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RESEARCH ARTICLE

BIOCHEMISTRY

PROTEIN PROFILES OF *IN VITRO* AND *IN VIVO* HALOTOLERANT RHIZOBIUM SPECIES ISOLATED FROM CAJANUS CAJAN PLANT.

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ABSTRACT

Salinity-induced changes in the protein profiles in *Rhizobium* sp. exhibited alterations in proteins, which either showed an enhanced rate of synthesis or a decline in the levels as compared with controls. The *in vitro* and *in vivo Rhizobium* of *Cajanus Cajan* plant produced high amount of protein from halophillic glyphosate (Hg) as compared to the Normal (N) plant. The proteomic diversity contributes the variation between the same species; its *in vitro* and *in vivo* protein profile contributes the isolated salt and herbicide tolerant *Rhizobium* sp. produced proteins to adapt in such stress conditions. The possible relationship between the rhizobial protein production & legume rhizobia symbioses related to stress is discussed.

KEY WORDS

Cajanus Cajan, Proteins, Rhizobium, SDS–PAGE*, Nodules* and *stress.*

INTRODUCTION

Salinity is one of the major environmental factors deleterious to plant growth and yield[1]. Increasing salt concentration may have detrimental effect on rhizobial population [2]. They have cellular adaptation strategies to environmental stress that protects organisms from various biotic and abiotic stresses like salinity and herbicidal effect on micro flora [3]. The adaptive responses include osmolytes, chemotaxis, mobility, competent transport of sugars and organic acids, production of secondary metabolites, nitrogen fixation and stress tolerance. Environmental stress induced modifications of protein synthesis have been observed in many micro organisms [4].The protein pattern of cultured tobacco (Nicotiana tabaccum L.var Wisconsin 38)[5] showed more abundance of two protein bands (32,000, and 20,000 Daltons) within salt adapted cells and one protein (26,000 Daltons) was unique to the salt cells, while salinity and water stresses induce a KDa protein called osmotin in Tobacco plant; while in some microorganisms osmotic stress proteins were also reported in Escherichia coli [4 & 6] and anabaena.

The productivity of *Cajanus cajan* (pigeon pea) a leguminous plant had declined due to salinity and also a wide use of herbicide which enhances soil salinity. This affects soil micro flora and ultimately the crop. Due to the increase in salinity, the plants and micro organisms develop strategies to cope with the environment [7]. Thus the response occurred due to stress in plant and bacteria results in formation of transcriptional action of many defense proteins leading to salt tolerance and yield stability 8. The plants defense against this salinity attack requires osmotic adjustment, and to a certain degree, this can be done through synthesis of intracellular solutes [9]. Also high salt concentration inhibits enzymes by impeding the balance of forces controlling the protein structure [9]. Hence implementation of biotechnology strategies are focused on to identify salt tolerance effectors and the regulatory components that control these during the stress episode.[8]

There are numerous options for genetic modification of plants to make them more tolerant to stress, and the strategies are developed through the molecular marker technique and biotechnology used for crop improvement. Further knowledge obtained about these stress tolerance will be additional resource of information of the plant in response to salinity, which will reveal how plants sense salt stress, transducer signals to mediate a defensive response and define the signal pathway outputs or effectors required for stress survival and alleviation. Molecular genetic and plant transformation advances have made it feasible to assess biotechnological strategies based on activated signal cascades, engineered biosynthetic pathways, targeted gene or protein expression or alteration of the stress responsiveness of genes for development of salt tolerant crops [10] although the study was to see the protein profile *in vitro* and *in vivo* of the halophytic *Rhizobuim* species grown in different stress conditions and various phenotypic and genotypic methodologies being used to identify and characterize bacteria., This paper reports the variation in the protein profiles, characterization on the basis of morphological, biochemical and molecular characters of the i*n vitro* and *in vivo* Rhizobuim species grown in different condition of salinity and herbicide (glyphosate) and the research should be

capable of improving the tolerance of non halophytic plants. Information on salt responsive protein/genes is crucial for improving salt tolerance through genetic engineering techniques.

MATERIAL AND METHODS

***Collection of samples**:*

The *Rhizobium* was isolated aseptically from the root nodules of *Cajanus cajan* (L.) Millsp. Plant of AKPH 2022 variety. The strains were identified and isolated according to Shende & Patil, 2005.[11]

Rhizobuim inoculums:

Fresh nodules were collected from Cajanus cajan plants. N plant was grown in normal condition, N_G was grown in presence of 4% glyphosate, H was grown in 300mM NaCl, H_G plant was grown in 300mM NaCl and 6% glyphosate.

Four different types of nodules were collected, crushed and inoculated in YM medium aseptically.

***Protein profiles in vitro and in vivo Rhizobuim strains**:*

a. ***Isolation of protein from Rhizobuim stains:*** Nodules were isolated from plants and were washed twice with normal phosphate buffer saline (pH 7.2). Nodules were homozanised in protein extraction buffer at 60^0C for 1 hour and centrifuged to collect extracted protein.
b. ***Protein estimation:*** Total protein contents in nodule of plants and Rhizobuim strain were tested by Lawry et al method[12]
c. Protein molecular weight markers used were 102KD, 78.8KD, 66KD, 22KD, 18KD, 6KD.
d. ***Preparation of protein samples for electrophoresis:*** *In vitro* and *in vivo* nodules and Rhizobuim strains of N, N_G, H and H_G were centrifuged at 10000 rpm for 10 min and the clear supernatant was taken for electrophoresis.

Polyacrylamide gel electrophoresis:

Polyacrylamide gel electrophoresis (PAGE) of the purified protein was carried out in presence of SDS according to the method of Laemmli, (1970).[13] The electrophoresis was carried out using 10.0% polyacrylamide gel in Tris glycine buffer pH 8.3 with a current of 15 mA for 3 hours. The gel was stained by commassie blue R-250 for 45 minutes and then destained till the colour was removed.

Molecular weight determination:

The molecular weight of the protein was measured by SDS-PAGE by the method of Laemmli, (1970)[13] using molecular weight standards. The molecular weight of salt stress proteins were compared with that of standard molecular weight markers used.

Then the graph was plotted of molecular weight marker against distance migrated by the markers on a semi log paper. The distance migrated by salt stress protein (SSP) was plotted on the above graph to find the molecular weight of SSP.

Also the proteomic diversity was clarified by Dendrogram.

Statistical analysis of DATA:

In case of SDS-PAGE the presence of band was scored as 1 and absence as 0. The bands with same mobility were treated as identical bands. The SDS-PAGE data were analyzed by software package MVSP (Multivariate Statistical Package) version 3.1. The similarity matrix was measured by Gower General similarity coefficient. The similarity matrix values were converted into Dendrogram using UPGMA (unweight pair group method with the arithmetic average) clustering method.

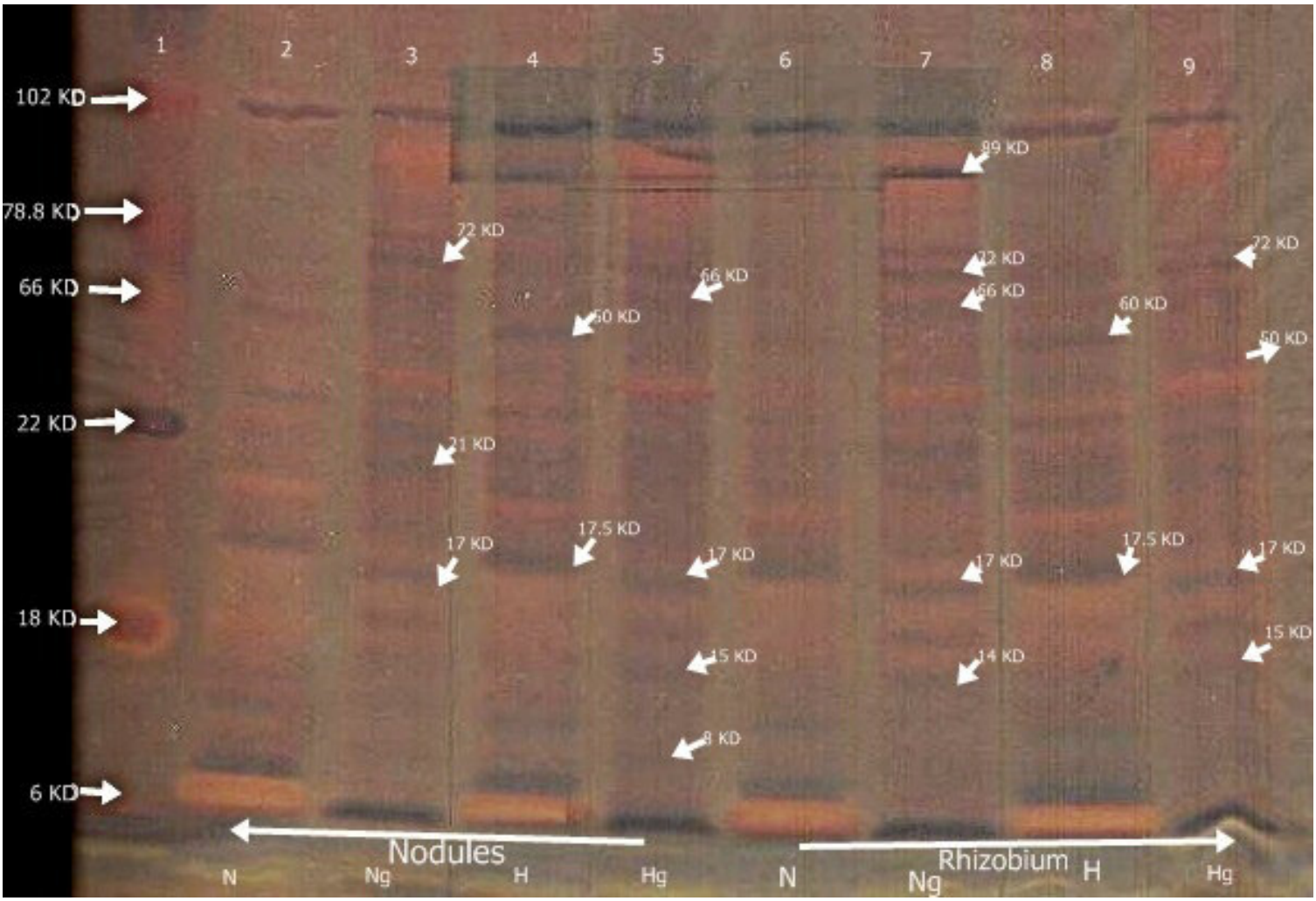

Figure 1

Stained SDS-PAGE of total soluble protein of Rhizobium strain isolated from nodules (in vivo) and rhizobium strain grown in YEM medium and incubated at 28 °C under control conditions .(lane 1) molecular mass standards (Kit MW-GF-1000, Sigma). (Lane 2) N proteins isolated from nodules of normal plants. (Lane 3) Ng proteins isolated from nodules of plants grown in presence of glyphosate 4% ai. (Lane 4) H proteins isolated from nodules grown in 300 mM NaCl. (Lane 5) Hg proteins isolated from nodules grown in presence of 6% ai glyphosate and 300 mM NaCl.

Stained SDS-PAGE of total soluble proteins of Rhizobium strains (lane 6) N, (lane 7) Ng, (lane 8) H & (lane 9) Hg grown in YEM medium and incubated at 28 °C under different halophillic and glyphosate conditions.

Graph 1

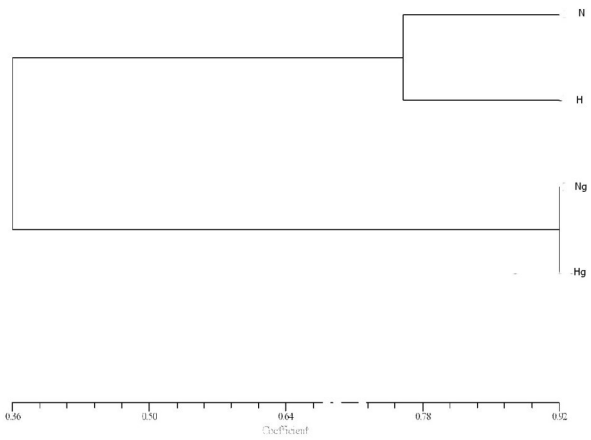

Fig.1. Dendrogram showing total soluble protein profiles of N, Ng, H & Hg and the result of a numerical comparison of the protein profiles of the Rhizobium isolated from the nodules of Cajanus cajan plant.

Graph 2

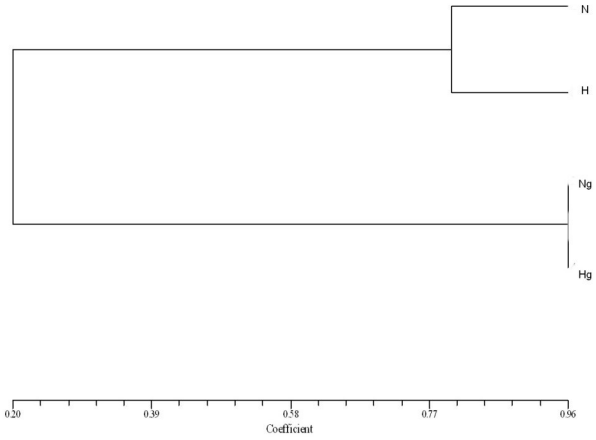

Fig.2. Dendrogram showing total soluble protein profiles of N, Ng, H & Hg and the result of a numerical comparison of the protein profiles of the Rhizobium strains of Cajanus cajan plant grown in YEM medium incubated at 28 °C under different halophillic and glyphosate conditions.

RESULTS AND DISCUSSIONS

The symbiont *Rhizobium* N, Ng, H & Hg strains were isolated from the healthy root nodules of *Cajanus cajan* plant. The identification of the bacteria was done according to Manual of Microbiological methods following Bergey's Manual [14]. The plants of different strains (N) were grown in normal conditions, (NG) in normal conditions containing 4% glyphosate, H in halophillic conditions with 300mM NaCl and HG with 300mM NaCl 6% containing glyphosate. The *Cajanus cajan* plants were grown in above aseptical conditions and the nodulation was rechecked.

The protein profiles of *in vitro* and *in vivo* Rhizobium showed modifications in the strains as compared with control and also the normal (N) strain which acts as its own respective control for other three strains obtained from saline and herbicidal condition. Also protein profile of N_G was compared with its other strain N and H_G was compared with N_G & H. The protein

concentration was highest in H and Hg Rhizobium strains, as it concludes that salinity enhances synthesis of protein. Rhizobium uses various mechanisms for osmotic adaptations under stress. These include accumulation of low molecular weight organic solutes: sugars, aminoacids, polyamines and accumulation of ions.[15 & 16].

Nodular Rhizobium Hg, strain showed more proteins of molecular masses 66 KD, 17 KD, 15 KD & 8 KD while H strain also had 60 KD & 17.5 KD. The isolated Hg strain was taken from the plant grown in presence of NaCl and glyphosate. It might utilize the mechanism of osmotic adaptation in presence of high levels of salt which results in the formation of specific protein in bacteria. Zahran et al [17] reported the appearance of new protein bands in (SDS-PAGE) profiles of rhizobia from woody legumes grown under salt stress. New proteins 72 KD, 66 KD, 21 KD, 15 KD & 8 KD also appeared in comparison between NG and Hg. these induced proteins might be due to herbicidal stress.

The variations in protein profile of Rhizobium strain could also be noticed in (fig 1) having 72 KD, 60 KD, 17 KD & 15 KD in Hg as compared to H, and Ng 89 KD, 72 KD, 66 KD, 17 KD & 14 KD as compared to its normal N strain which acts as control for other strain. In vitro and in vivo studies conclude that stress enhances proteins which function to maintain the intracellular pH and repair the ionic imbalance caused by osmotic stress. Although at stress, the plant & micro organism categories the cellular mechanisms of plant defense against the salinity attack, it requires osmotic adjustment and to a certain degree, this can be done through synthesis of intra cellular solutes and altering their energy metabolism [4 & 9]. Botsford [4] reported that the production of 41 proteins was increased at least 10-fold in salt stressed cells of E.coli. Zahran et al.[9] extended their work on halotolerant strain of cowpea Rhizobium and examined its cell morphology and ultrastructure under salt stress (1.64M NaCl). His finding concluded that the rhizobial cells responded to high salt-stress by changing their morphology, appearance of the cell as spiral or filament like structure, and the cell size greatly expanded, which severely affected the cell ultrastructure. The cell envelope was distorted and the homogeneous cytoplasm was disrupted. Even the rhizobia with altered morphology have been isolated from salt affected soils of Egypt.[18]

The Dendogram of protein diversifies the variation between the isolated Rhizobium strains from plants and rhizobia. In plants proteomic strain in Graph 1 conclude that strain N and Ng are close to each other as compared to H & Hg and also the normal strains form cluster which is 0.36 distance away from the H strains. Graph 2 of Rhizobium sp. N and Ng strains are very similar to each other forming a cluster of 0.8 coefficient and H and Hg strain which show 0.96 coefficient similarity form cluster which is 0.36 distance away from the N & Ng strains. However, successful Rhizobium-legume symbiosis under salt stress require the selection of salt tolerant rhizobia from those indigenous to saline soils. Thus this research strongly recommend the selection of salt tolerant and effective strains of rhizobia. In fact, and as indicated in certain reports, some strains of salt tolerant rhizobia are able to establish effective symbioses, while others formed ineffective symbioses. Inoculation of legumes by salt–tolerant strains of R. legminosarum bv. Trifolii and R. meliloti enhanced nodulation and N content under salt stress upto 1% NaCl [19]. Also under saline conditions, the salt tolerant strains of Rhizobium sp. formed more effective N2-fixing symbioses with soyabean than did the salt sensitive strains[20.] Evidence presented in the paper suggests a need to select plant genotypes that are tolerant to salt stress, thus studies on in vitro and in vivo protein profile contributes the isolated salt and herbicide tolerant Rhizobium sp.

It is inferred from the present finding that modification of salt stress protein with increasing

salt and herbicide concentration favours its role in maintenance of vital cellular functions and adaptation to stress tolerance. Thus the use of Novel approaches combining genetic, physiological and molecular techniques should provide excellent results in the near future.

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