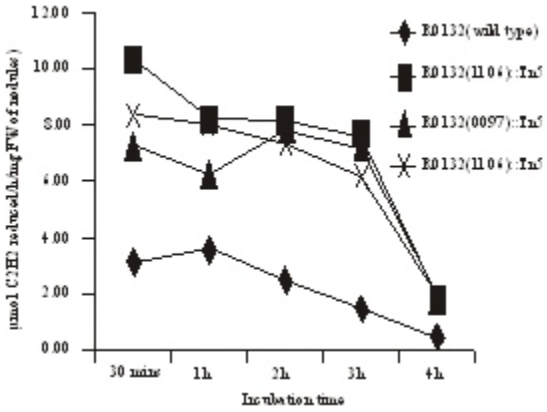

Fig. 1: ARA of *Vigna radiata* nodules inoculated with wild type and mutant strain in 10% C2H2 in air atmosphere

chloride is so detrimental for initiation of nitrogenase activity *in vitro* as were potassium nitrate and potassium nitrite. (Tubb, 1976) reported that significant nitrogenase activity was detected in *rhizobial* cultures grown with ammonia in excess of growth limiting condition. Similar observations were also reported by but observed that ammonium chloride was very much detrimental for initiation of nitrogenase activity clearly indicated that different nitrogen sources were detrimental for nitrogenase activity. However, the growth of *rhizobia* is not much impaired by application of different nitrogen sources.

This is a unique capacity found among wild type nitrogen fixing organism so far studied. The activity of nitrogenase though has been observed to be invariably lower than that observed in cultures grown with glutamate and glutamine, the growth in both nitrogen sources is normal. It was observed by that when cultures of *Rhizobium* species of *Cowpea miscellany* were provided with ammonium and glutamate nitrogen both contributed to the growth as is also observed in the present investigations. It is an indirect proof of capacity of bacterium to assimilate both sources of exogenously supplied nitrogen. However, a common feature could be concluded that the presence of glutamate some of the fixed nitrogen was excreted as ammonium and thus it was concluded that glutamate not only inhibited ammonium uptake but also resulted excretion of ammonium which was produced during nitrogen fixation (Dincturk *et al*., 2001).

The process of nitrogen fixation in presence of fixed nitrogen may be inhibited, therefore, it is often observed that nitrogenase is always repressed if nitrogen supplied as fixed nitrogen or inorganic nitrogen (Postgate, 1971). From the present observation as well as from the pre-existing literature it can therefore be very emphatically concluded that any nitrogen sources that are present in the soil will be inhibitory for induction of nitrogenase whether in free state living microbes or in symbiotic forms under symbiotic conditions e.g., *Rhizobium*. The nodulation itself has been reported to be very rare whenever external nitrogen is supplied externally in the form of inorganic fertilizers (Seneviratne *et al*., 2005).

CONCLUSION

The chemical agriculture though intensive has yielded fruitful results in terms of improving total productivity but over the years adversely affected the environment. Over use chemical fertilizers has seriously deteriorated the soil health. An efficient fertilizer, capable to fix atmospheric nitrogen, can play an important role in reducing chemical 'N' fertilizer. The present study aimed at selecting a suitable strains material compared with other carrier material tested viz; charcoal, vermiculite and FYM. Further studies, however, are needed to standardize the inoculum load, moisture content and temperature for improving the shelf life of biofertilizers.

Rhizobium leguminosarum has long been regarded as an important microorganism because of its symbiotic nature and ability to fix nitrogen. The newly-discovered role of this organism as a plant growth-promoting rhizobacterium is exciting, and will likely be greeted with appreciation. Rhizobial inoculants are used widely in agriculture to improve legume crop productivity; use of rhizobial inoculants for other crops could occur in the future.

Study of both types of Rhizobium-plant relationships, including use of mutants deficient in functional interactions, will broaden our knowledge about the individual organisms in the associations, and the overall ecology of plant-microbe interactions.

These rhizobial isolates are characterized and analyzed with regard to growth kinetics under different conditions viz., utilization of different carbon and nitrogen sources, and analysis of different molecules of wall component, nodulation efficiency test and activities of enzymes in nitrogen fixation.

Strains performance evaluation demonstrated that the R0132 (1106)::*Tn*5 inoculants *of Rhizobium leguminosarum* biovar *trifolii* is a prime candidate as a commercial inoculants. It benefited growth of *Vigna radiata* and it was more easily cultured on solid and liquid media than any of the other strains tested. Further tests are required, however, to refine the inoculation conditions as its performance was less consistent than that of the other inoculants. R0132 (1106)::*Tn*5 has the potential to be used in NEZ and NWZ

Endophytic bacteria are present to any cropping of India, as it was originally isolated under cool and wet conditions. system, and the identification of bacteria benefiting all crops in a crop rotation may have significant impact on the development of new agricultural practices. This study shows that specific strains R0132 (1106)::*Tn*5 of *Rhizobium leguminosarum* biovar *trifolii* can act as complimentary endophytes to Green gram or Mung bean, although they can depress or stimulate plant growth depending on environmental conditions.

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