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Symbiotic characterization of mutants defective in proline dehydrogenase in *Rhizobium* sp. *Cajanus* under drought stress condition

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ABSTRACT

Legume- Rhizobium nitrogen fixation is dramatically affected under drought and other environmental constraints. However, many efforts are going to unveil whether such regulation of nitrogen fixation is exerted, occur at nodule efficiency level. Proline has been reported to provide additional energy to support nitrogen fixation in legume root nodules. Proline dehydrogenase (ProDH) is a key enzyme, which catabolizes proline to yield energy. Therefore, in the present investigation symbiotic characterization of mutants of Rhizobium sp (Cajanus) defective in proline dehydrogenase under drought stress condition was undertaken. Ten wild type strains of Rhizobium sp (Cajanus) were screened for their ProDH activity and antibiotic resistance pattern. We selected one effective strain (Rspc-4) for Tn5 random mutagenesis. Out of 1500 transconjugants, twelve clones were adjudged as ProDH$^-$ mutants. Resistance to most of the antibiotics was similar in both parent and its ProDH$^-$ mutants. The proline dehydrogenase activity in cell and nodule extracts of mutant ProDH$^-$ was completely lacking. All the ProDH$^-$ mutants were non-nodulating (Nod$^-$) on pigeonpea plants. The other symbiotic parameters of host plants inoculated with the mutants under normal and drought stress conditions were significantly lower than that of plants infected with the parent strain.

Keywords: Proline dehydrogenase (ProDH), Tn5 mutagenesis, Rhizobium, drought stress.

INTRODUCTION

Symbiotic nitrogen fixation by legume-rhizobia associations plays an important role in sustaining crop productivity and maintaining soil fertility. Chemical synthesis of alternative N-fertilizers requires fossil fuel,but Rhizobium uses solar energy trapped by plant photosynthesis.Increased awareness of the importance of Rhizobium-legume symbioses in many agricultural and marginal land environments has prompted scientists from diverse disciplines to consider enhancing the efficiency of symbiotic nitrogen fixation in legumes.

In an effective Rhizobium-legume symbiosis, the host plant partitions photosynthates to the bacteroids to support nitrogen fixation. Evidence indicates that the nitrogen fixing capacity of the Rhizobium-legume symbiosis is influenced by the amount of photosynthates available to the bacteroids in the root nodule [9,10,21].

It has also been reported that in Rhizobium-legume symbiosis, the C4-Dicarboxylic acids have generally been considered the major carbon source exported from plant cells to the bacteroids, which support the nitrogen fixation process [20,7,3]. But it is not the exclusive energy source for the bacteroids. Little is known about the exact carbon sources utilized by micro-symbionts during nodule formation and invasion. It has been suggested that oxidation of amino acids, particularly glutamate and proline, imported by bacteroids from cytosol of infected cells, may supply additional energy needed to support nitrogen fixation in legume root nodules [14]. However, the impermeability of the plant and peribacteroid membranes to glutamate suggests that glutamate may not be available to the bacteroids in significant quantities [22,4].

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 [14] and exogenously applied proline stimulates nitrogen fixation rate as much as exogenous glutamate does and increase ProDH activity[25]. Moreover, if soybean plants were subjected to drought, then this resulted in accumulation of proline and ProDH activity was also high in the bacteroids [13].Both the compartmentation of ProDH within soybean nodules and its potential ability to contribute to the energy requirements of bacteroids suggests a role for this enzyme in nitrogen fixation and any defect in ProDH activity leads to an alternation of rhizobial nodulation [15].

However, Jimenez-Zurdo [11] isolated ProDH$^-$ mutants of *Rhizobium meliloti* and these mutants resulted alteration in nodulation efficiency and competitiveness on alfalfa roots. Pigeonpea (*Cajanus cajan* is a uriede- transporting legume and important kharif grain legume of Indian sub-continent [2].

It has been suggested that proline accumulation plays a role in protecting nodules from drought and increased ProDH activity in the face of drought stress might supply energy to help the cell survive under adverse conditions [15].

In present research work, we were trying to find out the role of proline dehydrogenase in symbiotic effectivity and competitive ability in legume plant (*Cajanus cajan*), in addition to help plants to overcome the drought stress condition.

Our approach to achieve this goal involved the comparison of ProDH activity in the cell cultures of wild type strains of *Rhizobium* sp. (*Cajanus*) and bacteroids of pigeonpea nodules induced by them. And isolation of ProDH$^-$ mutants, by Tn5 mutageneis and determine their ProDH activity. Experiment also includes the characterization of these ProDH$^-$ mutants for their symbiotic effectivity and competitive ability under normal and drought stress conditions.

MATERIALS AND METHODS

Bacterial Strains and Plant variety

Six Rhizobium strains (Rspc-1 to Rspc-6) infecting pigeon pea and used in the present investigation were from the Microbial Genetics Laboratory, department of Genetics, CCS Haryana Agricultural University, Hisar and four strains (PP201, PH9023, PT-300, PG-3) were procured from Department of Microbiology, CCS HAU, Hisar.Seeds of pigeon pea (cv. Manak) were obtained from the Pulses Section, Department of Plant Breeding, CCS HAU, Hisar.

Proline Dehydrogenase activity

The ProDH activity of all the ten wild type strains and ProDH- mutants was determined in cultured cells (In Yeast extract mannitol agar medium (YEMA) [23] as well as in the nodule extracts[5]. Preparation of Rhizobium cell extracts for ProDH activity was done by previously described method [11] while fractionation of nodules for proline dehydrogenase activity in nodule extracts was performed by the procedure as described [25].

One enzyme activity is defined as the amount of enzyme catalyzing the synthesis of one micromole (µM) of Proline-5-carboxylate per minute under standard assay conditions. Specific activity of the enzyme is defined as unit of enzyme per milligram of protein. Proteins were estimated in cell cultures[17].

Screening for antibiotic resistance
All the wild type strains used in present study were screened for resistance to antibiotics by taking various concentrations of antibiotics like neomycin, chloramphenicol, nalidixic acid etc., in YEMA medium. Based on antibiotic resistance pattern, the strain Rspc-4 was selected for Tn5 mutagenesis and symbiotic as well as competitive studies.

Genetic techniques
Tn5 mutagenesis was carried out with the E.coli strain S17-1 (pSUP Tn5: B-20) as the donor strain. Rhizobium strain Rspc-4 was grown at 30oc in YEM broth supplemented with chloramphenicol (100µg/ml) for 24 hrs, whereas E.coli strain (S 17-1) was grown in Luria Bertani (LB) broth containing kanamycin (50µg/ml) at 30o c for 12 hrs on shaker. Bacterial conjugation was performed as described previously[16]. Derivatives of Rspc-4 which contained Tn5 transpositions and failed to grow on unsupplimented proline minimal medium were further screened with both the antibiotics (chloramphenicol and neomycin) [11].

Symbiotic efficiency
These ProDH$^-$ mutants and parent strain (Rspc-4) were then characterized for their symbiotic phenotype by inoculation on to pigeon pea plants under pot culture conditions. Seeds of pigeon pea were surface sterilized with 0.1% mercuric chloride for 1 min followed by 5-6 washings with sterilized distilled water. The surface sterilized seeds were then inoculated with 3-day-old cultures of Rspc-4 and its Tn5 mutants (in YEM broth) by immersing them in Rhizobium cultures for 30 min and sown in pots with sterile sand (autoclaved for 3 hours). The surface sterilized but uninoculated seeds were treated as control. Half the strength of sterilized Sloger's mineral salt solution and sterile water was added to the plants alternatively. After 60 days of sowing, these plants were examined for the presence or absence of nodules and for the symptoms of nitrogen starvation. Symbiotic parameters like nodule number, nodule fresh and dry weight, root and shoot dry weight, Symbiotic competitveness and nitrogenase activity and total shoot nitrogen were recorded.

Nitrogen estimation
The total shoot nitrogen of the plants was estimated by microKjeldhal's method [1,19].

RESULTS AND DISCUSSION

Resistant to antibiotics and sodium azide in different Rhizobium strains
All the wild type Rhizobium strains were tested for minimum inhibitory concentration (MIC) of different antibiotics and sodium azide (Table-1). Resistance pattern varied with each individual strain. The range of MIC values was 25 to 110 µg/ml for neomycin, 150 to 200 µg/ml for chloramphenicol, 65 to 1000 µg/ml for naladixic acid while 75 to 120 µg/ml for sodium azide. Based on this resistance pattern, one strain (Rspc-4) was selected for Tn5 mutagenesis because this parent strain had low resistance to neomycin (25 µg/ml) and high resistance to chloramphenicol (150 µg/ml) which acts as a positive selection marker.

Table 1-Minimum Inhibitory concentration (MIC) of different antibiotics and sodium azide (µg/ml) in wild type strains of *Rhizobium* sp. (*Cajanus*)

Strain	Neomycin	Chloramphenicol	Nalidix acid	Sodium azide
PP201	100	175	1000	125
PH9023	110	150	650	125
PT300	100	150	650	100
PG-3	100	175	700	125
Rspc-1	110	175	700	100
Rspc-2	75	175	700	75
Rspc-3	50	150	900	75
Rspc-4	25	150	650	100
Rspc-5	50	200	700	75
Rspc-6	75	200	700	75

Proline dehydrogenase activity

All parent strains were characterized for proline dehydrogenase activity (ProDH) in cultured cells and in nodules extracts. ProDH activity in all the parent strains varied in both cell and nodule extracts. Rspc-4 strain had the maximum ProDH activity in cell extracts, whereas PG-3 had the minimum. ProDH activity in nodule extracts was minimum in Rspc-6 and maximum in Rspc-1 (Table-2). Based on the high ProDH activity in cultured cells of Rspc-4 and its low resistance to neomycin, this wild type strain was chosen for the isolation of ProDH$^-$ mutants.

Table 2 -Proline dehydrogenase activity of wild type strains in cultured cells and nodule extracts

Wild type strains	ProDH activity	
	In cell extract[1]	In nodule extract[2]
PP 201	4.75	4.34
PH 9023	4.92	4.37
PT 300	4.43	4.30
PG –3	3.44	3.39
Rspc - 1	6.44	7.19
Rspc - 2	3.52	4.08
Rspc - 3	3.94	4.09
Rspc - 4	6.89	4.24
Rspc - 5	4.02	3.69
Rspc - 6	4.38	3.24
CD at 5%	0.15	0.32

[1] n moles of P5C produced min^{-1} mg^{-1} of protein.
[2] n moles of P5C produced min^{-1} g^{-1} nodule fresh weight

Isolation of ProDH$^-$ mutants

Rhizobial strain Rspc-4 was used as recipient strain and E.coli S-17 (pSUP: B-20) as donor strain, to isolate ProDH$^-$ mutants by Tn5: mob random mutagenesis by biparental conjugation. Out of 1500 transconjugants tested, twelve clones were unable to grow on minimal medium supplemented with proline as sole carbon and nitrogen source (Figure A & B) as compared to parent strain (Rspc-4) and these were adjudged as putative ProDH$^-$ mutants. These results are consistent with other studies[27],found that proline utilizing mutants of *Pseudomonas putida*, unable to grow on minimal media because of a deletion of putA and putP genes. The frequency of ProDH$^-$ mutants in this study was found to be 0.8 percent. A frequency of 0.1% of ProDH$^-$ mutants was observed in *Sinorhizobium meliloti* [11].

Table 3 -Minimum Inhibitory concentration (MIC) of different antibiotics and sodium azide (*µ*g/ml) in parent strain Rspc-4 and its ProDH$^-$ mutants

Strain/ Mutant	Nm	Cm	Sm	Tc	Em	Rif	Nx	Azi
Rspc-4	25	150	100	10	40	6	650	100
ProDH$^-$1	200	600	75	15	40	6	250	50
ProDH$^-$2	150	700	50	15	40	6	250	50
ProDH$^-$3	150	600	50	15	40	6	250	40
ProDH$^-$4	200	700	75	15	40	6	250	50
ProDH$^-$5	200	600	50	15	40	6	250	40
ProDH$^-$6	200	700	50	15	40	6	250	50
ProDH$^-$7	150	700	75	15	40	6	250	50
ProDH$^-$8	200	600	75	15	40	6	250	50
ProDH$^-$9	200	700	75	15	40	6	250	50
ProDH$^-$10	150	600	100	15	40	6	250	50
ProDH$^-$11	200	600	100	15	40	6	250	50
ProDH$^-$12	200	700	100	15	40	6	250	50

Nm = Neomycin; Cm = Chloramphenicol; Tc = Tetracycline; Em = Erythromycin; Rif = Rifampicine; Nx = Naladixic acid; Sm = Streptomycin; Azi = Sodium azide

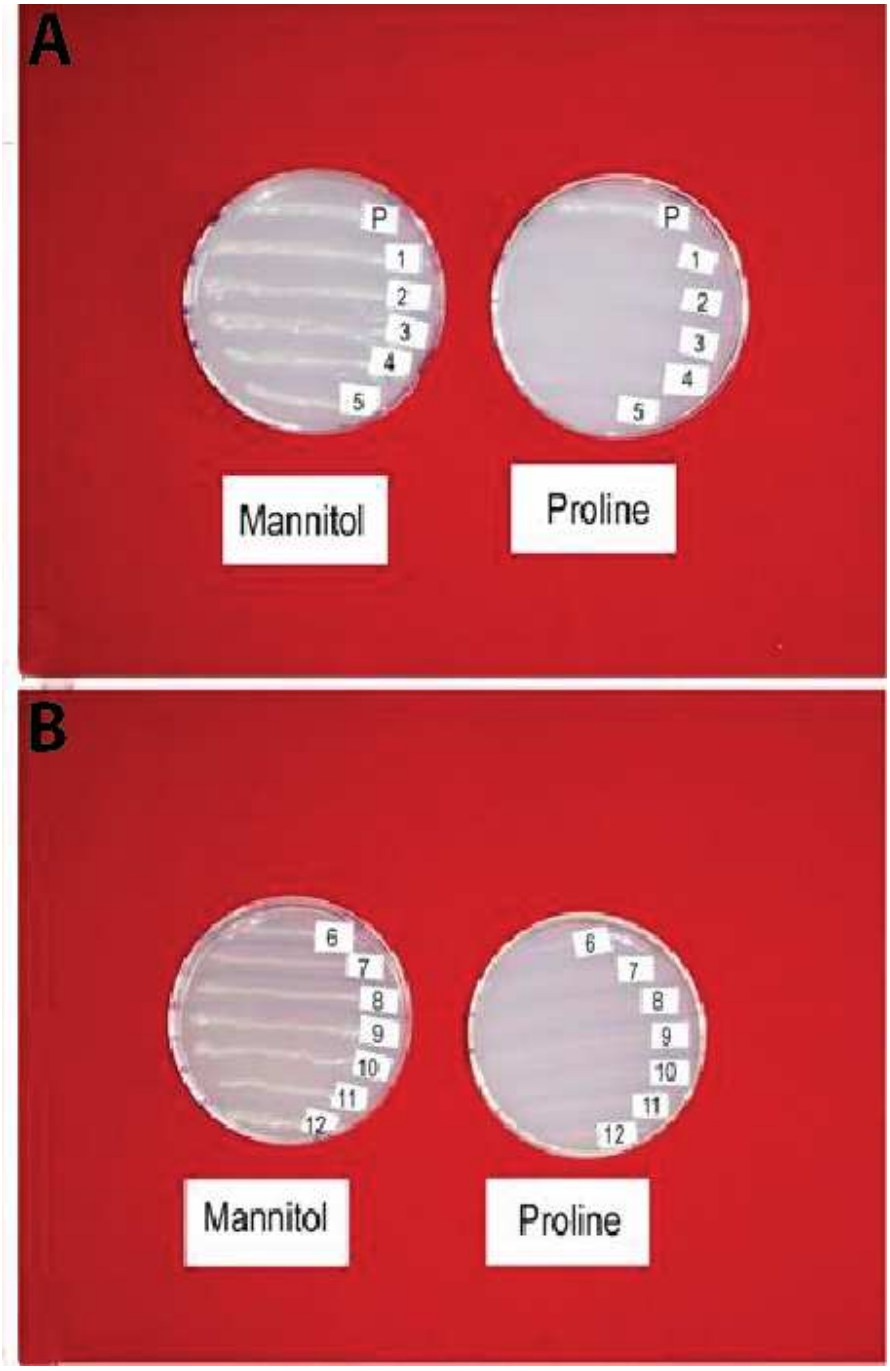


Figure A&B: Comparison of growth of parent strain and it's ProDH$^-$ (1 to 12) on MM medium and on MM+ Proline.
i. Parent strain (Rspc-4) shows growth on both the media
ii. ProDH$^-$ mutants (1 to 12) show no growth on MM+ Proline
ProDH$^-$ mutants (1 to 12) show no growth on MM+ Proline

Characterization of ProDH- mutants
Antibiotic resistance

All the ProDH$^-$ mutants (ProDH$^-$1 to ProDH$^-$12) along with their parent strain were characterized for their minimum inhibitory concentration (MIC) of different antibiotics and sodium azide. The mutants showed higher level of

neomycin resistance (150-200 µg/ml) as compared to parent strain Rspc-4, which had 25 µg/ml of resistance (Table-3). This higher resistance to neomycin in transconjugants may be explained by the insertion of Tn5 element in the Rhizobium chromosome. Expression of Km/Nm marker could have increased the level of resistance to this antibiotic (additive gene effect) in these transconjugants. The MIC of chloramphenicol in ProDH$^-$ mutants was also higher (600-700 µg/ml) than parent strain (150 µg/ml). As far as MIC values of naladixic acid and sodium azide are concerned, these were lower in mutants than that of parent strain. The higher MIC of chlorampenicol and lower MIC of naladixic acid and sodium azide may be due to insertion effect.

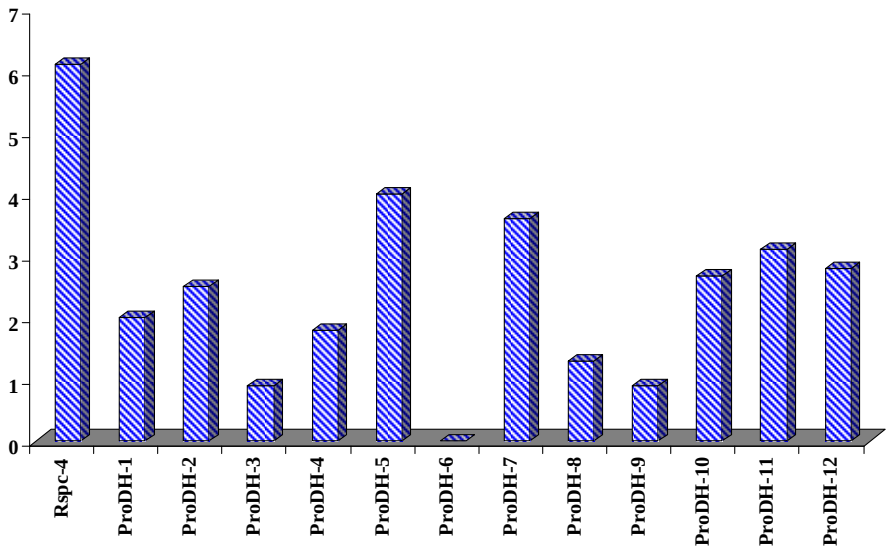
Proline dehydrogenase activity

The data on ProDH activity in cell extracts of parent strain and its mutants indicate that the activity in mutants was significantly lower than that of the parent strain (Rspc-4).There was complete abolition of ProDH activity in mutant strain ProDH$^-$6 (Table-4, Graph-1).The highest ProDH activity was observed in ProDH $^-$5 and lowest in both ProDH$^-$3 and ProDH$^-$9 mutant strains. Complete abolition of ProDH activity in mutants is consistent with other published data [11], where there was no ProDH activity in LM1 mutants of *Sinorhizobium meliloti* by Tn5 insertion.

The lower value of ProDH activity in other mutants might be due to the insertion in put P gene (for proline transport system) than in the put A gene. Similar proline non-utilizing mutants were found in E.coli and were suggested to be mutants on put p gene[24]. Tn5 insertion is sometimes unlikely to be very specific and there could be leakiness of the lesions.Similarly another study reported [6] isolation of several different carbohydrate mutants in *R.meliloti* (now *S.meliloti*) by Tn5 mutagenesis.

Table-4 Proline dehydrogenase activity in parent strain and its ProDH$^-$ mutants in cell extract

Strain/Mutant	ProDH activity (n moles of P5C produced min-1 mg-1 of protein)
Rspc-4	6.1
ProDH$^-$1	2
ProDH$^-$2	2.5
ProDH$^-$3	0.9
ProDH$^-$4	1.8
ProDH$^-$5	4
ProDH$^-$6	0
ProDH$^-$7	3.6
ProDH$^-$8	1.3
ProDH$^-$9	0.9
ProDH$^-$10	2.67
ProDH$^-$11	3.1
ProDH$^-$12	2.8



Graph-1 : Comparision of proline dehydrogenase activity between parent starian and mutants.

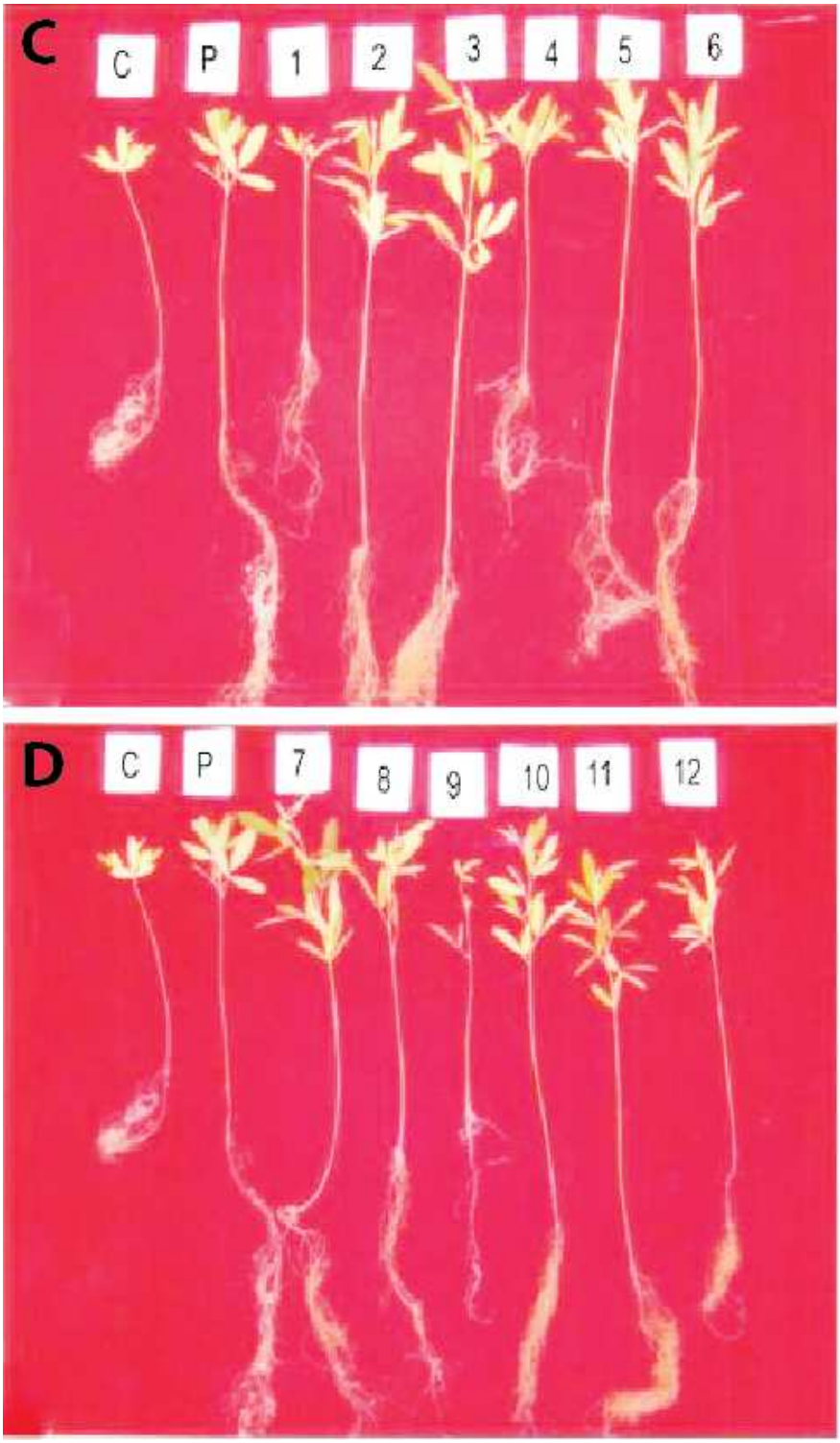


Figure A&B: Nodule status of plants inoculated with parent strain (Rspc-4) and its ProDH⁻ mutants (1 to 12) and uninoculated plant.

i. Nodule on the plant roots infected with Rspc-4 (P)
ii. Uninoculated control plant roots (C) without nodule
iii. Plant roots infected with ProDH⁻ mutants (1 to 12) without nodules

Symbiotic effectivity of ProDH$^-$ mutants

Symbiotic effectivity of parent strain and its mutants was tested in pots under normal and drought stress conditions in the net house. It was observed that all the ProDH$^-$ mutants did not include nodules (Nod$^-$) on the roots of pigeon pea plants, whereas the parent strain was highly nodulation (Nod$^+$) (Fig C&D).The non-nodulating nature of the ProDH$^-$ mutants indicates that proline and ProDH activity might be vital energy source in the infection process of this *Rhizobium* sp. (*Cajanus*) pigeon pea symbiosis. Almost similar results were found [16]on transposon Tn5-induced arginine auxotroph of *Sinorhizobium meliloti* that AK10 mutant, apart from having a Tn5 insertion in gene, appears to have another mutation in one of its symbiotic genes. Some auxotrophic mutations exert a pleiotropic effect and that the ability to nodulate is a secondary consequence of the initial lesion[18]. Thus, Nod$^-$ phenotype of ProDH$^-$ mutants might be due to pleiotropic effect of Tn5 insertion. Deficiency in the utilization of proline, affects rhizosphere colonization.

AAnother study [8] also observed that symbiotic plasmid genes essential to the catabolism of proline in *Rhizobium meliloti* are also required for efficient nodulation. In this way, from the results of this study, it can be surmised that proline dehydrogenase activity has some specific effect on nodulation. *Rhizobium etli* Tn5 insertion mutant LM01 unable to utilize glutamine was also affected in Nod factor production and nitrogen fixing efficiency[26].

Root fresh weight, root dry weight, Shoot fresh weight, shoot dry weight, percent shoot nitrogen and total shoot nitrogen of the plants infected with ProDH$^-$ mutants were significantly lower for all mutants than the plants inoculated with parent strain (Table 5 & 6)

Table 5-Symbiotic effectivity of wild type strain and its ProDH$^-$ mutants under normal conditions

Strain/Mutant	Nodule No.	Nodule fresh wt. (g/pl)	Root fresh wt. (g/pl)	Root dry wt. (g/pl)	Shoot fresh wt. (g/pl)	Shoot dry wt. (g/pl)	% shoot N	Total shoot N (g/pl)
Control	-	-	0.41	0.21	0.41	0.18	0.03	0.01
Rspc-4(P)	19	0.37	2.79	1.16	3.81	1.21	0.39	0.54
ProDH$^-$1	-	-	1.22	0.75	1.52	0.75	0.06	0.05
ProDH$^-$2	-	-	1.11	0.71	1.77	0.71	0.07	0.05
ProDH$^-$3	-	-	1.13	0.79	1.39	0.79	0.05	0.04
ProDH$^-$4	-	-	1.06	0.80	1.39	0.80	0.04	0.03
ProDH$^-$5	-	-	1.08	0.72	1.41	0.72	0.06	0.04
ProDH$^-$6	-	-	1.20	0.81	1.36	0.81	0.06	0.05
ProDH$^-$7	-	-	1.12	0.71`	1.39	0.71	0.04	0.02
ProDH$^-$8	-	-	1.09	0.61	1.40	0.61	0.06	0.04
ProDH$^-$9	-	-	1.07	0.71	1.33	0.71	0.05	0.03
ProDH$^-$10	-	-	1.09	0.60	1.37	0.60	0.05	0.03
ProDH$^-$11	-	-	1.04	0.63	1.33	0.63	0.05	0.02
ProDH$^-$12	-	-	1.25	0.64	1.32	0.64	0.05	0.03
CD at 5%	2.50	0.08	0.05	0.06	0.09	0.05	0.01	0.01

Table 6-Symbiotic effectivity of wild type strain Rspc-4 and its ProDH$^-$ mutants under drought stress conditions

Strain/ Mutant	Nodule No.	Nodule fresh wt.	Root fresh wt. (g/pl)	Root dry wt. (g/pl)	Shoot fresh wt. (g/pl)	Shoot dry wt. (g/pl)	% shoot N	Total shoot N (g/pl)
Control	0.00	0.00	0.45	0.25	0.40	0.19	0.04	0.01
Rspc-4(P)	15.00	0.46	2.65	1.11	3.15	1.22	0.38	0.46
ProDH$^-$1	-	-	1.06	0.63	1.54	0.76	0.07	0.06
ProDH$^-$2	-	-	1.13	0.46	1.20	0.71	0.07	0.05
ProDH$^-$3	-	-	1.14	0.49	1.47	0.80	0.06	0.04
ProDH$^-$4	-	-	1.12	0.47	1.53	0.80	0.05	0.04
ProDH$^-$5	-	-	1.11	0.41	1.69	0.73	0.06	0.04
ProDH$^-$6	-	-	1.14	0.45	1.48	0.82	0.07	0.06
ProDH$^-$7	-	-	1.13	0.38	1.54	0.72	0.05	0.04
ProDH$^-$8	-	-	1.17	0.43	1.41	0.61	0.06	0.04
ProDH$^-$9	-	-	1.00	0.29	1.36	0.72	0.06	0.04
ProDH$^-$10	-	-	1.13	0.35	1.42	0.61	0.06	0.03
ProDH$^-$11	-	-	1.08	0.29	1.31	0.63	0.05	0.04
ProDH$^-$12	-	-	1.14	0.43	1.35	0.65	0.06	0.04
CD at 5%	5.10	0.10	0.30	0.06	0.42	0.05	0.01	0.02

Symbiotic competitiveness of parent strain and its ProDH⁻ mutants

The observations on symbiotic competitiveness of parent strain and its ProDH⁻ mutants in co-inoculation experiments indicate that the parent strain (Rspc-4) occupied almost all the nodules (nearly 100%) formed on the roots of pigeon pea. The range being 79 to 100 percent (Table 7).The nodule occupancy of mutants was in the range of 0 to 6 percent. Nodule occupancy is positively correlated with all the symbiotic characters at 1 and 5 percent level of significance (Table 8).Under drought stress conditions, nodule occupancy by ProDH⁻ mutants was zero(Table 9).Symbiotic characters are positively and significantly correlated with nodule occupancy under drought stress conditions also. Drought tension affects the biological performance of the plant so that water deficiency reduces the biological weight of the plant[28].

These results show that ProDH is involved in the competition of micro-symbiont for nodule occupancy. Similarly another study [12] found that an impaired praline metabolism in Rhizobium meliloti leads to reduced nodule efficiency and competitiveness on alfalfa roots.

Table 7 Competition between parent strain and its ProDH⁻ mutants as co-inoculants under normal conditions

Strain/Mutants	Nodule occupancy (%)	
	Parent strain	Mutant
Control	00	00
Rspc-4(P)	100	00
P+ProDH-1	98	2
P+ProDH-2	94	5
P+ProDH-3	98	2
P+ProDH-4	92	3
P+ProDH-5	90	6
P+ProDH-6	94	1
P+ProDH-7	92	3
P+ProDH-8	96	2
P+ProDH-9	94	3
P+ProDH-10	96	2
P+ProDH-11	100	0
P+ProDH-12	79	2
CD at 5%	12.49	12.89

Table 8-Correlation of nodule occupancy of Rspc-4 (parent strain) and its ProDH⁻ mutants with different N_2 fixing parameters under normal conditions

	Nodule No.	Nodule fresh wt.	Root fresh wt.	Root dry wt.	Shoot fresh wt.	Shoot dry wt.	% Shoot N	Total shoot N	Nodule occupancy	
									Parent	Mutant
Nodule No.	1.00	1.00 **	0.922**	1.000**	0.849**	0.886**	0.689**	0.995**	0.995**	0.857**
Nodule fresh wt.			0.922**	1.000**	0.849**	0.886**	0.689**	0.995**	0.995**	0.857**
Root fresh wt.				0.922**	0.971**	0.910**	0.884**	0.943**	0.948**	0.982**
Root dry wt.					0.849**	0.886**	0.689**	0.995**	0.995**	0.857**
Shoot fresh wt.						0.888**	0.932**	0.874**	0.885**	0.982**
Shoot dry wt.							0.821**	0.911**	0.917**	0.910**
% shoot N								0.732**	0.751**	0.933**
Total shoot N									0.999**	0.890**
Nodule Occupancy										
Parent										0.897**
Mutant										1.00.

**Significant at 5 per cent level.*
***Significant at 1 per cent level*

Table 9 Competition between parent strain and its ProDH$^-$ mutants as co-inoculants under drought stress conditions

Strain/Mutants	Nodule occupancy (%)	
	Parent strain	Mutant
Control	-	-
Rspc-4(P)	100	-
P+ProDH-1	100	-
P+ProDH-2	99	-
P+ProDH-3	100	-
P+ProDH-4	98	-
P+ProDH-5	100	-
P+ProDH-6	97	-
P+ProDH-7	100	-
P+ProDH-8	100	-
P+ProDH-9	100	-
P+ProDH-10	96	-
P+ProDH-11	100	-
P+ProDH-12	99	-
CD at 5%	2.28	-

CONCLUSION

From the present study,it can be surmised that proline dehydrogenase activity has some specific effect on nodulation.The non-nodulating nature of proline dehydrogenase negative mutants indicates that proline and proline dehydrogenase enzyme activity might be vital as extra energy source in the infection process of this *Rhizobium* sp.(*Cajanus*)-pigeonpea symbiosis. Results also showed that proline dehydrogenase is involved in the competition of micro-symbiont for nodule occupancy.

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