" ROLE OF PROLINE DEHYDROGENASE IN NODULATION EFFECTIVITY AND COMPETITIVENESS OF MUNGBEAN - *RHIZOBIUM* SYMBIOSIS"

BY

SUMAN
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Thesis submitted to the Chaudhary Charan Singh Haryana Agricultural University,Hisar,in partial fulfilment of the requirements for the degree of

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IN

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DEDICATED
TO MY
LOVING FAMILY

CERTIFICATE - I

This is to certify that this thesis entitled: "**Role of Proline Dehydrogenase in Nodulation Effectivity and competitiveness of Mungbean —*Rhizobium* symbiosis**" submitted in partial fulfilment of the requirements for the degree of **Master of Science in Microbiology** of Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Suman** under my supervision and guidance and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE - II

This is to certify that this thesis entitled : **"Role of Proline Dehydrogenase in Nodulation Effectivity and competitiveness of Mungbean —*Rhizobium* symbiosis"** submitted by **Suman**, to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, in partial fulfilment of the requirements for the degree of **Master of Science in Microbiology** has been approved by the Student's Advisory Committee, after an oral examination on the same.

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Suman

[SUMAN]

CONTENTS

LIST OF ABBREVIATIONS

Pro	—	Proline
P5CR	—	Pyrroline-5-carboxylate reductase
ProDH	—	Proline dehydrogenase
PPP	—	Pentose phosphate pathway
P5C	—	Pyrroline-5-carboxylate
R5P	—	Ribose 5 phosphate
PRPP	—	Phosphoribosyl pyrophosphate
PRA	—	Phosphoribosylamine
AAT	—	Aspartate aminotransferase
GS	—	Glutamine synthetase
GOGAT	—	Glutamate synthase
P5CDH	—	Δ' Pyrroline-5-carboxylate dehydrogenase
OCD	—	Ornithine cyclo deaminase
PEP	—	Phosphoenol pyruvate
TCA	—	Tricarboxylic acid
NADPH	—	Reduced nicotinamide adenine dinucleotide phosphate
NADP	—	Nicotinamide adenine dinucleotide phosphate
$FADH_2$	—	Reduced flavin adenine dinucleotide
FAD	—	Flavine adenine dinucleotide
OAT	—	Ornithine amino transferase
Glu	—	Glutamate
Gln	—	Glutamine
ATP	—	Adenosine triphosphate
Pro.O	—	Proline oxidase

INTRODUCTION

Legumes are capable of fixing nitrogen in symbiotic association with *Rhizobium/Bradyrhizobium* and can fix nitrogen in the range of 50-300 kg per hectare per year (Philips, 1980). This amount of nitrogen is sufficient to support plant growth and yield. If greater reliance on biological N_2 fixation is made it may reduce the application of extensive nitrogenous fertilizers which otherwise imbalance ecosystem and create environmental pollution. These facts have stimulated scientists all over the world to exploit the process of biological nitrogen fixation.

In *Rhizobium* - legume symbiosis the C_4 dicarboxylic acids have generally been considered the major carbon source exported from plant cells to the bacteroids that support the nitrogen fixation process (Ranson *et al.*, 1981; Finan , 1983; Bolton *et al.*,1986). However, it has not been established to be the exclusive or even the most efficient energy source under different environmental conditions. Further little is known about the carbon sources used by rhizobia during nodule formation and invasion process. It has been suggested that oxidation of amino acids particularly glutamate and proline imported by the bacteroids from the cytosol of infected

cells may supply additional energy needed to support nitrogen fixation in legume root nodules (Kohl *et al.*, 1988). However, the impermeability of the plant and peribacteroid membranes to glutamate suggests that glutamate may not be available to the bacteroids in significant quantities (Udvardi *et al.*, 1990, Day and Copeland, 1991). Proline is usually catabolized in prokaryotic cells into pyrroline-5-carboxylate (P5C) by means of proline dehydrogenase (ProDH) enzyme. It has been reported that ProDH is associated with bacteroids (Kohl *et al.*, 1988). It has been observed that exogenously applied proline stimulates nitrogen fixation rate as much as exogenous succinate or glutamate does and increases ProDH activity (Zhu *et al.*, 1992). Moreover, if soybean plants were subjected to drought then this resulted in accumulation of proline and ProDH activity was also high in the bacteroids (Kohl *et al.*, 1991). Both the compartmentation of ProDH within soybean nodules and its potential ability to contribute to the energy requirements of the bacteroids suggests a role for this enzyme in nitrogen fixation. Any defect in ProDH activity impairs its effect on nodule formation and results in an alteration of rhizobial nodulation.

However, Jimenez-Zurdo *et al.*(1995) isolated ProDH mutants of *Rhizobium meliloti* and these mutants resulted alteration in nodulation efficiency and competitiveness on alfalfa roots. Mungbean (*Vigna radiata* L. Wilczek) is a ureide transporting legume (Norhayati *et al.*, 1988; Peoples *et al.*, 1988) and important kharif grain legume of Indian subcontinent. Nodulation competitiveness by the inoculant strains is usually low in mungbean (Dudeja *et al.*, 1995). Therefore to improve nodule occupancy and efficacy

the present investigation was undertaken with the following objectives:

1. To assess the involvement of ProDH in nodulation effectivity of mungbean - *Rhizobium* symbiosis.
2. To assess the role of ProDH deficient mutants in competitiveness in mungbean - *Rhizobium* symbiosis.

REVIEW OF LITERATURE

The presence of native rhizobia is a major problem for inoculant performance under agricultural field conditions. *Rhizobium* and *Bradyrhizobium* inoculation would be beneficial only when the inoculant strain is able to displace the native rhizobia and forms nodules. Rhizobial strains improved for any symbiotic property can not have consistent agricultural impact untill the problem of indigenous competitiveness is solved. Nodulation competitiveness appears to be a complex process and is a result of many interacting mechanisms. A particular strain may be highly competitive due to many factors, some of which are not yet fully understood. Host cultivar, soil properties and the nature of competing strains influence the success of inoculant strains in nodule formation. The possible phenotypes of a rhizobial strain which may directly or indirectly determine the competitiveness are : motility, polysaccharide production, alternation in nodule formation efficiency genes *nfe* (Sanjuan and Olivares, 1989), rhizopine production (Murphy and Saint, 1992), bacteriocin production (Triplett *et al.*, 1994), hydrogenase uptake system (Dudeja *et al.*, 1995) and ProDH activity in bacteroids (Jimenez- Zurdo *et al.*, 1995). One of the major problem being

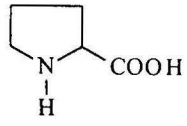
faced in northern region of India is of low nodule occupancy by inoculant *Rhizobium* strains (Dudeja and Khurana, 1988; Khurana *et al.*, 1991; Dudeja *et al.*, 1995; Sheoran *et al.*, 1997; Pathak *et al.*, 1998). To overcome this problem it is important either to select, construct highly competitive rhizobia and this requires the identification of the competitive genes present on rhizobia and than construction of super bug, but its release into the environment and further its performance under the natural ecosystem will be a question mark. Although there are many factors which play their unique role in competitiveness but in the present review the role of ProDH activity in nodulation effectivity and competitiveness is being emphasised.

2.1 PROLINE METABOLISM IN *RHIZOBIUM* AND ROOT NODULES OF LEGUMES

Nitrogen fixation in the legume- *Rhizobium* symbiosis requires an energy source to be available to the microsymbiont (bacteroid). To meet this need the plant supplies reduced carbon compounds which are capable of being oxidized under the microaerobic conditions [10 to 20 mmol m^{-3} free dissolved O_2 prevailing in infected root nodule cells] (Appleby, 1974; Layzell et al., 1990). The consensus view is that 'Evidence supporting C_4 dicarboxylates as major carbon source exported to the bacteroids has accumulated (Witty *et al.*, 1988). Day and Copeland (1991) state their conclusion more categorically for malate as the sole carbon source supplied to infected cells during nitrogen fixation and ammonia assimilation. However Kohl *et al.* (1988) indicated that there may be a significant energetic role

for proline under certain circumstances. Based on high levels of proline synthesizing activity in the host cell cytosol and on proline oxidizing activity in bacteroids of soybean nodules, it was further proposed that proline might be important as an energy source for N_2 fixation in bacteroids. The essential elements of this hypothesis are that one product of pyrroline -5- carboxylate reduction , proline is imported into bacteroids where it is dehydrogenated, while the other reaction product, $NADP^+$ drives the oxidative limb of the pentose phosphate pathway.

Proline represents a unique class of molecules among amino acids. With its peptide bond within the pyrrolidone ring, proline confers rigidity and three dimensional stability of protein (Phang *et al.*, 1973).



L-PROLINE

Although proline can be a source of α - ketoglutarate to serve as either substrate for TCA cycle or as means of repleting cycle intermediates, experimental evidences suggests that the critical role played by proline in insects involves redox transfer (Balboni, 1978). Proline is usually catabolized in prokaryotic cell via pyrroline -5-carboxylate by means of proline dehydrogenase (EC 1.5.9.98) yielding NADPH or by proline oxidase (EC 1.4.3.2). Proline and pyrroline-5-carboxylate provide a mechanism for the intercompartmental and intercellular transfer of redox potential. The transfer of redox potential alters the ratio of $NADP^+$/NADPH by activating

certain metabolic pathways. The end point of this regulation is the formation of purine ribonucleotides (Phang, 1985).

Pyrroline-5-carboxylate reductase (E.C. 1.5.1.2) catalyzes the final step in proline biosynthesis (Krueger *et al.*, 1986). The enzyme has been partially purified and characterized from various animal source (Adams and Goldstone, 1960; Greenberg, 1962), bacteria (Adams and Goldstone, 1960; Costilow and Cooper, 1978; Pahlich *et al.*, 1981), Neurospora (Costilow and Cooper, 1978; Matzurzawa and Ishiguro, 1980) and Saccharomyces (Matzurzawa and Ishiguro, 1980). The purified reductase oxidizes proline under extreme pH conditions (pH 10.3) thus supporting the assumption that in reality the proline dehydrogenase of higher plants is a pyrroline -5-carboxylate reductase (Mazelis and Creveling, 1974; Rena and Splittstoesser, 1975). An enzyme has been purified to homogencity from barley seedlings which has proline dehydrogenase activities. The purification achieved is 39000 fold as calculated from the proline dehydrogenase activity. The subunit molecular weight of proline is 30 kilodaltons. The specific activity of ProDH enzyme in barley seedlings was found to be very high (500 units / mg of protein) and enzyme after final purification is stable for 1 month when stored at -80°C in 5% glycerol with no activity loss (Krueger *et al.*, 1986).

Purification and properties of ProDH was studied by Meile *et al.* (1982) in *Pseudomonas aeruginosa*. ProDH enzyme has been shown to be membrane bound flavoprotein transferring electron carriers but not to molecular oxygen (Scarpulla and Scoffer, 1978). ProDH and P5CDH have

been demonstrated to be located in the mitochondria of mammals (Johnson and Strecker, 1962) and plants (Stewart and Lee, 1974; Huang and Cavalieri, 1979). Both enzyme activities have been found to be associated with the particulate fraction of crude extracts from *E. coli* (Scarpulla and Scoffer, 1978) and *Salmonella typhimurium* (Menzel and Roth, 1981). Solubilization and purification of two membrane bound enzyme activities involved in proline catabolism from *Salmonella typhimurium* resulted in a homogenous proline preparation catalyzing both the ProDH and P5CDH reaction (Menzel and Roth, 1981). The enzymatic properties of mutants defective in proline catabolism suggested that a single enzyme is involved in proline as well as in ornithine catabolism and that its synthesis is induced by proline but not by ornithine (Meile *et al.*, 1982).

Proline dehydrogenase which catalyses reaction of the oxidation of proline to glutamic acid, was purified from *Pseudomonas aeruginosa.* Mutants of *Pseudomonas aeruginosa* deficient in the utilization of L- proline as the only carbon and nitrogen source have been found to be defective either in proline dehydrogenase activity or in both proline dehydrogenase and Δ'- pyrroline- 5-carboxylate dehydrogenase (Meile *et al.*, 1982).

Nitrogen fixation in legume is a symbiotic process in which bacteria of the genus *Bradyrhizobium* or *Rhizobium* infect root cells and form specialized organs (nodules) within which N_2 is reduced to NH_4^+. Fixation of nitrogen is an energy intensive process, requiring a total of 25-30 ATP per N atom fixed. Of this total, 12-14 ATP per N are required within the bacteroids to reduce nitrogen. As much as 10-30 per cent of the total

photosynthetic capacity of the plant is used to support this process (Schubert, 1982). The energy yielding metabolities supplied by the host to the bacteroid, the symbiotic form of the bacterium is not known. However one attractive suggestion is that bacteroids import and oxidize a nitrogenase compound, such as glutamate (Kahn *et al.*, 1985). The nitrogen fixed in the bacteroid is exported as ammonium to the infected host cells where it is packaged for export to the rest of the plant.

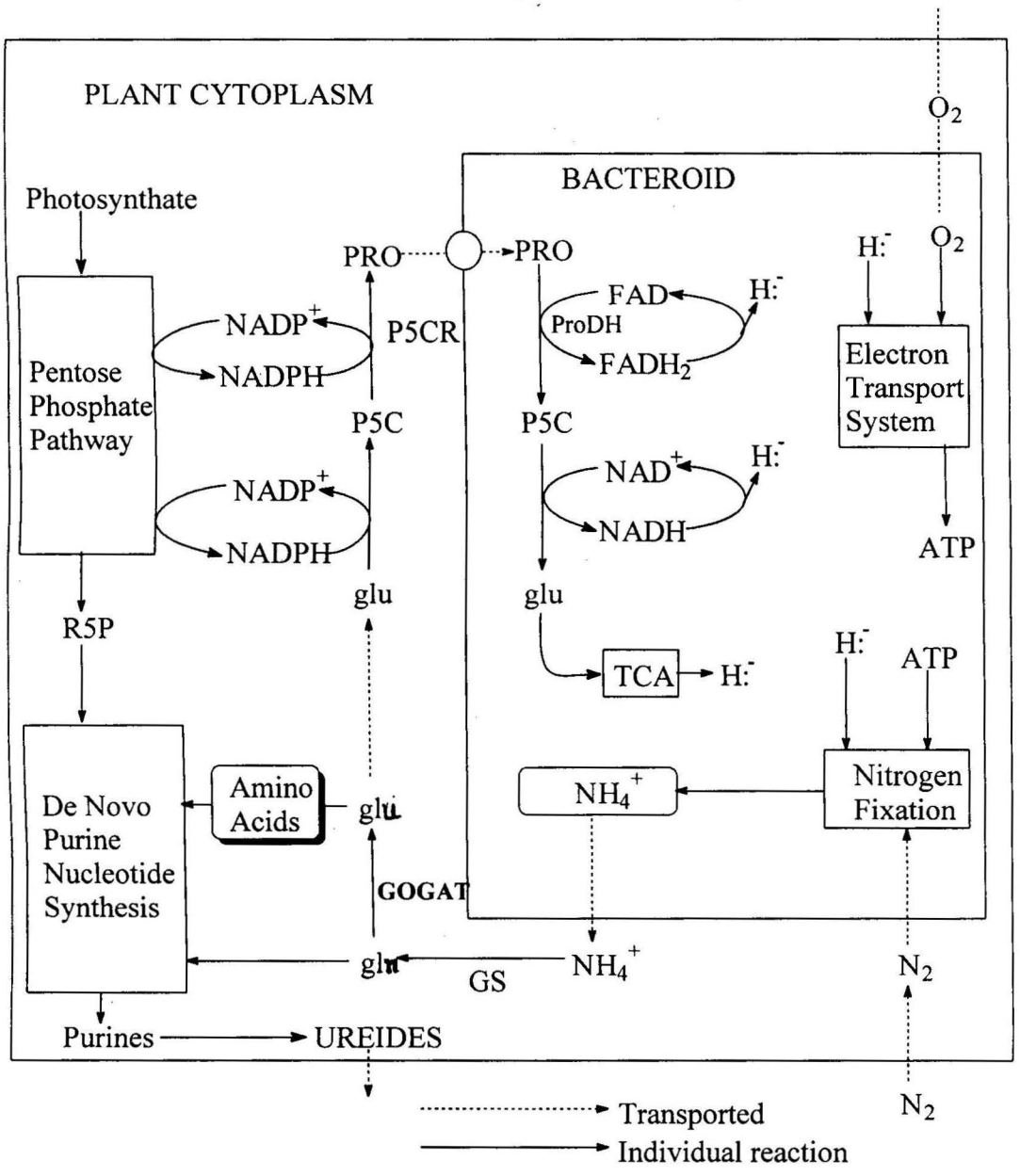
In addition to other carbon sources whether amino acids play a role in the nutrition of rhizobia in the soil or symbiotic state is unknown. It has been suggested that amino acids from the plant cytosol may be carbon sources for bacteroids and attention has focussed mainly on the possible role of glutamate (O'Gara and Shanmugam, 1976; Tubb, 1976; Kahn *et al.*, 1985). However the impermeability of the peribacteroid membrane at least in soybean nodules suggest that in some organisms glutamate may not be made available to the bacteroids in significant quantities. It has been suggested that proline synthesized in the plant cytosol by pyrroline-5 - carboxylate reductase is a carbon source for soybean root nodule bacteria and is metabolized via proline dehydrogenase (Kohl *et al.*, 1988). However, little attention was given in the study to the catabolism route for proline degradation or to the regulation of the enzymes of proline utilization by bradyrhizobia. In *Clostridium sporogenes* (Costilow and Cooper, 1978) and *Bradyrhizobium japonicum* (Kohl *et al.*, 1988) proline is reported to be catabolized by means of proline dehydrogenase. It was reported that isolated cowpea bacteroids of NGR234 were unable to transport L

(^{14}C) proline (<0.5 nmol min^{-1} mg protein^{-1}) although they were able to take up (^{14}C) succinate (16.5 nmol min^{-1} mg protein^{-1}) (Glenn *et al.*, 1991). Cell free extracts of cowpea bacteroids lacked detectable proline oxidase or pyrroline- 5 - carboxylate dehydrogenase activity. It is thus unlikely that cowpea bacteroids have any capacity to metabolize L-proline, consistent with the observation of Jin *et al.* (1990) that cowpea nodule cytosol has little or no proline. Legumes of temperate origin (e.g. peas) export nitrogen as amides (principally asparagine), whereas legumes of tropical origin (e.g. soybeans) export the ureides, allantoin and allantoic acid. Ureide biogenesis proceeds by way of synthesis of purine nucleotides (Reynolds *et al.*, 1982). This pathway is the same as that found in microorganisms, fungi and animals. The purines so formed are then oxidatively degraded to ureides (Reynolds *et al.*, 1982). Kohl *et al.* (1988) had reported very high levels of P5CR activity in the host plant cytoplasm in soybean nodules. Any cell that has high levels of P5CR activity but does not have high requirements for proline for biosynthetic purposes is left with the significant problem of disposing of the proline. One possible solution is that proline carries hydride ions from the cytoplasm into the mitochondria or possibly from one cell to another (Hagedom *et al.*, 1982; Hagedom and Phang, 1986). The transfer of hydrids ions provides a mechanism for the intercompartmental transfer of redox potential (Phang, 1985). The crucial features of this mechanism are P5CR activity in the cytoplasm and proline dehydrogenase activity in the other compartment. Kohl *et al.* (1988) had reported that bacteroid preparations exhibited ProDH activity comparable to that for rat

liver mitochondria and higher than that reported for plant mitochondrial ProDH activity which was atmost only a small fraction of that observed in the bacteriods.

The novel compartmentation of ProDH within soybean nodules raises the distinct possibility that together, P5CR and ProDH play an important role in the transfer of redox potential from the plant cytoplasm to the bacteroids. This hypothesis was presented diagrammatically (shown on next page Fig. 1) by Kohl *et al.* (1988). Its central feature is that energy needed by the bacteroids to support nitrogen fixation is supplied by the plant as proline. NADPH produced in the host plant cytoplasm is used to reduce P5C to proline. The latter is imported by the bacteroids, where dehydrogenation of proline catalyzed by ProDH is coupled to the bacteroid electron transport system and oxidative phosphorylation to produce ATP required for nitrogen fixation. If proline is oxidized all the way to CO_2 (via P5C, glutamate, oxoglutarate, and the tricarboxylic acid cycle) 35-38 ATP equivalents may be produced (a number sufficient to reduce 2-3 N). If either P5C or glutamate moves from the bacteroid to the host cytosol then correspondingly fewer ATP equivalents would be produced from proline carbon skeleton. It also shows that nitrogen being fixed into NH_4^+, and the NH_4^+ entering the plant host cytosol, where this toxic ion is converted to glutamine and then glutamate by the action of glutamine synthetase and glutamate synthase. While most of the fixed nitrogen from these amino acids is eventually incorporated into ureides, some glutamate might also be available for the synthesis of P5C and thence proline.

Fig 1 ROLE OF ProDH IN NITROGEN FIXING, UREIDE EXPORTING NODULES. (Kohl *et al.*, 1988)



Another feature of this hypothesis is that increased flux through the PPP allows the fixed nitrogen to be packaged as ureides for export from the nodule to the plant shoot. Finally this hypothesis is consistent with the proposal given by Kahn *et al.* (1985) that energy may be supplied to the bacteroid as a compound that contains nitrogen as well as carbon skeleton.

On the basis of levels and location of P5CR and ProDH in soybean nodules. Kohl *et al.* (1988) proposed that the activity of these enzymes of proline metabolism results in the transfer of cytoplasmic reducing equivalents from the host plant to its symbiotic partner. These results are also consistent with the regulatory role proposed for P5CR in animal cells. $NADP^+$ (produced as a consequence of making the proline needed to satisfy the energy demands of nitrogen reduction within the bacteroid) drives PPP which in turn results in higher rate of synthesis of R5P and ultimately purine ribonucleotides. This enhanced rate of *de novo* purine ribonucleotide synthesis would allow the nitrogen fixed within the nodule to be processed into ureides for export from the nodule to the rest of the plant.

The ProDH activity has been found to be inhibited 78 per cent in the presence of 4 mM $NADP^+$, but was not inhibited by proline or NAD^+ (Kohl *et al.*, 1988). Based on these results it was suggested that P5CR might have two major roles in addition to the production of the considerable quantity of proline needed for biosynthesis, particularly of cell wall glycoproteins. These possible roles are, the production of reduced carbon skeleton (proline) whose subsequent oxidation contributes to fueling energy intensive biological nitrogen fixation, the production of $NADP^+$ and the

assimilation of biologically fixed N_2 into ureides, the major form in which biologically fixed nitrogen is transported from soybean root nodules to the plant shoot (Schubert, 1981; Reynolds *et al.*, 1982). Futher it was proposed that the high P5CR activity in soybean nodules might function to increase the activity of the PPP, thus in turn increase the rate of production of R5P. Yeh and Phang (1988) have shown that P5C increases the synthesis of purine ribonucleotides by enhancing the activity of the PPP, giving rise to higher concentration of R5P. Since soybean nodules export fixed N primarily in the form of ureides, the synthesis of which requires *de novo* purine ribonucleotide synthesis, a possible role for the high activity of P5CR observed in soybean nodules is the regulation of the PPP by increasing the level of $NADP^+$ thereby stimulating formation of R5P, purines and ureides. About half of ProDH activity was in bacteroids, the symbiotic form of *Bradyrhizobium japonicum* which is responsible for the energy intensive reduction of dinitrogen. Very little activity was present in plastids or mitochondria (less than 2% of the total activity). The latter being the site of ProDH activity in other plant and animal tissues.

The development of activities of enzymes involved in the initial assimilation of fixed NH_4^+ (G S and GOGAT) as well as most of those involved in ribonucleotide and ureide synthesis, have been shown to parallel that of nitrogenase activity (C_2H_2 reduction) within soybean nodules (Schubert, 1981; Reynolds *et al.*, 1982). Essential house-keeping enzymes required for nodule growth and maintenance but not unique to nodules or required for the process of nitrogen fixation might be expected to follow a slightly

different pattern. In this context, it was of interest to determine, the developmental profile of activities of enzymes of the PPP and proline metabolism. PPP and P5CR activities were higher in ureide exporting nodules would provide additional support for a role of P5CR activity in stimulating purine and hence ureide synthesis (Kohl *et al.*, 1991). The activity of ProDH was reported to be higher in bacteroids from ureide than from amide exporting nodules (Kohl *et al.*, 1990).

The very rapid induction of extremely high levels of PPP and P5CR activities supports the premise that these activities play an important role in nodule development and metabolism perhaps in the initial events involved in nodule formation and in production of proline (which requires NAD(P)H, possibly provided by the PPP) for energy transfer to bacteroids (Kohl *et al.*, 1991).

Proline is also reported to be accumulated under salt stress in mungbean (Sheoran and Garg, 1978). Proline has shown to accumulate with drought not only in the plant tissue of the nodule but also in the symbiotic nitrogen fixing bacterial (bacteroid) nodule fraction (Kohl *et al.*, 1991), the site of most nodule ProDH activity (Kohl *et al.*, 1988).

Surprisingly, ProDH activity increases in bacteroids when soybean plants are drought stressed (Kohl *et al.*, 1991) in contrast to barley mitochondria in which one response to drought is a decrease in ProDH activity (Stewart *et al.*, 1977). These observations have led to hypothesize that proline synthesized in the plant fraction of soybean root nodules can serve as a supplementary carbon and energy source for bacteroids during drought when

other energy sources may be limited (Kohl *et al.*, 1994). Pedersen *et al.* (1996) observed that higher acetylene reduction rate is supported by proline in bacteroids from drought stressed nodules compared to bacteroids from control nodules of soybean (*Glycine max* L.).

2.2 ROLE OF ProDH IN NODULATION EFFECTIVITY

Kohl *et al.* (1990) have reported that magnitude of ProDH activity in ureide as well as amide exporting nodules is sufficiently high to supply a significant fraction of the energy requirement for N_2 fixation. This conclusion is based on a comparison of the energy needed to support the observed nitrogenase rates with the energy produced by the oxidation of proline to CO_2 at a rate given by the ProDH assay. So it can be assumed that (a) the rate of N_2 fixation is one fourth that of acetylene reduction (b) that 24 molecules of ATP are used per molecule of N_2 fixed (Schubert, 1982). (c) that 30 molecules of ATP are produced per proline oxidized to 5 molecules of CO_2. Both the localization in bacteroids and its potential ability to contribute to the energy needs of the bacteroid suggest a role for ProDH activity in nitrogen fixation. The marked increase in activity during soybean ontogeny and high levels of ProDH support a proposed role for ProDH in supplying at least a part of the energy needs of the bacteroids, especially late in growth.

In isolated organelles from soybean nodules, little ProDH activity was detected in the mitochondria, its usual locus in plant cells (Stewart, 1981). It was proposed that the high P5CR activity in nodules might function to increase the activity of the oxidative limb of the PPP by producing

NADP+ while the ProDH activity in the bacteroid might supply part of the energy needed for N_2 fixation (Kohl *et al.*, 1988). Supplying L - proline to the root system of intact soybean (*Glycine max* L.) plants stimulated acetylene reducing activity to the same extent as did supplying succinate. Feeding L - proline also caused an increase in bacteroid ProDH activity that was highly correlated with increase in acetylene reducing activity (Zhu *et al.*, 1992). They further indicated that proline crosses both plant and bacteroid membranes under in vivo experimental conditions and are consistent with a significant role for proline as an energy source in support of bacteroid functioning .

A possible role for proline as a source of energy to the bacteroid has also been proposed (Kohl *et al.*, 1988). Evidence supporting role for proline in N_2 fixation is that in *Klebsiella*, the expression of the *gln* A (the structural gene for glutamine synthetase), *nif* (N_2 fixation) and *put* (proline utilization) operon are all regulated by the ntr, (nitrogen regulatory) system (Magasanik, 1982). However the significance of proline as a compound supplying energy to the bacteroid was called into question by the results of Day *et al.* (1990). Who found that proline was not taken up at a rapid rate by isolated peribacteroid units. Zhu *et al.* (1992) had also provided evidence that exogenous proline was taken up from the surrounding medium and metabolized by bacteroids in nodules of intact soybean plants. Thus proline must have crossed the peribacteroid membrane at rates fast enough to influence metabolic events with in intact plants, despite the slow uptake of proline as compare to malate and succinate into isolated peribacteroid

units. In the *Rhizobium* legume symbiosis the C_4 dicarboxylic acids have generally been considered the major carbon source exported from plant cells to the bacteroids that support the nitrogen fixation process (Bolton *et al.*, 1986). However it has not been established to be the exclusive or even the most efficient energy source under different environmental conditions and little in known about the carbon sources used by the microsymbiont during nodule formation and invasion. It has been suggested that oxidation of amino acids imported by the bacteroids from the cytosol of infected cells may supply additional energy needed in legume root nodules and attention has focussed mainly on possible role of glutamate and proline (Kohl *et al.*, 1988). However the impermeability of the plant and peribacteroid membrane to glutamate suggests that this amino acid may not be made available to the bacteroids in significant quantities (Udvardi *et al.* ,1990; Day and Copeland, 1991)

Tn 5 mutagenesis has been used by number of workers for isolation of carbohydrate mutants of *Rhizobium* (Ranson *et al.*, 1981; Walton and Moseley, 1981; Thurn and Chatterjee, 1985). Carbohydrate mutants were isolated from *Rhizobium meliloti* LS-30 using the translocatable drug resistance elements Tn 5 (Duncin, 1981).

The efficiency of a variety of common mutagen in producing mutation in *Rhizobium trifolii* P_3 was examined. Transposon mutagenesis with Tn5 yielded same frequency and range of auxotrophs as did N- methyl, N'-nitro N- nitrosoguadine (Walton and Moseley, 1981). Using Tn5 mutagenesis Jimenez - Zurdo *et al.* (1995) obtained a mutant derivative strain unable

to catabolize either ornithine or proline . DNA hybridization studies showed that the mutant carries a single Tn5 insertion within a chromosomally located gene as deduced from a partial nucleotide sequence encodes a proline dehydrogenase enzyme . Mutants *Rhizobium etli*, a bacterium that establishes symbiosis with *Phaseolus vulgaris* were obtained by general Tn5 mutagenesis these mutants were unable to grow on plate containing asparagine as the nitrogen and carbon source (Zepeda *et al.*, 1997).

Jimenez - Zurdo *et al.* (1995) described the isolation and characterization of a *Rhizobium meliloti* Tn5 mutant affected in ornithine and proline utilization and lacking ProDH activity. Nodulation properties of ProDH$^-$ mutants and its possible effect on nodule development, plant vigor and nitrogen fixation in nodulated alfalfa plants was also investigated. The importance of proline catabolism was studied not only in the bacteroids but also in the effectivity, competitiveness and nutrition of free living rhizobia.

2.3 ROLE OF ProDH ACTIVITY IN COMPETITIVENESS

Soils invariably contain some rhizobia nodulating the particular plants when legumes are regularly grown. The number of nodules per plant is used as an index for *Rhizobium* species efficiency. However this does not reflect the true efficiency. Since the count may include nodules formed by indigenous rhizobia also. Thus to distinguish the inoculum strain from any indigenous rhizobia and to screen the success of individual occupancy with different cultivars of legumes in different soils and agronomic conditions, a suitable methodology in required. Intrinsic antibiotic resistance pattern is very well known method to assess competitiveness (Sharma and Khurana,

1988; Khurana *et al.*, 1991). Sensitivity to different antibiotics at a range of concentration varied between species and it was suggested that such variation may be a useful taxonomic character. Antibiotic resistant strains have been used for rhizobial ecological studies (Obaton, 1973; Amarger, 1981). The disadvantage of this method is that selected lines may vary in other important characteristics including competitiveness for nodule formation (Bromfield and Jones, 1979) and ability to fix N_2 (Schwnighamer and Dudman, 1973). However Sharma *et al.* (1991) observed no change in competitiveness of streptomycin resistant *Rhizobium* species (*cicer*).

Jimenez - Zurdo *et al.* (1995) isolated characterized ProDH$^-$ mutant of *Rhizobium meliloti* obtained by Tn 5 mutagenesis impaired in nodulation efficiency and competitiveness on alfalfa roots. They termed the ProDH mutants as LMI which show disability to use proline as its sole carbon and nitrogen source resulted from the Tn 5 transposon insertion with in a chromosomally located gene that encodes a ProDH enzyme. Because the mutant exhibited no other growth defect and its nodulation ability is recovered to parental level when the ProDH activity is supplied by genetic complementation, thus they also concluded that mutant's symbiotic defect are a direct result of the loss of ProDH enzyme activity. This is found to be first report describing *Rhizobium meliloti* ProDH enzyme mutant.

Different reports have suggested that at least in soybean root nodules, proline catabolism may have particular importance in the bacteroid metabolism and one hypothesized role for proline is as a source of reduced carbon for bacteroids, especially in environmentally stressed nodules

(Zhu *et al.*, 1992; Kohl *et al.* ,1994). On the other hand studies of the transport and catabolism of L- proline in free living cells and bacteroids of cowpea. *Rhizobium* NGR 234 have shown that in this bacterium proline is metabolized via P5C and glutamate by means of proline oxidase and P5CDH (Glenn *et al.*, 1991).

It has recently been reported that symbiotic plasmid gene essential to the catabolism of proline are also required for efficient nodulation (Goldman *et al.*, 1994).

Jimenez-Zurdo *et al.* (1995) reported that in 1:1 coinoculation mixtures, *Rhizobium meliloti* parent strain appeared to be more competitive than ProDH$^-$ mutant, as deduced from their respect·percentages of nodule occupancy (only 6 per cent of nodules analyzed were found to be occupied by the ProDH$^-$ mutant strain). Thus competition assays demostrated that in *R. meliloti* ProDH is required for competitiveness on alfalfa (*Medicago sativa*) roots.

In this regard ProDH would be a key enzyme in the catabolism of different metabolites, including some amino acids and derivative compounds that lead to proline formation and are available during the infection process and blocking any step of these degradation pathways would results in an alteration of rhizobial nodulation efficiency and competitiveness. Thus one can not rule out that proline may have a physiological effect on nitrogen fixing metabolism in the *Rhizobium*- legume symbiosis in open field and additional work would be required to elucidate this question.

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS

All chemicals and media components were of analytical grade obtained from SRL, Merck (India) and Sigma (U.S.A.).

O-aminobenzaldehyde was prepared in the Department of Chemistry and Biochemistry, College of Basic Sciences and Humanities, Chaudhary Charan Singh Haryana Agricultural University, Hisar as per procedure described by Smith and Opie (1967).

3.1.2 ANTIBIOTIC SUPPLEMENTS

Stock solutions of Kanamycin (Km), Nalidixic acid (Nal), Chloramphenicol (Cm), Neomycin (Nm), Streptomycin (Sm) and Tetracyclin (Tc) were prepared in double distilled water and were micro filter sterilized.

These antibiotics were added to pre cooled media to have final required concentration.

3.1.3 INSTRUMENTS USED

1. Branson Sonifier 250
2. Cooling centrifuge C-24 (REMI)

3. Gerhardt Kjeldatherm and Gerhardt Vapodest 20.

4. U.V. VIS Spectrophotometer Model SL-150 (ELICO).

3.1.4 SEEDS

Seeds of mungbean cv. Asha used in present studies were obtained from the Pulse Section, Department of Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

3.1.5 BACTERIAL STRAINS

Rhizobium and *Bradyrhizobium* strains infecting mungbean used in present investigation were procured from Department of Microbiology, Chaudhary Charan Singh Haryana Agricultural University, Hisar.

MH1, MH2, MH3, MH4, MH5, Mo2, Mo6, M29 Hup+, MH202, M29 SCI, PP 9038, S-24, G-14, PP 2015-2, VBM-39, LMR-207, PMR-1, DM-3, M-3-92, MRM-4, M-10-92, CC-1021, G-1305, M-11, G-112, G-20 and CP3.

E. Coli S17-1 (p SUP Tn5 :B-20) strain was used for Tn5 mutagenesis.

3.1.6 MEDIA

1. Yeast extract mannitol agar (YEMA) medium (Fred, 1932)

Components	gl^{-1}
Mannitol	10.0
Yeast Extract	00.5
$K_2HPO_4.3H_2O$	00.5
$MgSO_4.7H_2O$	00.2
NaCl	00.1
Agar Agar	20.0

pH	6.5-7.0

In one litre YEMA 10ml of Congo red (1:400) was added whenever required.

2. Luria Bertini (LB) medium (Maniatis *et al.*; 1982)

Components	gl^{-1}
Tryptone	10.0
Yeast Extract	5.0
Sodium Chloride	2.0
Dextrose	1.0
Agar Agar	15.0
pH	7.4

3. Tryptone yeast extract (TY) medium (Beringer, 1974)

Components	gl^{-1}
Tryptone	5.0
Yeast Extract	1.0
$CaCl_2.6H_2O$	0.5
Agar Agar	20.0
pH	7.0

4. Rhizobial minimal medium (Jimenez-Zurdo *et al.*, 1995)

Components	gl^{-1}
$MgSO_4.7H_2O$	0.15
$CaCl_2.2H_2O$	0.05
$FeCl_3$ (anhydrous)	0.06
NaCl	0.05

K_2HPO_4	00.3
KH_2PO_4	00.3
Glutamic acid	1.1
Mannitol	10.0
Biotin	0.002
Thiamine HCl	0.001
Calcium pantothenate	0.001
Agar Agar	20.0
pH	6.8

Mannitol and glutamic acid were replaced with proline (0.2% w/v) as source of carbon and nitrogen in the media.

5. Sloger's nitrogen free nutrient solution (Sloger, 1969)

Components	gl^{-1}
$K_2HPO_4.3H_2O$	0.2
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$MnCl_2.H_2O$	0.004
$CuSO_4.5H_2O$	0.0002
Na_2 EDTA	0.03
H_3BO_3	0.00025
$FeSO_4.7H_2O$	0.02
$ZnSO_4$	0.1
Na_2MoO_4	0.01
$CaHPO_4$	10.0
pH	7.0

3.2 METHODS

3.2.1 ProDH ACTIVITY IN *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS INFECTING MUNGBEAN

All the available *Rhizobium* and *Bradyrhizobium* strains infecting mungbean were used to determine the ProDH activity under cultural conditions.

3.2.1.1 PREPARATION OF RHIZOBIAL CELL EXTRACTS FOR ProDH ACTIVITY (Jimenez- Zurdo *et al.*, 1995)

Rhizobium cultures were grown to exponential phase in minimal media broth containing glutamate and mannitol, supplemented with proline or ornithine (0.2% w/vol.) for preparation of enzyme extract. Growth thus obtained was centrifuged at 6000 rpm for 10 min. at 4°C and pellet suspended in 3 ml of 20 mM potassium phosphate buffer (pH=7.0). Cell suspensions were sonicated in 3 cycles of 30 sec. bursts at 35 W with a microprobe and resulting extracts (with cell debris not removed) were used as a source of enzyme or as digested sample for protein estimation.

3.2.1.2 ESTIMATION OF ProDH ENZYME ACTIVITY (Dessaux *et al.*, 1986)

The reaction mixture constituted a total volume of 3 ml* (0.6 m mol potassium phosphate buffer pH = 6, 1.3 m mol. proline, 4.0 µM O-aminobenzaldehyde). Reaction was initiated by addition of bacterial extract and stopped by addition of 0.4 ml of 20 per cent Trichloroacetic acid. The precipitate was eliminated by centrifugation and absorbance was read at 440 nm.

* (1.9 ml, 1 ml, 0.1 ml respectively)

3.2.1.3 PROTEIN ESTIMATION (Lowry *et al.*, 1951)

Proteins were estimated in cell culture. 5 ml of alkaline copper sulphate solution was added to 1 ml of digested sample, mixed well and allowed to settle at room temperature for 10 minutes. 0.5 ml of Folin's reagent was added and mixed thoroughly. Blank was run by taking 1 ml of distilled water instead of sample solution. The colour was read after 30 min in spectrophotometer at 660 nm. Standard curve was prepared by taking bovine serum albumin as the standard protein.

3.2.1.4 ENZYME ACTIVITY AND SPECIFIC ACTIVITY

One enzyme activity is defined as the amount catalyzing the synthesis of one micro-mole (μM) of proline per minute under standard transfer assay conditions.

Specific activity of the enzyme is defined as unit of enzyme per milligram of protein.

3.2.2 SCREENING OF *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS FOR ANTIBIOTIC RESISTANCE

All the strains used in present study were screened for antibiotic resistance pattern using various concentraion of antibiotic like Sm^{250}, Sm^{400}, Cm^{20}, Cm^{200}, Tc^{10}, Nm^{50}, Nm^{100}, Nal^{50} and Km^{50} in YEMA medium. Based on this, strains were selected for Tn5 mutagenesis and competitive studies

3.2.3 Tn5 MUTAGENESIS TO SELECT ProDH ACTIVITY VARIANTS

Rhizobium strains viz. Mo2, Mo6, G-14 and PP9038 were selected for Tn5 mutagenesis as these strains were having Nal^{50} resistance and were sensitive to Km^{50}. Tn5 mutagenesis was carried out with

E. coli S17-1 (pSUP Tn5 : B-20) strain. All four *Rhizobium* strains mentioned above were grown in trypton yeast extract (TY) broth supplemented with nalidixic acid (50µg ml^{-1}) at 28±1°C for 24 hours and each was mated separately with *E. Coli* strain grown in Luria Bartini (LB) broth containing Kanamycim (50µg ml^{-1}) at 37°C for 12 hours on shaker. Cultures were centrifuged in 1.5 ml eppendrof tubes washed with TY broth and resuspended in the ratio of 5:1 (*Rhizobium : E. coli*), centrifuged and resuspended in 30-40 µl of TY broth. Conjugal mixture (25 µl) was put on TY plates as a spot and incubated for 16-24h at 28±1°C. Cells were removed from the spot on TY plates, resuspended in 5ml TY broth and was vortexed. Serial dilutions were plated on yeast extract mannitol agar (YEMA) medium plates supplemented with kanamycin (50µg ml^{-1}) and nalidixic acid (50µg ml^{-1}) . Plates were incubated at 28±1°C for 72h. As control *Rhizobium* and *E. coli* were also plated on YEMA and TY medium containing both the antibiotics. About 1000 transconjugants were screened for ProDH activity using mannitol minimal media supplemented with proline (Jimenez-Zurdo *et al.*,1995) containing both antibiotics. Mutants showing growth on control plate containing mannitol and proline were replicated on mininal media plate having proline as carbon source to select ProDH mutants. Mutants which didnot show any growth or showing abundant growth on proline plates were selected as ProDH mutants. Such mutants were selected from control plates and were reconfirmed on proline plates. These mutants were further used in the present study.

3.2.4 EFFICACY OF *RHIZOBIUM* STRAINS AND Tn5 MUTANTS DIFFERING IN ProDH ACTIVITY

All the strains showing variation in ProDH activity viz. Mo2, Mo6, G-14, PP9038 and Tn5 mutants were inoculated the plants grown under chillum jar and pot culture conditions to determine efficacy of N_2 fixation.

3.2.4.1 CHILLUM JAR AND POT CULTURE EXPERIMENT

Seeds of mungbean cv. Asha were surface sterilized with absolute alcohol for 5 min. followed by repeated washing with sterilized distilled water. The surface sterilized seeds were then inoculated with 3 day old cultures of different *Rhizobium* strains and Tn5 mutants grown on YEMA broth. The growth of each strain in YEM broth was transferred to surface sterilized seeds in separate petriplates. The seeds were allowed to soak in the broth for 30 min and sown under unsterilized pot culture conditions as well as in autoclaved (3 hr, 20 psi) chillum jar assemblies containing acid washed river sand (Dahiya and Khurana, 1981). The lower assembly of each chillum jar was filled with quarter strength Jensen's nitrogen free mineral salt solution (Sloger, 1969). The surface sterilized but uninoculated seeds were sown as control. The jar assemblies were then kept in net house under day light conditions. Quarter strength sterilized Sloger's mineral salt solutions and sterilized water were used for watering the plants as and when required. Tap water was used for watering the plants under pot culture conditions. Sampling was done after 45 days of sowing and checked for parameters i.e. nodulation, enzyme activity, plant dry weight, root dry weight and total shoot nitrogen.

3.2.4.2 FRACTIONATION OF NODULES FOR ProDH ACTIVITY

(Zhu *et al.*, 1992)

Nodules were detached, rinsed with tap water, blotted dried, weighed and were gently crushed in a chilled pestle and mortar using grinidng buffer [Tricine (8.0 pH) 100 mM, Sucrose = 400 mM, $MgCl_2$ = 2.5 mM]. 2 ml g^{-1} fresh weight of nodules to minimize leaching of metabolites from bacteroids. The homogenate was filtered through muslincloth. The filterate was centrifuged at 500 rpm for 5 minutes to remove cell debris. The supernatent was centrifuged at 12000 rpm for 15 minutes. Pellet was washed once and resuspended in same volume of grinding buffer and used for assay of ProDH enzyme activity. ProDH activity was determined by the same procedure as used for rhizobial cell extracts.

3.2.4.3 NODULE FRESH WEIGHT , ROOT AND SHOOT DRY WEIGHT

Nodule were detached, counted and fresh weight of nodule was determined. The root and shoot portion were dried in oven at 80°C till constant weight and dry weight of root and shoot were determined

3.2.4.4 ESTIMATION OF TOTAL PLANT NITROGEN

Total plant nitrogen contents of the plants were estimated by using Gerhardt Kjeldatherm unit based on Kjeldahl's steam distillation method (Breman, 1960).

REAGENTS :

i) Digestion mixture

Potassium sulphate
Copper sulphate } 10:4:1
Selenium dioxide

ii) Sodium hydroxide 40 per cent

iii) Mixed indicator (It was prepared by mixing 0.5 g of bromocresol green and 0.1 g of methyl red in 100 ml of ethanol and bluish purple colour was neutralized with few drops of 1N NaOH as to get pink colour).

iv) Sulphuric acid N/ 50

v) 2 per cent Boric acid (2 g boric acid was dissolved in 89 ml of distilled water thoroughly and added 10 ml of ethanol and 1 ml of mixed indicator).

vi) Concentrated sulphuric aicd

PROCEDURE : Finely ground dried plant material (150 mg) was taken in Gerhardt Kjeldatherm tubes alongwith 1 g of digestion mixture and 10 ml of concentrated H_2SO_4 and digestion was carried out on Gerhardt Kjeldatherm. Tubes were transferred to Gerhardt Vapodest 20 for distillation one by one, followed by titration using Burette Digital II. Nitrogen contents were calculated from the volume of N/50 H_2SO_4 used, as follows:

1 ml of N/50 H_2SO_4 used = 280 µg of nitrogen

3.2.5 COMPETITIVENESS OF *RHIZOBIUM* STRAINS DIFFERING IN ProDH ACTIVITY

Competitive ability of *Rhizobium* strains Mo6 (Sm^{400}) PP9038 (Tc^{20}+Cm^{200}) and ProDH mutants (Nal^{50} + Km^{50}) was determined under sterilized as well as under unsterilized conditions. To determine the

complementation between different ProDH mutants and their comparative nodule occupancy, mutants/parents were coinoculated in 1:1 ratio under sterilized chillum jar conditions. Nodule occupancy by different ProDH mutants was assessed under pot culture conditions. All the nodules after 60 days of plant growth were detached and used for determining nodule occupancy. Nodules were surface sterilized using 0.2 per cent (w/v) $HgCl_2$ for 5 min and subsequently with alcohol (95%) for 5 min and washed 6-7 times with sterilized distilled water. For testing the presence of Mo6 (Sm^{400}) in nodules, each of the nodule was crushed and nodule sap was streaked on YEMA plates containing 400 μg ml^{-1} streptomycin. For assessing the presence of strain PP9038 (TC^{20} + Cm^{200}) nodule sap was streaked on YEMA plates containing tetracyclin (20 μg ml^{-1}) and chloramphenicol (200 μg ml^{-1}). Similarly to determine the nodule occupancy by ProDH mutants, nodule sap was streaked on YEMA plates containing Nalidixic acid (50 μg ml^{-1}) and Kanamycin (50 μg ml^{-1}). Sap from each of the nodule was also streaked on YEMA plates without any antibiotics and were used as controls. Plates were incubated at 30°C for 3-6 days. Colonies appearing on plates with antibiotics as compared to control plates were scored.

3.2.6 CORRELATION AMONG DIFFERENT PARAMETERS

Correlations of ProDH activity in bacteroid fraction and cell culture with different parameters affecting nodulation were determined using statistical methods.

RESULTS

Symbiotic nitrogen fixation by *Rhizobium* species in association with leguminous plants is fueled by carbon sources which are supplied by the plants. The evidence from studies of mutants of various species of *Rhizobium* (Peterson and La Rue, 1981; Finan, 1983) and supported by other studies (Sarosa *et al.*, 1984; Arwas *et al.*, 1985) concluded that the tricarboxylic cycle acids succinate and malate are the principle energy source for the symbiotic N_2 fixing bacteroids in the root nodules of legumes. An hypothesis involving proline as a carbon and nitrogen source for bacteroids in nodules have been proposed (Kohl *et al.*, 1988). Proline has been reported to provide additional energy to support nitrogen fixation in legume root nodules particularly in case of ureide transporting legumes. Proline dehydrogenase (ProDH) is a key enzyme, which catabolises proline to yield energy. Therefore, in the present investigation, role of ProDH in effectiveness and competitiveness of rhizobia infecting an ureide transporting legume, mungbean, which shows low competitiveness with the inoculant strains was undertaken.

4.1 SCREENING OF *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS INFECTING MUNGBEAN FOR ProDH ACTIVITY AND THEIR EFFICACY IN SYMBIOTIC ASSOCIATION WITH MUNGBEAN

Mungbean is an ureide transporting legume (Norhayati *et al.*, 1988; Peoples *et al.*, 1988) and particularly in ureide transporting legume it has been observed that ProDH activity is related to its N_2 fixing efficiency in symbiotic host association. Therefore in the present study all the available rhizobial strains infecting mungbean were screened under cultural conditions for ProDH activity and then its relationship with ProDH activity under symbiotic condition as well as with N_2 fixing efficiency was determined. To asses the activity of ProDH in cell extracts of rhizobial strains, all the cultures were grown in complete minimal media containing mannitol and glutamate and supplemented with proline as potential indicator of the enzyme activity. Enzyme activity ranged from 86.7 to 286.7 nmoles of P5C produced min^{-1} mg^{-1} of protein for different *Rhizobium* strains (Table 1) MH2 and CP3 strains showed lowest enzyme activity i.e. 86.7 and 106.7 nmoles of P5C produceed $min^{-1}mg^{-1}$ of protein where as PP2015-2 and MH5 showed highest enzyme activity i.e. 266.7and 286.7 nmoles of P5C produced min^{-1} mg^{-1} of proteins respectively in cell extracts and differences in ProDH activity shown by different rhizobial strains were statistically significant.

Efficacy of all these strains was determined under pot culture conditions. Different parameters considered for N_2 fixing efficacy of mungbean host

Table 1 **ProDH activity in cell extracts of *Rhizobium* and *Bradyrhizobium* strains infecting mungbean**

S. No.	Strains	ProDH activity*
1	MH1	133.3
2	MH2	86.7
3	MH3	130.0
4	MH4	203.3
5	MH5	286.7
6	Mo2	123.3
7	Mo6	146.7
8	M29Hup+	236.7
9	MH202	136.7
10	M29SCI	146.7
11	PP9038	240.0
12	S-24	240.0
13	G-14	250.0
14	PP2015-2	266.7
15	VBN39	246.7
16	LMR207	236.7
17	PMRI	250.0
18	DM3	240.0
19	M-3-92	246.7
20	MRM4	207.7
21	M-10-92	223.3
22	CC-1021	200.0
23	G-1305	210.0
24	M-11	210.0
25	G-112	170.0
26	G-20	170.0
27	CP3	106.7
SE (m)		17.91
CD at 5%		35.90

ProDH - Proline dehydrogenase
P5C -Pyrroline-5-carboxylate
* nmoles of P5C produced $min^{-1}mg^{-1}$ of protein

activity in bacteroid fraction of nodules (Table 2). Results showed that nodule number ranged from 16 to 45 nodules per plant for different *Rhizobium* and *Bradyrhizobium* strains.M-10-92 showed 16 nodules per plant where as strains Mo6, CP3 and PP9038 recorded highest number of nodules i.e. 45,44 and 41 nodules per plant respectively, whereas Mo2 and G-14 recorded 31 and 26 nodules per plant respectively. Nodule fresh weight ranged from 183 to 733 mg pl^{-1} whereas strain G-20 recorded lowest nodule fresh weight and highest nodule fresh weight was shown by PP9038 strain. In comparison to highest and lowest nodule fresh weights, moderate nodule fresh weight was recorded by strains Mo2 (346 mg pl^{-1}) and G-14(389 mg pl^{-1}). Similary PP9038 recorded highest root dry weight (416 mg pl^{-1}) and also total shoot nitrogen (78.9 mg pl^{-1}). Strain LMR 207 recorded maximum shoot dry weight (3102 mg pl^{-1}) whereas strains G-1305 and Mo2 recorded low shoot dry weight (1160 and 1696 mg pl^{-1}) respectively. ProDH activity in bacteroid fraction ranged from 11.4 to 56.7 nmoles of P5C produced min^{-1} g^{-1} nodule fresh weight in different strains used in present study (Table 2). Highest ProDH activity in symbiotic state was observed in case of strain S24 (56.7 nmoles of P5C produced $min^{-1}g^{-1}$ nodule fresh weight) followed by strains Mo2 and G-20, while strain DM-3 showed lowest ProDH activity. Differences in N_2 fixing parameters observed in case of different strains were statistically significant.

To establish whether there exist any correlation between the ProDH activity in free living and symbiotic rhizobial strains and their nitrogen fixing efficacy, correlation coefficients were determined. Results showed

Table 2 **Efficacy of *Rhizobium* and *Bradyrhizobium* strains in symbiotic associati with mungbean under pot culture conditions**

Rhizobial strains	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity i bacteroi
MH1	31	300	259	1586	33.0	30.9
MH2	31	421	299	1188	27.5	15.6
MH3	33	313	257	2147	55.0	22.9
MH4	18	304	145	23.76	59.6	21.2
MH5	21	228	183	1712	42.2	28.6
Mo2	31	346	276	1696	29.6	47.3
Mo6	45	324	234	2747	62.5	44.2
M29Hup+	37	479	388	2831	65.6	24.5
MH202	18	225	209	1590	40.0	17.2
M29SCI	23	228	199	2070	47.9	43.5
PP9038	41	732	416	2937	78.9	30.7
S-24	26	295	231	2896	68.3	56.7
G-14	26	389	080	2242	56.6	27.4
PP2015-2	25	543	222	2114	61.2	14.4
VBN39	24	400	243	1516	36.4	17.2
LMR207	21	266	191	3102	67.0	29.0
PMRI	38	594	239	2586	69.4	42.5
DM3	28	561	365	2249	29.7	11.4

Contd..

Rhizobial strains	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
M-3-92	18	243	176	2016	48.0	28.2
MRM4	38	519	333	2594	57.8	35.3
M-10-92	16	293	214	3087	69.0	20.6
C-1021	27	275	179	2827	64.0	21.6
G-1305	20	360	220	1160	34.5	17.5
M-11	38	257	218	2894	68.5	36.2
G-112	32	447	240	2174	55.9	25.8
G-20	17	183	107	1428	46.2	46.5
P3	44	405	160	2229	46.5	23.4
Uninoculated	10	094	211	1698	12.3	00.8
E (m)	1.82	22.25	15.65	145.09	2.24	2.97
D at 5%	3.64	44.59	31.36	290.76	4.49	5.96

* nmoles of P5C produced $min^{-1}g^{-1}$ nodule fresh wt

that ProDH activity in cell extracts was positively correlated with nodule fresh weight, shoot dry weight and total shoot nitrogen but not with nodule number and root dry weight (Table 3). This correlation was statistically significant at 1 per cent level of significance. Further number of nodules, shoot dry weight and total shoot nitrogen were positively correlated with the ProDH activity in bacteroids but correlation with nodule number and total shoot nitrogen were significant at 1 per cent level of significance and with shoot dry weight at 5% level of significance (Table 4). Correlations among parameters other than ProDH activity were also observed (Tables 3&4). Nodule number was correlated with nodule fresh weight, root dry weight shoot dry weight and total shoot nitrogen where as nodule fresh weight showed correlation only with shoot dry weight and total shoot nitrogen. Shoot dry weight was also positively correlated with total shoot nitrogen where as root dry weight was positively correlated with shoot dry weight and total shoot nitrogen but this was not significant at 1per cent or 5 per cent level of significance .

4.2 SELECTION OF Tn5 MUTANTS UNABLE TO UTILIZE PROLINE AS CARBON AND NITROGEN SOURCE

Tn5 mutagenesis has been used by number of workers for isolation of carbohydrate mutants of *Rhizobium* and particularly mutants unable to catabolize either ornithine or proline. Such mutants have been used to establish the relation of ProDH activity with N_2 fixing efficacy or nodulation competitiveness. Therefore rhizobial strains infecting mungbean were used to select Tn5 mutants unable to utilize proline as carbon or nitrogen source.

Table 3 Correlation of ProDH activity in rhizobial cell extracts with different N_2 fixing parameters under unsterilized conditions

	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})
ProDH activity in cell extracts (nmoles of P5C produced min^{-1} mg^{-1} of protein)	0.0231	0.2863**	0.0670	0.3916**	0.4509**
Nodule number (pl^{-1})		0.6089**	0.4525**	0.3711**	0.3937**
Nodule fresh wt. (mg pl^{-1})			0.6359**	0.2556*	0.4713**
Root dry wt. (mg pl^{-1})				0.1834	0.2063
Shoot dry wt. (mg pl^{-1})					0.8460**

Correlation coefficient at 5% = 0.217 (*)
Correlation coefficient at 1% = 0.283 (**)

Table 4 **Correlation of ProDH activity in bacteroid fraction with different N_2 fixing parameters under unsterilized conditions**

	ProDH activity in cell extracts (nmoles of P5C produced min^{-1} mg^{-1} of protein.)	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})
ProDH activity in bacteroids (nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.)	-0.0747	0.3391**	-0.0177	-0.0726	0.2822*	0.3170**
Nodule number (pl^{-1})			0.6089**	0.4525**	0.3711**	0.3937**
Nodule fresh wt. (mg pl^{-1})				0.6359**	0.2556*	0.4713**
Root dry wt. (mg pl^{-1})					0.1834	0.2063
Shoot dry wt. (mg pl^{-1})						0.8460**

Correlation coefficient at 5% = 0.217 (*)
Correlation coefficient at 1% = 0.283 (**)

Table 5 shows the inharent antibiotic resistance pattern of *Rhizobium* strains used in the present studies. Almost all the strains showed resistance to Sm^{400} except CC1021 and G-14 which showed resistance to Sm^{250} while strain PP9038 showed resistance to Tc^{20}+ Cm^{200} and strain CP3 was resistant to Tc^{20}+ Cm^{50} . All *Rhizobium* strains were also screened for their sensitivity to Km ^{50} and resistance to Nal^{50} but only four strains namely Mo2, Mo6, PP9038 and G-14 showed the desired antibiotic pattern. Therefore for Tn5 mutagenesis, these four strains were used and mutants were screened on minimal media containing mannitol and glutamate or proline as the only carbon and nitrogen source. Tn5 induced mutants unable to grow on minimal medium containing only proline as source of carbon and nitrogen, were obtained only from two *Rhizobium* strains Mo6 and PP9038. Mutants of Mo6 *Rhizobium* strain i.e. Mo6 Tn5 ProDH 20, Mo6 Tn5 ProDH 21, Mo6 Tn5 ProDH 22 and Mo6 Tn5 ProDH 23 showed no growth on prolin e plates (Fig. 2) and similarly mutants of PP9038 *Rhizobium* strain i.e. PP9038 Tn5 ProDH 30 and PP9038 Tn5 ProDH 31 also showed no growth on proline plates (Fig. 3). These mutants appeared to be defective in ProDH activity, as they were found not to utilize proline as source of carbon and thus were designated as $ProDH^-$ mutants. Other mutants of PP9038 *Rhizobium* strain which seemed to utilize proline more efficiently than ProDH deficient mutants but comparable to parent *Rhizobium* strain were also selected. These mutants were designated as PP9038 Tn5 ProDH 32 and PP9038 Tn5 ProDH 33 (Fig. 3). Similarly other ProDH Tn5 mutants selected were Mo6 Tn5 ProDH 24, Mo6 Tn ProDH 25, PP9038 Tn5 ProDH 34 and PP9038

Table 5 **Antibiotic resistance of *Rhizobium* and *Bradyrhizobium* strains infecting mungbean**

Rhizobial strains	Antibiotic resistance pattern
MH1	Sm^{400}
MH2	Sm^{400}
MH3	Sm^{400}
MH4	Sm^{400}
MH5	Sm^{400}
Mo2	$Sm^{400} + Nal^{50}$
Mo6	$Sm^{400} + Nal^{50}$
M29Hup+	Sm^{400}
MH202	Sm^{400}
M29SCI	Sm^{400}
PP9038	$Tc^{20} + Cm^{200} + Nal^{50}$
S-24	Sm^{400}
G-14	$Sm^{250} + Nal^{50}$
PP2015-2	Sm^{400}
VBN39	Sm^{400}
LMR207	Sm^{400}
PMRI	Sm^{400}
DM3	Sm^{400}
M-3-92	Sm^{400}
MRM4	Sm^{400}
M-10-92	Sm^{400}
CC-1021	Sm^{250}
G-1305	Sm^{400}
M-11	Sm^{400}
G-112	Sm^{400}
G-20	Sm^{400}
CP3	$Tc^{20} + Cm^{50}$
E.coli S17-1 (pSUP Tn5 : B-20)	Km^{50}

Fig 2 **Growth of ProDH deficient mutants of Mo6 *Rhizobium* strain on proline or mannitol mininal media plates**

[P] Mo6 parent *Rhizobium* strain
[1] Mo6 Tn5 ProDH 20 [2] Mo6 Tn5 ProDH 21
[3] Mo6 Tn5 ProDH 22 [4] Mo6 Tn5 ProDH 23

Fig. 3 **Growth of ProDH deficient and proline utilizing mutants of PP9038*Rhizobium* strain on proline or mannitol minimal media plates**

[P] PP9038 parent *Rhizobium* strain
[1] PP9038 Tn5 ProDH 30 [2] PP9038 Tn5 ProDH 31
[3] PP9038 Tn5 ProDH 32 [4] PP9038 Tn5 ProDH 33

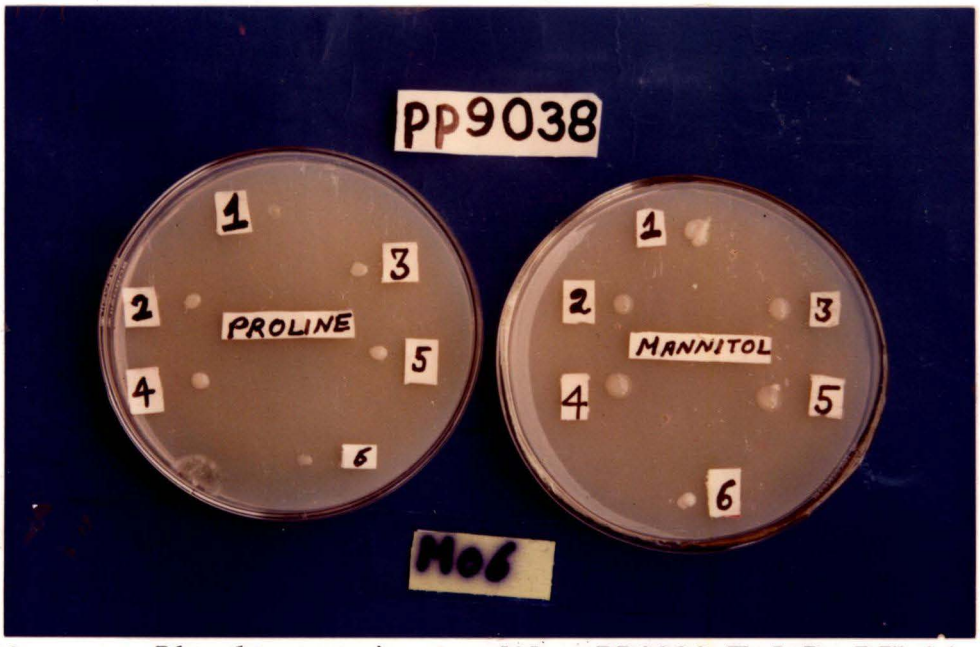
Tn5 ProDH 35 which showed comparatively more growth on proline plates then their respective parent *Rhizobium* strain (Fig. 4). Similarly mutants of Mo2 and G-14 *Rhizobium* strains Mo2Tn5 ProDH 44, Mo2 Tn5 ProDH 45, G-14 Tn5 ProDH 54 and G-14 Tn5 ProDH 55 showed more growth in comparison to their respective parent *Rhizobium* strain (Fig. 5). Mutants other than ProDH deficient mutants were selected to compare the differences if any in N_2 fixing parameters while comparing the efficiency of ProDH$^-$ mutants with ProDH$^+$ mutants.

Further to confirm these results, ProDH activity in cell extract of these mutants was determined. ProDH activity was undetectable in all the ProDH deficient mutants of Mo6 and PP9038 *Rhizobium* strains viz. Mo6 Tn5 ProDH 20, Mo6 Tn5 ProDH 21, Mo6 Tn5 ProDH 22, Mo6 Tn5 ProDH 23, PP9038 Tn5 ProDH 30 and PP9038 Tn5 ProDH 31 under same cultural conditions as provided to the parent (Table 6). ProDH activity of other rhizobial Tn5 mutants was comparable to their respective parents.

4.3 EFFICACY OF RHIZOBIAL Tn5 MUTANTS VARYING IN ProDH ACTIVITY IN SYMBIOTIC ASSOCIATION WITH MUNGBEAN

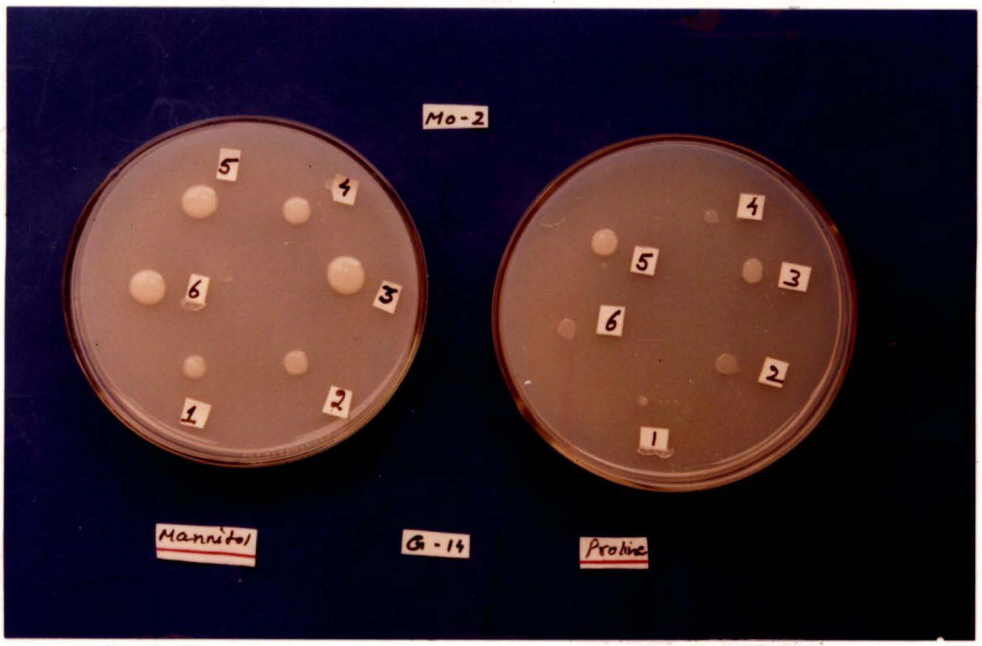
Using Tn5 mutagenesis of four rhizobial strains, mutants lacking ProDH activity, having comparable or more activity than their respective parents were selected. Such mutants were excellent material to generate information and to pinpoint the role of ProDH in providing energy or carbon source to free living or symbiotic rhizobia. Therefore efficacy of these mutants, alongwith parent and in combination to know whether mutants lacking in ProDH activity could be supplemented with another

Fig 4 **Growth of ProDH mutants (other than ProDH⁻ mutants) of PP9038 and Mo6 strains on proline or mannitol mininal media plates**



[1] PP9038 parent *Rhizobium* strain
[2] PP9038 Tn5 ProDH 34
[3] PP9038 Tn5 ProDH 35
[4] Mo6 Tn5 ProDH 24
[5] Mo6 Tn5 ProDH 25
[6] Mo6 parent *Rhizobium* strain

Fig. 5 **Growth of ProDH mutants (other than ProDH⁻ mutants) of Mo2 and G-14 strains on proline or mannitol mininal media plates**



[1] G-14 parent *Rhizobium* strain
[2] G-14 Tn5 ProDH 54
[3] G-14 Tn5 ProDH 55
[4] Mo2 parent *Rhizobium* strain
[5] Mo2 Tn5 ProDH 44
[6] Mo2 Tn5 ProDH 45

Table 6 ProDH activity in cell extracts of different ProDH Tn5 mutants

S. No.	*Rhizobium* strains/ Mutants	ProDH activity*
1	**Mo6 parent**	146.7
2	Mo6 Tn5 ProDH 20	D
3	Mo6 Tn5 ProDH 21	D
4	Mo6 Tn5 ProDH 22	D
5	Mo6 Tn5 ProDH 23	D
6	Mo6 Tn5 ProDH 24	160.0
7	Mo6 Tn5 ProDH 25	160.0
8	**PP9038 parent**	240.0
9	PP9038 Tn5 ProDH 30	D
10	PP9038 Tn5 ProDH 31	D
11	PP9038 Tn5 ProDH 32	240.0
12	PP9038 Tn5 ProDH 33	206.7
13	PP9038 Tn5 ProDH 34	236.7
14	PP9038 Tn5 ProDH 35	226.7
15	**Mo2 parent**	123.3
16	Mo2 Tn5 ProDH 44	130.0
17	Mo2 Tn5 ProDH 45	136.7
18	**G-14 parent**	250.0
19	G-14 Tn5 ProDH 54	266.0
20	G-14 Tn5 ProDH 55	276.7
	SE (m)	15.92
	CD at 5%	32.40

D : Not detectable

* nmoles of P5C produced $min^{-1}mg^{-1}$ of protein

coinoculant for nodule formation and its effectivity, chillum jar and pot culture experiments were conducted using mungbean as host.

Results showed that Mo6 parent formed 37 nodules per plant with 569 mg pl^{-1} nodule fresh weight, 497 mg pl^{-1} root dry weight, 2182 mg pl^{-1} shoot dry weight and 62.9 mg pl^{-1} total shoot nitrogen where as mutants lacking ProDH activity Mo6 Tn5 ProDH 20, Mo6 Tn5 ProDH 21, Mo6 Tn5 ProDH 22 and Mo6 Tn5 ProDH 23 formed only 19-23 nodules per plant with 234-391 mg pl^{-1} nodule fresh weight, 164-191 mg pl^{-1} root dry weight, 1176-1279 mg pl^{-1} shoot dry weight and 19.8 - 24.3 mg pl^{-1} total shoot nitrogen (Table 7). Similar trend was observed for strain PP9038 and its mutants PP9038 Tn5 ProDH 30 and PP9038 Tn5 ProDH 31 lacking ProDH activity (Table 7). There was significant decrease in nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen for all the ProDH Tn5 mutants of Mo6 and PP9038 showing no detectable level of ProDH activity when compared with their respective parent strains. The decrease in total shoot nitrogen in case of Tn5 mutants deficient in ProDH activity was to the extent of 3 folds in case of strain Mo6 and 2 folds in case of strain PP9038.

Measurements of ProDH activity in bacteroid fraction showed that it was undetectable in bacteroid fraction of Tn5 mutants of *Rhizobium* strains Mo6 and PP9038 (lacking in ProDH activity under cultural conditions) whereas Mo6 and PP9038 parent strains recorded 25.7 and 34.1 nmoles of P5C produced $min^{-1}g^{-1}$ nodule fresh weight of activity respectively (Table 7). Statistically significant more nodule number, nodule fresh weight,

Table 7 **Efficacy of *Rhizobium* strains Mo6 and PP9038 and their Tn5 mutants with no detectable ProDH activity in symbiotic association with mungbean under chillum jar conditions**

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
Mo6 parent	37	569	497	2182	62.9	25.7
Mo6 Tn5 ProDH 20	23	234	173	1269	20.4	D
Mo6 Tn5 ProDH 21	20	311	164	1279	24.3	D
Mo6 Tn5 ProDH 22	19	244	176	1242	20.1	D
Mo6 Tn5 ProDH 23	19	264	191	1176	19.8	D
PP9038 Parent	37	716	332	2837	70.0	34.1
PP9038 Tn5 ProDH 30	22	273	279	1836	30.7	D
PP9038 Tn5 ProDH 31	25	297	243	1994	30.3	D
Uninoculated	00	00	156	1133	06.5	00
SE (m)	3.15	36.26	43.24	178.23	1.52	0.35
CD at 5%	6.68	76.87	91.66	377.85	3.22	0.74

D- Not detectable

* nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

root and shoot dry weight, total shoot nitrogen and ProDH activity were shown by parent as compared to ProDH⁻ mutants. However, when Tn5 mutants having comparable ProDH activity to their parents were used, no significant differences in nodule number, nodule fresh weight, root dry wieght, shoot dry weight and total shoot nitrogen were observed (Table 8). Tn5 mutants of all four parents Mo6, PP9038, Mo2 and G-14 viz. Mo6 Tn5 ProDH 24, Mo6 Tn5 ProDH 25, PP9038 Tn5 ProDH 34, PP9038 Tn5 ProDH 35, Mo2 Tn5 ProDH 44, Mo2 Tn5 ProDH 45, G-14 Tn5 ProDH 54, and G-14 Tn5 ProDH 55 behaved similarly. Similar trend was observed for ProDH activity in bacteroid fraction of Tn5 mutants and their respective parent strains. However, as compared to uninoculated control the differences were statistically significant.

Correlation of ProDH activity in cell extracts with different nitrogen fixing parameters in case of all the four parents Mo6, PP9038, Mo2 and G-14 and their Tn5 mutants under sterilized conditions is shown in Table 9. The ProDH activity in cell extracts was correlated with nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen at 1 per cent level of significance. Similarly ProDH activity in bacteroid fraction of nodules under sterilized conditions was correlated with nodule number, nodule fresh weight and total shoot nitrogen at 1 per cent level of significance and with root and shoot dry weight at 5 per cent level of significance (Table 10). All other N_2 fixing parameters were also correlated with each other at 1 per cent level of significance (Tables 9&10). ProDH activity in cell extracts was also correlated with

ble 8 Efficacy of rhizobial Tn5 mutants with variable ProDH activity in symbi association with mungbean under chillum jar conditions

izobium ains/ itants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
o6 parent	37	569	497	2182	62.9	25.7
o6 Tn5 ProDH 24	34	498	433	2012	65.0	30.0
o6 Tn5 ProDH 25	35	475	416	2280	62.7	27.4
P9038 parent	37	716	332	2837	70.0	34.1
P9038 Tn5 ProDH 32	31	502	274	2425	55.9	26.5
P9038 Tn5 ProDH 33	35	622	282	2411	56.0	26.7
P9038 Tn5 ProDH 34	41	724	402	2978	75.6	35.7
P9038 Tn5 ProDH 35	39	706	412	2945	72.8	33.9
lo2 parent	30	273	164	1555	37.8	57.7
lo2 Tn5 ProDH 44	31	325	239	1656	39.9	51.5
lo2 Tn5 ProDH 45	32	295	243	1690	42.2	58.5
-14 parent	31	433	204	1832	47.9	27.5
-14 Tn5 ProDH 54	34	472	254	1994	50.7	36.0
-14 Tn5 ProDH 55	32	431	247	2014	50.5	32.5
Jninoculated	00	00	156	1133	06.5	00
E (m)	4.00	49.46	27.14	73.09	2.69	3.75
D at 5%	8.72	107.78	79.13	189.26	5.87	8.18

* nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

Table 9 **Correlation of ProDH activity in cell extracts of rhizobial strains an their Tn5 mutants with different N_2 fixing parameters under sterilize conditions**

	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})
ProDH activity in cell extracts (nmoles of P5C produced min^{-1} mg^{-1} of protein)	0.7213**	0.7208**	0.4858**	0.4019**	0.7437**
Nodule number (pl^{-1})		0.7684**	0.4724**	0.4937**	0.7366**
Nodule fresh wt. (mg pl^{-1})			0.6006**	0.6224**	0.7909**
Root dry wt. (mg pl^{-1})				0.7113**	0.7437**
Shoot dry wt. (mgpl^{-1})					0.8524**

Correlation coefficient at 5% = 0.250 (*)
Correlation coefficient at 1% = 0.325 (**)

Table 10 Correlation of ProDH activity in bacteroidal fraction of rhizobial strains and their Tn5 mutants with different N_2 fixing parameters under sterilized conditions

	ProDH activity in cell extracts (nmoles of P5C produced min^{-1} mg^{-1} of protein)	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})
ProDH activity in bacteroids (nmoles of P5C produced min^{-1} g^{-1}. nodule fresh wt.)	0.6802**	0.6854**	0.3734**	0.2871*	0.3262*	0.5950**
Nodule number (pl^{-1})			0.7684**	0.4724**	0.4937**	0.7366**
Nodule fresh wt. (mg pl^{-1})				0.6006**	0.6224**	0.7909**
Root dry wt. (mg pl^{-1})					0.7113**	0.7437**
Shoot dry wt. (mg pl^{-1})						0.8524**

Correlation coefficient at 5% = 0.250 (*)
Correlation coefficient at 1% = 0.325 (**)

ProDH activity in bacteroid fraction at 1 per cent level of significance as shown in Table 10.

These results were further confirmed under unsterilized conditions in pot culture. ProDH deficient mutants viz. Mo6 Tn5 ProDH 20, Mo6 Tn5 ProDH 21,Mo6 Tn5 ProDH 22 and Mo6 Tn5 ProDH 23 formed 17-21 nodules per plant with 174-191 mg pl^{-1} nodule fresh weight, 98-104 mg pl^{-1} root dry weight, 1476-1754 mg pl^{-1} shoot dry weight and 23.2 - 31.9 mg pl^{-1} total shoot nitrogen. These values were much lower than that observed with Mo6 parent strain which produced 45 nodules per plant, 324 mg pl^{-1} nodule fresh weight, 234 mg pl^{-1} root dry weight, 2747 mg pl^{-1} shoot dry weight and 63.5 mg pl^{-1} total shoot nitrogen (Table 11). Similarly significant decrease in nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen was observed in case of ProDH mutants of PP9038 in comparison to parent strain. ProDH activity in bacteroid fraction, ranged from 0.4 to 0.5 and 0.3 to 0.4 nmoles of P5C produced $min^{-1}g^{-1}$ nodule fresh weight in case of ProDH mutants of *Rhizobium* strains Mo6 and PP9038, respectively. However, parent strains Mo6 and PP9038 recorded 44.2 and 30.7 nmoles of P5C produced min^{-1} g^{-1} nodule fresh weight, respectively.

Similarly when other Tn5 mutants having ProDH activity comparable to the parents were used, there observed no significant difference in any N_2 fixing parameter between the parent strains and their respective proline utilizing mutants (Table 12). ProDH activity in bacteroid fraction of proline utilizing Tn5 mutants also observed no appreciable increase in the activity

Table 11 **Efficacy of *Rhizobium* strains Mo6 and PP9038 and their Tn5 mutants with no detectable ProDH activity in symbiotic association with mungbean under pot culture conditions**

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
Mo6 parent	45	324	234	2747	62.5	44.2
Mo6 Tn5 ProDH 20	21	181	104	1597	23.2	00.4
Mo6 Tn5 ProDH 21	19	191	098	1757	31.9	00.5
Mo6 Tn5 ProDH 22	17	183	093	1628	26.4	00.4
Mo6 Tn5 ProDH 23	19	174	103	1467	23.9	00.3
P9038 parent	41	732	416	2937	78.9	30.7
P9038Tn5 ProDH 30	25	255	231	1703	35.5	00.4
P9038Tn5 ProDH 31	24	219	205	1900	42.8	00.3
Uninoculated	10	094	211	1698	12.3	00.8
SE (m)	1.73	9.00	11.39	152.07	2.19	1.45
CD at 5%	3.66	19.09	24.14	322.38	4.63	3.08

nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

Table 12 Efficacy of rhizobial Tn5 mutants with variable ProDH activity in symbiotic association with mungbean under pot culture conditions

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
Mo6 parent	45	324	234	2747	62.5	44.2
Mo6 Tn5 ProDH 24	43	296	223	2720	64.9	42.0
Mo6 Tn5 ProDH 25	41	325	224	2682	65.5	40.5
PP9038 parent	41	732	416	2937	78.9	30.7
PP9038Tn5 ProDH 32	31	530	427	2375	56.9	28.1
PP9038Tn5 ProDH 33	30	521	431	2315	56.0	30.2
PP9038Tn5 ProDH 34	44	536	447	2545	74.0	38.2
PP9038Tn5 ProDH 35	42	514	453	2480	75.6	39.0
Mo2 parent	31	346	276	1696	29.6	47.3
Mo2 Tn5 ProDH 44	34	357	268	1750	30.9	53.3
Mo2 Tn5 ProDH 45	34	360	267	1700	30.4	54.7
G-14 parent	26	389	080	2242	56.6	27.4
G-14 Tn5 ProDH 54	28	409	108	2334	58.7	32.4
G-14 Tn5 ProDH 55	30	415	101	2326	56.5	34.0
Uninoculated	10	094	211	1698	12.3	00.8
SE (m)	1.57	14.03	12.05	43.94	1.69	3.39
CD at 5%	3.44	30.57	26.25	95.75	3.69	7.38

* nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

as compared to their respective parents.

Correlation coefficient of the experiment conducted under pot culture conditions was also determined. Correlation of ProDH activity in cell extracts of *Rhizobium* strains and their Tn5 mutants is shown in Table 13. The ProDH activity in cell extracts was positively correlated with all the N_2 fixing parameters at 1 per cent level of significance. In case of ProDH activity in bacteroid fraction it was also positively correlated with nodule number, nodule fresh weight and total shoot nitrogen at 1 per cent level of significance (Table 14). Other N_2 fixing parameters were found to be correlated with each other at 5 per cent as well as 1 per cent level of significance (Table 13 and 14). ProDH activity in bacteroid fraction was found to be positively correlated with ProDH activity in the cell extracts of *Rhizobium* strains and their Tn5 mutants at 1 per cent level of significance.

4.4 EFFECTIVITY AND COMPETITIVENESS OF *RHIZOBIUM* STRAINS AND THEIR Tn5 MUTANTS VARYING IN ProDH ACTIVITY USED AS COINOCULANTS IN MUNGBEAN

C_4 dicarboxylic acids have generally been considered the major carbon source exported from plant cells to the bacteroids that support the N_2 fixation process. But little is known about the carbon source used by the *Rhizobium* during nodule formation and nodule invasion process. It has been suggested that glutamate and proline may supply the additional energy. In the present study, the role of ProDH enzyme activity in forming the nodules by a strain was investigated. In this Tn5 mutants lacking in ProDH activity, alongwith other mutants and parent strains were used

coinoculant for nodule formation and its effectivity, chillum jar and pot culture experiments were conducted using mungbean as host.

Results showed that Mo6 parent formed 37 nodules per plant with 569 mg pl^{-1} nodule fresh weight, 497 mg pl^{-1} root dry weight, 2182 mg pl^{-1} shoot dry weight and 62.9 mg pl^{-1} total shoot nitrogen where as mutants lacking ProDH activity Mo6 Tn5 ProDH 20, Mo6 Tn5 ProDH 21, Mo6 Tn5 ProDH 22 and Mo6 Tn5 ProDH 23 formed only 19-23 nodules per plant with 234-391 mg pl^{-1} nodule fresh weight, 164-191 mg pl^{-1} root dry weight, 1176-1279 mg pl^{-1} shoot dry weight and 19.8 - 24.3 mg pl^{-1} total shoot nitrogen (Table 7). Similar trend was observed for strain PP9038 and its mutants PP9038 Tn5 ProDH 30 and PP9038 Tn5 ProDH 31 lacking ProDH activity (Table 7). There was significant decrease in nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen for all the ProDH Tn5 mutants of Mo6 and PP9038 showing no detectable level of ProDH activity when compared with their respective parent strains. The decrease in total shoot nitrogen in case of Tn5 mutants deficient in ProDH activity was to the extent of 3 folds in case of strain Mo6 and 2 folds in case of strain PP9038.

Measurements of ProDH activity in bacteroid fraction showed that it was undetectable in bacteroid fraction of Tn5 mutants of *Rhizobium* strains Mo6 and PP9038 (lacking in ProDH activity under cultural conditions) whereas Mo6 and PP9038 parent strains recorded 25.7 and 34.1 nmoles of P5C produced $min^{-1}g^{-1}$ nodule fresh weight of activity respectively (Table 7). Statistically significant more nodule number, nodule fresh weight,

able 7 **Efficacy of *Rhizobium* strains Mo6 and PP9038 and their Tn5 mutants with no detectable ProDH activity in symbiotic association with mungbean under chillum jar conditions**

hizobium rains/ utants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
lo6 parent	37	569	497	2182	62.9	25.7
lo6 Tn5 ProDH 20	23	234	173	1269	20.4	D
lo6 Tn5 ProDH 21	20	311	164	1279	24.3	D
lo6 Tn5 ProDH 22	19	244	176	1242	20.1	D
lo6 Tn5 ProDH 23	19	264	191	1176	19.8	D
P9038 Parent	37	716	332	2837	70.0	34.1
P9038 Tn5 ProDH 30	22	273	279	1836	30.7	D
P9038 Tn5 ProDH 31	25	297	243	1994	30.3	D
ninoculated	00	00	156	1133	06.5	00
E (m)	3.15	36.26	43.24	178.23	1.52	0.35
D at 5%	6.68	76.87	91.66	377.85	3.22	0.74

)- Not detectable

nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

root and shoot dry weight, total shoot nitrogen and ProDH activity were shown by parent as compared to ProDH$^-$ mutants. However, when Tn5 mutants having comparable ProDH activity to their parents were used, no significant differences in nodule number, nodule fresh weight, root dry wieght, shoot dry weight and total shoot nitrogen were observed (Table 8). Tn5 mutants of all four parents Mo6, PP9038, Mo2 and G-14 viz. Mo6 Tn5 ProDH 24, Mo6 Tn5 ProDH 25, PP9038 Tn5 ProDH 34, PP9038 Tn5 ProDH 35, Mo2 Tn5 ProDH 44, Mo2 Tn5 ProDH 45, G-14 Tn5 ProDH 54, and G-14 Tn5 ProDH 55 behaved similarly. Similar trend was observed for ProDH activity in bacteroid fraction of Tn5 mutants and their respective parent strains. However, as compared to uninoculated control the differences were statistically significant.

Correlation of ProDH activity in cell extracts with different nitrogen fixing parameters in case of all the four parents Mo6, PP9038, Mo2 and G-14 and their Tn5 mutants under sterilized conditions is shown in Table 9. The ProDH activity in cell extracts was correlated with nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen at 1 per cent level of significance. Similarly ProDH activity in bacteroid fraction of nodules under sterilized conditions was correlated with nodule number, nodule fresh weight and total shoot nitrogen at 1 per cent level of significance and with root and shoot dry weight at 5 per cent level of significance (Table 10). All other N_2 fixing parameters were also correlated with each other at 1 per cent level of significance (Tables 9&10). ProDH activity in cell extracts was also correlated with

Table 8 Efficacy of rhizobial Tn5 mutants with variable ProDH activity in symbiotic association with mungbean under chillum jar conditions

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
Mo6 parent	37	569	497	2182	62.9	25.7
Mo6 Tn5 ProDH 24	34	498	433	2012	65.0	30.0
Mo6 Tn5 ProDH 25	35	475	416	2280	62.7	27.4
PP9038 parent	37	716	332	2837	70.0	34.1
PP9038 Tn5 ProDH 32	31	502	274	2425	55.9	26.5
PP9038 Tn5 ProDH 33	35	622	282	2411	56.0	26.7
PP9038 Tn5 ProDH 34	41	724	402	2978	75.6	35.7
PP9038 Tn5 ProDH 35	39	706	412	2945	72.8	33.9
Mo2 parent	30	273	164	1555	37.8	57.7
Mo2 Tn5 ProDH 44	31	325	239	1656	39.9	51.5
Mo2 Tn5 ProDH 45	32	295	243	1690	42.2	58.5
G-14 parent	31	433	204	1832	47.9	27.5
G-14 Tn5 ProDH 54	34	472	254	1994	50.7	36.0
G-14 Tn5 ProDH 55	32	431	247	2014	50.5	32.5
Uninoculated	00	00	156	1133	06.5	00
SE .(m)	4.00	49.46	27.14	73.09	2.69	3.75
CD at 5%	8.72	107.78	79.13	189.26	5.87	8.18

* nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

Table 9 **Correlation of ProDH activity in cell extracts of rhizobial strains and their Tn5 mutants with different N_2 fixing parameters under sterilized conditions**

	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})
ProDH activity in cell extracts (nmoles of P5C produced min^{-1} mg^{-1} of protein)	0.7213**	0.7208**	0.4858**	0.4019**	0.7437**
Nodule number (pl^{-1})		0.7684**	0.4724**	0.4937**	0.7366**
Nodule fresh wt. (mg pl^{-1})			0.6006**	0.6224**	0.7909**
Root dry wt. (mg pl^{-1})				0.7113**	0.7437**
Shoot dry wt. ($mgpl^{-1}$)					0.8524**

Correlation coefficient at 5% = 0.250 (*)
Correlation coefficient at 1% = 0.325 (**)

Table 10 Correlation of ProDH activity in bacteroidal fraction of rhizobial strains and their Tn5 mutants with different N_2 fixing parameters under sterilized conditions

	ProDH activity in cell extracts (nmoles of P5C produced min^{-1} mg^{-1} of protein)	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})
ProDH activity in bacteroids (nmoles of P5C produced min^{-1} g^{-1}. nodule fresh wt.)	0.6802**	0.6854**	0.3734**	0.2871*	0.3262*	0.5950**
Nodule number (pl^{-1})			0.7684**	0.4724**	0.4937**	0.7366**
Nodule fresh wt. (mg pl^{-1})				0.6006**	0.6224**	0.7909**
Root dry wt. (mg pl^{-1})					0.7113**	0.7437**
Shoot dry wt. (mg pl^{-1})						0.8524**

Correlation coefficient at 5% = 0.250 (*)
Correlation coefficient at 1% = 0.325 (**)

ProDH activity in bacteroid fraction at 1 per cent level of significance as shown in Table 10.

These results were further confirmed under unsterilized conditions in pot culture. ProDH deficient mutants viz. Mo6 Tn5 ProDH 20, Mo6 Tn5 ProDH 21,Mo6 Tn5 ProDH 22 and Mo6 Tn5 ProDH 23 formed 17-21 nodules per plant with 174-191 mg pl^{-1} nodule fresh weight, 98-104 mg pl^{-1} root dry weight, 1476-1754 mg pl^{-1} shoot dry weight and 23.2 - 31.9 mg pl^{-1} total shoot nitrogen. These values were much lower than that observed with Mo6 parent strain which produced 45 nodules per plant, 324 mg pl^{-1} nodule fresh weight, 234 mg pl^{-1} root dry weight, 2747 mg pl^{-1} shoot dry weight and 63.5 mg pl^{-1} total shoot nitrogen (Table 11). Similarly significant decrease in nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen was observed in case of ProDH mutants of PP9038 in comparison to parent strain. ProDH activity in bacteroid fraction, ranged from 0.4 to 0.5 and 0.3 to 0.4 nmoles of P5C produced $min^{-1}g^{-1}$ nodule fresh weight in case of ProDH mutants of *Rhizobium* strains Mo6 and PP9038, respectively. However, parent strains Mo6 and PP9038 recorded 44.2 and 30.7 nmoles of P5C produced min^{-1} g^{-1} nodule fresh weight, respectively.

Similarly when other Tn5 mutants having ProDH activity comparable to the parents were used, there observed no significant difference in any N_2 fixing parameter between the parent strains and their respective proline utilizing mutants (Table 12). ProDH activity in bacteroid fraction of proline utilizing Tn5 mutants also observed no appreciable increase in the activity

Table 11 **Efficacy of *Rhizobium* strains Mo6 and PP9038 and their Tn5 mutants with no detectable ProDH activity in symbiotic association with mungbean under pot culture conditions**

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
Mo6 parent	45	324	234	2747	62.5	44.2
Mo6 Tn5 ProDH 20	21	181	104	1597	23.2	00.4
Mo6 Tn5 ProDH 21	19	191	098	1757	31.9	00.5
Mo6 Tn5 ProDH 22	17	183	093	1628	26.4	00.4
Mo6 Tn5 ProDH 23	19	174	103	1467	23.9	00.3
PP9038 parent	41	732	416	2937	78.9	30.7
PP9038Tn5 ProDH 30	25	255	231	1703	35.5	00.4
PP9038Tn5 ProDH 31	24	219	205	1900	42.8	00.3
Uninoculated	10	094	211	1698	12.3	00.8
SE (m)	1.73	9.00	11.39	152.07	2.19	1.45
CD at 5%	3.66	19.09	24.14	322.38	4.63	3.08

* nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

Table 12 Efficacy of rhizobial Tn5 mutants with variable ProDH activity in symbiotic association with mungbean under pot culture conditions

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	ProDH* activity in bacteroids
Mo6 parent	45	324	234	2747	62.5	44.2
Mo6 Tn5 ProDH 24	43	296	223	2720	64.9	42.0
Mo6 Tn5 ProDH 25	41	325	224	2682	65.5	40.5
PP9038 parent	41	732	416	2937	78.9	30.7
PP9038Tn5 ProDH 32	31	530	427	2375	56.9	28.1
PP9038Tn5 ProDH 33	30	521	431	2315	56.0	30.2
PP9038Tn5 ProDH 34	44	536	447	2545	74.0	38.2
PP9038Tn5 ProDH 35	42	514	453	2480	75.6	39.0
Mo2 parent	31	346	276	1696	29.6	47.3
Mo2 Tn5 ProDH 44	34	357	268	1750	30.9	53.3
Mo2 Tn5 ProDH 45	34	360	267	1700	30.4	54.7
G-14 parent	26	389	080	2242	56.6	27.4
G-14 Tn5 ProDH 54	28	409	108	2334	58.7	32.4
G-14 Tn5 ProDH 55	30	415	101	2326	56.5	34.0
Uninoculated	10	094	211	1698	12.3	00.8
SE (m)	1.57	14.03	12.05	43.94	1.69	3.39
CD at 5%	3.44	30.57	26.25	95.75	3.69	7.38

* nmoles of P5C produced min^{-1} g^{-1} nodule fresh wt.

as compared to their respective parents.

Correlation coefficient of the experiment conducted under pot culture conditions was also determined. Correlation of ProDH activity in cell extracts of *Rhizobium* strains and their Tn5 mutants is shown in Table 13. The ProDH activity in cell extracts was positively correlated with all the N_2 fixing parameters at 1 per cent level of significance. In case of ProDH activity in bacteroid fraction it was also positively correlated with nodule number, nodule fresh weight and total shoot nitrogen at 1 per cent level of significance (Table 14). Other N_2 fixing parameters were found to be correlated with each other at 5 per cent as well as 1 per cent level of significance (Table 13 and 14). ProDH activity in bacteroid fraction was found to be positively correlated with ProDH activity in the cell extracts of *Rhizobium* strains and their Tn5 mutants at 1 per cent level of significance.

4.4 EFFECTIVITY AND COMPETITIVENESS OF *RHIZOBIUM* STRAINS AND THEIR Tn5 MUTANTS VARYING IN ProDH ACTIVITY USED AS COINOCULANTS IN MUNGBEAN

C_4 dicarboxylic acids have generally been considered the major carbon source exported from plant cells to the bacteroids that support the N_2 fixation process. But little is known about the carbon source used by the *Rhizobium* during nodule formation and nodule invasion process. It has been suggested that glutamate and proline may supply the additional energy. In the present study, the role of ProDH enzyme activity in forming the nodules by a strain was investigated. In this Tn5 mutants lacking in ProDH activity, alongwith other mutants and parent strains were used

Table 13 Correlation of ProDH activity in cell extracts of rhizobial and their Tn5 mutants with different N_2 fixing parameters under unsterilized conditions

	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})
ProDH activity in cell extracts (nmoles of P5C produced min^{-1} mg^{-1} of protein)	0.6469**	0.7630**	0.4641**	0.6651**	0.8225**
Nodule number (pl^{-1})		0.7041**	0.6320**	0.6567**	0.7313**
Nodule fresh wt. (mg pl^{-1})			0.8182**	0.6529**	0.7689**
Root dry wt. (mg pl^{-1})				0.6168**	0.6069**
Shoot dry wt. (mg pl^{-1})					0.8172**

Correlation coefficient at 5% = 0.250 (*)
Correlation coefficient at 1% = 0.325 (**)

and nodule occupancy by different inoculants was determined using antibiotic resistance markers.

To avoid competition from native rhizobial population the experiment was first conducted under sterilized chillum jar conditions. Table 15 shows the effectiveness and competitiveness of *Rhizobium* strain Mo6 and its Tn5 mutants. As observed earlier, in this experiment also, similar trend was observed when parent strain Mo6 or its mutant alone was used. However, coinoculation of parent with Tn5 mutant resulted in comparable nodulation, root dry weight, shoot dry weight and total plant nitrogen to as observed by the parent strain. Such additive effect was not observed when only Tn5 mutants were used as coinoculant. Measurement of nodule occupancy showed that as there was no competition from native rhizobial population, so all the nodules were formed by the inoculant parent strain or Tn5 mutant. But when Tn5 mutant defective in ProDH activity was coinoculated with the parent strain than majority of the nodules (93-94%) were formed by the parent strain as compared to the mutants (defective in ProDH activity). Similar trend was observed when both *Rhizobium* strain PP9038 and its Tn5 mutants were used as single or coinoculant (Table 16).

Further to confirm these finding the experiment was conducted under unsterilized pot culture conditions. The performance of Tn5 mutants either lacking in ProDH activity or having comparable activity to parents were assessed in the presence of native rhizobial population able to infect mungbean. Decrease in N_2 fixing parameters was observed when both ProDH$^-$ mutants of *Rhizobium* strains Mo6 (Table 17) or PP9038 (Table 18) were used

Table 15 Efficiency and competitiveness of *Rhizobium* strain Mo6 and its Tn5 mutants varying in ProDH activity used as coinoculants in mungbean under chillum jar conditions

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	Nodule occupancy (%)	
						Rhizobium strain	Tn5 mutants
Mo6 parent	33	462	365	1781	43.7	96.7	-
Mo6 Tn5 ProDH 20	23	234	146	0961	20.3	-	97.9
Mo6 Tn5 ProDH 21	24	234	144	0929	20.5	-	97.8
Mo6 Tn5 ProDH 22	22	223	148	1060	19.0	-	99.8
Mo6 Tn5 ProDH 24	35	472	380	1809	42.5	-	98.5
Mo6+Mo6 Tn5 ProDH 20	31	450	384	1781	48.0	92.8	6.9
Mo6+Mo6 Tn5 ProDH 21	33	449	348	1748	43.5	93.7	5.4
Mo6+Mo6 Tn5 ProDH 22	32	460	353	1824	45.3	93.7	5.6
Mo6 Tn5 ProDH 20+ Mo6 Tn5 ProDH 21	21	211	146	0952	19.6	-	94.5
Mo6 Tn5 ProDH 21+ Mo6 Tn5 ProDH 22	22	201	144	0995	22.5	-	96.2
Uninoculated	00	00	103	0924	07.4	-	-
SE (m)	1.02	8.36	11.54	34.90	0.55	-	-
CD at 5%	2.13	17.45	24.06	72.81	1.15		

ProDH activity used as coinoculants in mungbean under chillum jar conditions

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	Nodule occupancy (%)	
						Rhizobium strain	Tn5 mutants
PP9038 parent	37	687	321	2143	54.1	97.1	-
PP9038 Tn5 ProDH 30	20	254	220	1534	29.8	-	96.0
PP9038 Tn5 ProDH 31	21	259	223	1638	28.9	-	94.5
PP9038 Tn5 ProDH 34	39	730	345	2271	56.2	-	96.2
PP9038+ PP9038 Tn5 ProDH 30	36	725	291	2166	61.1	84.7	9.6
PP9038+ PP9038 Tn5 ProDH 31	39	727	307	2207	53.9	86.2	8.5
PP9038 Tn5 ProDH 30+ PP9038 Tn5 ProDH 31	23	270	222	1565	28.5	-	90.6
Uninoculated	00	00	103	924	07.4	-	-
SE (m)	1.18	20.61	11.56	63.49	1.13	-	-
CD at 5%	2.54	44.22	24.79	136.19	2.42	-	-

activity used as coinoculants in mungbean under pot culture conditions

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	Nodule occupancy (%)	
						Rhizobium strain	Tn5 mutants
Mo6 parent	36	461	300	2358	52.1	87.8	-
Mo6 Tn5 ProDH 20	20	198	129	1152	25.4	-	82.5
Mo6 Tn5 ProDH 21	15	179	131	1258	23.2	-	84.5
Mo6 Tn5 ProDH 22	20	198	129	1050	25.4	-	82.6
Mo6 Tn5 ProDH 24	38	472	293	2256	50.8	-	88.6
Mo6+Mo6 Tn5 ProDH 20	35	458	331	2460	54.3	83.7	6.3
Mo6+Mo6 Tn5 ProDH 21	37	462	322	2390	54.6	84.0	5.4
Mo6+Mo6 Tn5 ProDH 22	36	460	426	2510	57.0	86.7	5.8
Mo6 Tn5 ProDH 20+ Mo6 Tn5 ProDH 21	19	200	147	1006	24.9	-	87.6
Mo6 Tn5 ProDH 21+ Mo6 Tn5 ProDH 22	19	201	134	1208	21.8	-	88.4
Uninoculated	05	048	191	1007	13.3	-	-
SE (m)	1.11	5.41	7.57	63.03	0.85	-	-
CD at 5%	2.33	11.29	15.78	131.48	1.77	-	-

Table 18 **Efficiency and competitiveness of *Rhizobium* strain PP9038 and its Tn5 mutants varying in ProDH activity used as coinoculants in mungbean under pot culture conditions**

Rhizobium strains/ Mutants	Nodule number (pl^{-1})	Nodule fresh wt. (mg pl^{-1})	Root dry wt. (mg pl^{-1})	Shoot dry wt. (mg pl^{-1})	Total shoot nitrogen (mg pl^{-1})	Nodule occupancy (%)	
						Rhizobium strain	Tn5 mutants
PP9038 parent	37	669	279	2156	61.3	80.8	-
PP9038 Tn5 ProDH 30	21	234	183	1490	32.8	-	84.7
PP9038 Tn5 ProDH 31	21	225	181	1475	38.3	-	86.7
PP9038 Tn5 ProDH 34	38	672	301	2267	61.9	-	88.0
PP9038+ PP9038 Tn5 ProDH 30	39	732	273	2132	60.7	76.3	6.0
PP9038+ PP9038 Tn5 ProDH 31	37	736	267	1979	56.6	75.0	6.4
PP9038 Tn5 ProDH 30+ PP9038 Tn5 ProDH 31	20	212	176	1419	28.6	-	87.5
Uninoculated	05	048	191	1007	13.3	-	-
SE (m)	1.09	22.33	12.40	36.14	1.01	-	-
CD at 5%	2.34	47.89	26.59	77.52	2.17	-	-

as coinoculants and no significant difference in N_2 fixing parameters was observed when ProDH mutant was inoculated alongwith their respective parent *Rhizobium* strain in comparision to parent *Rhizobium* strain when used alone as inoculant . Under unsterilized conditions using *Rhizobium* strain Mo6 and its ProDH$^-$ mutant in equal proportion, parent strain Mo6 showed nodule occupancy of 84 to 87 per cent, whereas ProDH mutant showed nodule occupancy of 5 to 6 per cent (Table 17). Nodule occupancy of parent *Rhizobium* strain PP9038 was 75-76 per cent, and its ProDH mutant showed nodule occupancy of only 6 per cent (Table 18). However, when both Tn5 mutants defective in ProDH activity alone were used as inoculants than the nodule occupancy was 83 to 89 and 85 to 88 per cent by Mo6 Tn5 mutants and PP9038 Tn5 mutants, respectively.

DISCUSSION

The symbiotic partnership between bacteria of the genus *Rhizobium*, *Bradyrhizobium* and legumes is well known. The importance of this relationship is much appreciated since long and currently also as sustainable agriculture relies greatly on renewable resources and on farm situation, nitrogen contributions are achieved largely through biological nitrogen fixation (BNF). In legumes the rhizobia induces nitrogen fixation nodules on the roots of leguminous plants. In this process dinitrogen which is chemically inert, is reduced to ammonia and is available to the plants. This process is an energy intensive enzymatic reaction requiring 16-25 ATP per molecule of dinitrogen fixed. To meet this need the plant supplies reduced carbon compounds to the bacteroids. Generally C_4 dicarboxylates are considered as major carbon source exported to bacteroids (Witty *et al.*, 1988). Kohl *et al.* (1988) reported that oxidation of amino-acids imported by the bacteroids from the cytosol of infected cells may supply additional energy needed to support nitrogen fixation in legume root nodules. Attention has been focussed mainly on possible role of proline, particularly in ureide transporting legumes like soybean. Proline is usually catabolized via P5C and glutamate

by means of ProDH. This conversion of proline into P5C depends on ProDH enzyme in *Rhizobium* strains. So if this system is more effective than one can expect supply of more energy to nodules and thereby fixing nitrogen more efficiently and even during nodule formation energy need may be fulfilled by this mechanism. Therefore, more activity of the key enzyme ProDH and availability of more energy are expected to influence the process of nitrogen fixation particularly effectiveness and competitiveness.

Thus considering the importance of the problem, role of ProDH activity in nodulation effectivity and competitiveness in mungbean (*Vigna radiata* L. Wilczek) host an ureide transporting legume (Norhayati *et al.*, 1988; Peoples *et al.*, 1988) was undertaken. In the present study screening of *Rhizobium* and *Bradyrhizobium* strains infecting mungbean showed that all the strains varied in ProDH activity under cultural conditions when supplemented with proline. Similarly ProDH activity in cell extracts of cowpea - *Rhizobium* NGR 234 has been reported when the cells were grown in presence of L-proline (Glenn *et al.*, 1991). But when cells were grown on sucrose or succinate supplemented medium no detectable activity of ProDH enzyme was present. Jimenez-Zurdo *et al.* (1995) had also reported the presence of ProDH activity in cell extracts of *Rhizobium meliloti* infecting alfalfa (*Medicago sativa*) grown in presence of proline in addition to other carbon sources.

Efficacy of rhizobial strains infecting mungbean was studied under unsterilized conditions. All the strains varied in their efficacy of fixing nitrogen as assessed through various N_2 fixing parameters. ProDH activity

in different rhizobial cell extracts was positively correlated to different N_2 fixing parameters like nodule fresh weight, shoot dry weight and total shoot nitrogen. Similarly bacteroidal ProDH activity was also positively correlated with nodule number and total shoot nitrogen at 1 per cent level of significance. Jimenez-Zurdo *et al.* (1995) also reported a positive role of ProDH activity in N_2 fixation of *R. meliloti* in symbiotic association with alfalfa (*Medicago sativa*). ProDH activity in bacteroid fraction was also varied when inoculated with different strains.

Kohl *et al.* (1990) reported that magnitude of ProDH activity in bacteroids of soybean nodules was sufficiently high during most of the time course to supply a significant fraction of energy requirement for N_2 fixation. It was suggested that ProDH activity should be higher in ureide exporting nodules of soybean (*Glycine max*), cowpea (*Vigna unguiculata*), garden bean (*Phaseolus vulgaris*), pigeonpea (*Cajanus cajans*) than in amide exporting nodules as peanut (*Arachis hypogaea*), alfalfa (*Medicago sativa*) and peas (*Pisum sativum*). Level of ProDH activity in bacteroid fractions of mungbean, an ureide exporting legume was comparable to other ureide transporting legume, but was higher than the amide transporting legumes.

Tn5 mutagenesis has been used by number of workers for isolation of carbohydrate mutants of *Rhizobium* (Duncin, 1981; Ranson *et al.*, 1981; Walton and Moseley, 1981; Thurn and Chatterjee, 1985) Efficacy of this technique has been compared with other chemical mutagens and this technique has been reported to yield same frequency and range of auxotrophs as chemical mutagens. Therefore, in the present study Tn5 mutagenesis was used to

generate proline auxotrophs of 4 rhizobial strains Mo2, Mo6, G14 and PP9038. Two strains Mo6and PP9038 yielded mutants showing no growth on proline plates, while other mutants showed comparable or even more growth than the parent strains. Further measurement of ProDH activity in cell extracts showed no activity in mutants showing no growth on proline plates while others showed activity comparable to the parent strains.

Similarly, Jimenez-Zurdo *et al.* (1995) also isolated mutants of *Rhizobium meliloti* infecting alfalfa unable to catabolise proline by Tn5 mutagenesis. ProDH activity in such mutants was not detectable. Mutants altered in degradation of asparagine of *Rhizobium etli*, a bacterium that establishes symbiosis with *Phaseolus vulgaris* has also been reported, using Tn5 mutagenesis (Zepeda *et al.*, 1997).

ProDH mutants of mungbean rhizobia were then used for determining the efficacy under sterilized as well as under unsterilized conditions to exactly pin point the role of ProDH in nitrogen fixation. A significant decrease in N_2 fixing parameters like nodulation, root dry weight, shoot dry weight, total shoot nitrogen in ProDH mutants with undetectable level of ProDH activity in cell extracts as well as in bacteroids was observed as compared to parent strains Mo6 and PP9038. However, under unsterilized conditions negligible ProDH activity in bacteroidal fraction could be due to formation of low number of nodules by the native rhizobial population. Indicating that native rhizobial population has very low ProDH activity. Further ProDH activity in bacteroid fraction of ProDH$^-$ mutants, was found to be absent as in case of cell extracts of ProDH$^-$ mutants. This could

be one of the possibility for lower efficacy of ProDH$^-$ mutants as these might be limiting the energy supply in bacteroids.

Jimenez-Zurda *et al.* (1995) also reported that ProDH$^-$ mutants strain nodulated 100 per cent of plants 1 day later and induced fewer nodules per plant than the parental strain. Results obtained under present studies are in equivalence with these results, thereby all the plants showed nodulation when inoculated with parent or mutant strain. Further the results are also in accordance that the plants nodulated by ProDH$^-$ mutants showed less number of nodules.

Chin *et al.* (1991) reported no significant difference in nodulation by parent and proline mutants in relation to proline catabolism and biosynthesis. In contradiction to above report Jimenez-Zurdo *et al.* (1995) reported that ProDH activity is required for nodulation efficiency of *Rhizobium meliloti* on alfalfa roots and less number of nodules are formed by ProDH$^-$ mutants in comparison to parent *Rhizobium* strains. It was further reported that ProDH$^-$ mutants exhibited no other growth defect and its nodulation ability is recovered to the parental level when the ProDH activity is supplied by genetic complementation. Thereby they concluded that symbiotic defects in ProDH$^-$ mutants were the direct result of the loss of ProDH enzyme activity. Our results also accord with the results obtained by these workers. But in the present studies genetic complementation was not performed. However, co-inoculation of ProDH$^-$ mutants with the parent strain resulted in 100 per cent nodulation, which also suggest either complementation or replacement in the nodules by another coinoculant strain.

Contrary to this Tn5 derived mutants of *R. leguminosarum* bv. *viciae* affected in proline catabolism and biosynthesis were found to be unaffected in their ability to form nodules with wild type levels of nitrogenase activity (Chien *et al.*, 1991). Possibly, ProDH was not playing much role in amide transporting legume like pea. Further, it was shown that ProDH activity is required for nodulation efficiency of *R. meliloti* on alfalfa roots (Jimenez-Zurda *et al.*,1995). Goldman *et al.* (1994) also reported that symbiotic plasmid genes essential to the catabolism of proline are also required for efficient nodulation of *Rhizobium meliloti* on alfalfa roots.

Nodule occupancy of antibiotically marked inoculant parent strain and ProDH deficient mutants on mungbean showed equal acceptance of both the inoculants when used alone. But when parent strain and ProDH deficient mutants were used as coinoculant, a significant decrease in nodule occupancy of ProDH deficient mutant was observed. Indicating that ProDH deficient mutants were competing poorly with the parent strain for nodulation and maximum number of nodules were occupied by the parent *Rhizobium* strain under sterilized as well as under unsterilized conditions. Nodule occupancy of ProDH deficient mutant and parent *Rhizobium* strain was comparable when used individually as inoculant strain under unsterilized conditions. Indicating that ProDH deficient mutants were able to compete native rhizobial population. Probably the native rhizobial population was low in the soil as indicated by formation of only 5 nodules under uninoculated conditions. Thereby native rhizobial population may be poor competitor as compared to ProDH deficient mutants. But when effective parent strain

was inoculated with the ProDH deficient mutant as coinoculant this resulted in a significant decrease in nodule occupancy by the ProDH deficient mutant in comparison to parent *Rhizobium* strain. When mungbean was inoculated with both ProDH deficient mutants, than both mutants complemented and formed majority of the nodules. Actually this could not be confirmed whether these nodules were formed by one or another ProDH deficient mutant or jointly, because there was no separate antibiotic marker to identify the ProDH deficient mutants in a coinoculated mixture.

Similar observations have been reported elsewhere under coinoculation mixtures, parent strain GRM8 (*Rhizobium meliloti*) appeared to be more competitive than LMI (ProDH$^-$ mutant) deduced from their respective percentages of nodule occupancy as only 6 per cent of the nodules analyzed were found to be occupied by ProDH$^-$ mutants (Jimenez-Zurdo *et al.*, 1995) indicating that ProDH activity is also required for competitiveness of *Rhizobium meliloti* on alfalfa roots and *Rhizobium* sp. on mungbean.

ProDH activity in cell extracts as well as bacteroid fraction of different *Rhizobium* and *Bradyrhizobium* strains or their Tn5 mutants infecting mungbean was found to be positively correlated with N_2 fixing parameters like nodule number, nodule fresh weight,root dry weight, shoot dry weight and total shoot nitrogen. Results of rhizobial strains infecting mungbean shows that if ProDH activity in cell extracts was absent as in case of ProDH deficient mutants then this was also reflected in bacteroid fraction. Thus resulting in lower efficacy of ProDH deficient mutants as compared to parent *Rhizobium* strains having ProDH activity. Indicating that ProDH activity was essential

for nodulation effectivity atleast in mungbean - an ureide transporting legume. Results also indicated a positive role of ProDH activity in nodulation competitiveness by a inoculant strain/mutant and if a strain is having low or no ProDH activity then this may be competed out by another strain having higher ProDH activity. Thus role of ProDH activity might be essential in nodulation effectivity and competitiveness of mungbean - *Rhizobium* symbiosis.

SUMMARY

1. Twenty seven rhizobial strains infecting mungbean were screened for ProDH activity in cell extracts and all the strains varied in activity.
2. ProDH activity in cell extracts of different rhizobial strains showed statistically significant (1% level) positive correlation with N_2 fixing parameters like nodule fresh weight, shoot dry weight and total shoot nitrogen.
3. ProDH activity in bacteroid fraction also showed positive correlation with N_2 fixing parameters like nodule number and total shoot nitrogen.
4. Tn5 mutagenesis of four *Rhizobium* strains Mo2, Mo6, PP9038 and G-14 resulted in proline auxotrophs of two strains which were deficient in ProDH activity.
5. Absence of ProDH activity in culture cell extracts of mutants was also found to be reflected in bacteroid fraction which were deficient in ProDH activity.
6. ProDH deficient mutants affected different N_2 fixing parameters such as nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen under sterilized as well as under

unsterilized conditions.

7. Proline utilizing Tn5 mutants having comparable or more ProDH activity than their respective parents recorded slight increase in N_2 fixing parameters and ProDH activity in bacteroid fraction but statistically it was not significant.

8. ProDH activity in cell extracts as well in bacteroid fraction of ProDH mutants was positively correlated with N_2 fixing parameters like nodule number, nodule fresh weight and total plant nitrogen. ProDH activity in cell extracts was also positively correlated with ProDH activity in bacteroid fraction at 1 per cent level of significance.

9. ProDH deficient mutants and parent rhizobial strains infecting mungbean showed comparable nodule occupancy when used individually as inoculant. However, ProDH deficient mutants were poor competitor when coinoculated in 1:1 ratio with parent strains.

10. ProDH deficient mutants complemented with each other and formed majority of the nodules.

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* Original not seen.

ROLE OF PROLINE DEHYDROGENASE IN NODULATION EFFECTIVITY AND COMPETITIVENESS OF MUNGBEAN - *RHIZOBIUM* SYMBIOSIS

by

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Proline has been reported to provide additional energy to support nitrogen fixation in legume root nodules particularly in case of ureide transporting legumes. Proline dehydrogenase (ProDH) is a key enzyme, which catabolises proline to yield energy. Therefore, in the present investigation, role of ProDH in effectiveness and competitiveness of rhizobia(infecting an ureide transporting legume, mungbean) which shows low competitiveness with the inoculant strains was undertaken. Twenty seven rhizobial strains infecting mungbean were screened for ProDH activity in cell extracts and all strains varied in activity. ProDH activity in cell extracts of different rhizobial strains showed positive correlation with nodule fresh weight, shoot dry weight and total shoot nitrogen. ProDH activity in bacteroid fraction showed positive correlation with nodule number and total shoot nitrogen. Tn5 mutagenesis of four *Rhizobium* strains Mo2, Mo6, PP9038 and G-14 resulted in proline auxotrophs of two strains which were deficient in ProDH activity. Absence of ProDH activity in culture cell extracts of mutants was also found to be reflected in bacteroid fraction which were deficient in ProDH activity. ProDH deficient mutants affected different N_2 fixing parameters such as nodule number, nodule fresh weight, root dry weight, shoot dry weight and total shoot nitrogen under sterilized as well as under unsterilized conditions. Proline utilizing Tn5 mutants having comparable or more ProDH activity than their respective parents recorded slight increase in N_2 fixing parameters and ProDH activity in bacteroid fraction but statistically it was not significant. ProDH activity in cell extracts as well in bacteroid fraction of ProDH mutants was positively correlated with N_2 fixing parameters like nodule number, nodule fresh weight and total plant nitrogen. ProDH activity in cell extracts was also positively correlated with ProDH activity in bacteroid fraction at 1 per cent level of significance. ProDH deficient mutants and parent rhizobial strains infecting mungbean showed comparable nodule occupancy when used individually as inoculant. However, ProDH deficient mutants were poor competitor when coinoculated in 1:1 ratio with parent strains. ProDH deficient mutants complemented with each other and formed majority of the nodules.