EFFECT OF OSMOLYTES ON THE GROWTH OF RHIZOBIA NODULATING *Cajanus cajan* (PIGEON PEA)

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ABSTRACT

Glycines, Proline, Sucrose and Trehalose have been found to confer a big level of osmotic tolerance. In stress conditions, these osmoprotectants accumulate in the *Rhizobium* to overcome the osmotic shocks and proliferate in the given habitat. The accumulation of these osmolytes raises the internal osmotic pressure, which protects against stress. In this paper these osmoprotectants were determined from the nodules of *Cajanus cajan* plant grown in presence of NaCl and Glyphosate. It was found that, these osmolytes were accumulated in more quantity with increase in stress. Effect of these osmolytes on the growth of *Rhizobium* was also studied.

Key words: Osmolytes, Rhizobium and *Cajanus cajan*.

INTRODUCTION

Biological nitrogen fixation is markedly affected by many stresses originated from human activities and natural factors such as salinity, air pollution, drought, temperature, light intensities, biocides and nutritional limitations. Among these stresses salinity is a serious threat to agriculture. In India saline and alkaline soil covers more than 9 million hector and is rapidly expanding[1]. To overcome such stresses plants develop and evolve strategies to adapt and cope up with the osmotic stress. The stress resistance implies all morphological and physiological measures required to inhibit or ameliorate such stresses[2].

The osmotic strength of the environment is one of the physical parameter that determines the ability of an organism to proliferate in a given habitat. Plants and bacteria have evolved such sophisticated mechanism for balancing their osmotic strength with that of the surrounding. Exposure of cells with high osmotic pressure results in the rapid efflux of intracellular water, which leads to a decrease in cell volume and a decrease in turgor pressure. The accumulation of osmolytes or compatible solutes raises the internal osmotic pressure, which protects against osmotic stress and salt inactivation. Such compounds are termed as osmoprotectants[3].

Several environmental conditions are limiting factors to the growth and activity of nitrogen fixing plants. The *Rhizobium*-legume symbioses are more sensitive to salt and osmotic stress[4]. Under osmotic stress bacteria commonly accumulate potassium ions and organic solutes in their cytoplasm to build up internal osmotic strength and prevent diffusion of water out of cell. Landford (1986) pointed out that the organic solutes used by bacteria comprise only a type of compounds namely amino acids, sugars, polyols and betaine[5,6].

Rhizobium utilizes the mechanism of osmotic adaptation. Several Scientists have examined effect of salt stress on nodulation and nitrogen fixation by *Rhizobium*. Among the most prominent compatible solutes accumulated in plants and bacteria in response to stress are Proline, Glycine, Ecotine, Betaine, Glutamate, Sucrose, Trehalose, Choline etc.

These osmotically active solutes cause an increase in the osmolarity of the cytoplasm, which is accompanied by influx of water into the cells of plant and bacteria, thus providing the necessary turgor pressure for expansion of cell wall. The turgor pressure has been proposed to regulate the rate of cell elongation. In absence of active adjustments by the cell, the cytoplasmic volume would shrink until the water content of the interior equates to that of the exterior. Elevation in the content of various intracellular molecules may be inhibitory to cellular stresses, alterations in the volume would not be adequate to adapt in such osmotic stress[7]. Thus to respond to such stresses plant and bacteria increase the concentration of limited number of solutes. Bernard (1986) observed the presence of high levels of salts, intracellular glutamate and K^+ in the cells of *R. melilote* in hypersalinity. The concentration of glycine and betaine increases more in salt tolerant strains than in sensitive strains[8].

Trehalose accumulation to higher levels in cells of *R. leguminosarum* has been shown by Breedveld (1991)[9]. Another osmoprotectants ecotine was as effective as glycine and betaine in improving the growth of *Rhizobium* under osmotic conditions[10]. *Rhizobium*-legume symbiosis under stress thus requires the osmotolerant *Rhizobium* strains. Graham (1992) reported that salt tolerant strains of *Rhizobium* represent only a small percentage of all the strains isolated and identified so far[11].

The present paper deals with the effect of osmolytes on the growth of halophilic weedicide tolerant rhizobia *in vitro* in yeast extract mannitol medium and the accumulation of these osmolytes in nodules induced by these rhizobia.

MATERIAL AND METHODS

1. Source**:** Pigeon pea [*Cajanus cajan* (L.) Millsp.] seeds of AKPH2022 variety were used.
2. *Rhizobium* strains isolated and identified in our laboratory designated as **N** and **H** were used (Shende and Patil , 2005)
3. Growth conditions**:** *Rhizobium* N strains were grown in YM broth in presence of 100mM NaCl at PH 6.0. This *Rhizobium* exhibited weedicide Glyphosate tolerance at 4% ai. *Rhizobium* H strain exhibited optimum growth at pH 8.0 in presence of 300-400 mM NaCl and could tolerate glyphosate at 6% ai.
4. Nodulation of *C. cajan* in presence and in absence of Glyphosate**:** The method described by Chiddarwar (1997) was followed for nodulation (12). The soil supplemented with 4% and 6% Glyphosate ai was used for development of nodules by N and H *Rhizobium* strains respectively, running suitable controls without Glyphosate.
5. Growth of N and H *Rhizobium* in liquid Y M broth in presence and absence of glyphosate and osmolytes**:** *Rhizobium* N and H strains were grown in liquid Y M broth supplemented with osmolytes such as Glycine, Proline, Sucrose and Trehalose. Effect of these osmolytes on growth of N and H strain in presence of 4% and 6% Glyphosate ai respectively was also studied. Osmolytes were mixed separately in sterile YM broth at 10, 20, 30, 40 &50 mM concentration. Twenty-four hour grown liquid inoculum of N and H *Rhizobium* containing approximately $109*10^9$ cells /ml was used. Fresh 0.1ml inoculum was transferred aseptically to 5 ml Y M broth. Growth of the *Rhizobium* was measured, after 3 days incubation at 28 ± 2ºC, at 520 nm.

Determination of osmolytes in nodules:

1. Proline & Glycine**:** 50mg nodules were crushed very carefully in 5ml sterile distilled water. The contents of the tubes were centrifuged at 9668g for 15 min. The pellet obtained was washed and suspended in isotonic salt solution and then recentrifuged. The amino acids from the resulting pellet were extracted by suspending in 5ml of 5% TCA at 0°C for 24 hrs with occasional blending in a vortex mixture. The pellets were then centrifuged at 9668g for 30min. The supernatant was used for amino acid analysis.
The proline was determined by Acid Ninhydrin method[12] and Glycine was determined by Sorenson's formal titration method[13].

2. Sucrose**:** 100 ul of conc HCl was added to 50 mg nodules. The nodules were crushed in test tube with glass rod in 5ml sterile distilled water, hydrolysis was carried out at 90ºC for 5min, 0.25ml of 5N KOH was added to neutralize the acid after cooling. The next procedure was followed as described by Schaffer[14].

3. Trehalose**:** For determination of Trehalose the method of Parrou (1998) was used[15].

Statistical analysis:

Results represented in Table - 1 is the mean of three replicates and analysed by applying single linear regression equation.

Table - 1: Determination of osmolytes in nodules of *C. cajan*

Nodules	Glycine(mg)	Proline (ug)	Sucrose (mg)	Trehalose(mg)
N	1.31mg	3.86ug	0.1mg	0.17mg
Ng	1.89	11.5	0.149	0.26
H	2.47	15	0.28	0.30
Hg	3.25	16.2	0.54	0.5

N: Normal Nodules, **Ng**: Normal nodules grown in presence of 4% glyphosate, **H**: Halophilic nodules grown in saline conditions & **Hg**: Halophilic nodules grown in presence of 6 % glyphosate & saline conditions.

RESULTS AND DISCUSSION

Root nodules were harvested after 40 days of inoculation of seedlings of *C.cajan* by *Rhizobium* N and H strain. The nodules were designated as N nodules obtained from *C.cajan* roots, where *Rhizobium* N was grown in its natural normal conditions and Ng nodules were harvested from *C.cajan* roots receiving N *Rhizobium* and 4% ai glyphosate. Similarly H nodules were obtained from plants receiving *Rhizobium* H strain in its normal growth conditions and Hg nodules were harvested from *C.cajan* roots receiving *Rhizobium* H and 6% ai glyphosate.

Results presented in Table 1 indicate the accumulation of osmolytes in presence and absence of stress of glyphosate. Comparison of the accumulated osmolytes between N and H nodules shows that the halophilic (H strain) *Rhizobium* in its normal growth conditions accumulated approximately 1.9 times more of Glycine than the nodules developed by normal (N strain) *Rhizobium* strain. Similarly, Proline content was 4 times more in H nodules. Sucrose accumulation was three times more, where as Trehalose content was also high in H nodules than its N counterpart.

Comparison of accumulation of osmolytes between N and Ng nodules and that with H and Hg nodules reflects the accumulation of osmolytes in more quantity in Ng than N, also between H and Hg indicating their proportional accumulation in nodules with respect to the increase in stress. Thus the accumulated osmolytes appear to help the nodulating rhizobia to grow & establish themselves well in the form of nodules in the roots in stress conditions. This was confirmed by the fact that, when the osmolytes were included in the growth medium, there was little alteration in the growth of N and H *Rhizobium* in presence and absence of glyphosate stress (Fig- 1,2,3&4). These results clearly support the idea, that the osmolytes help the Rhizobia to tolerate the weedicide stress. These results were similar to those of Pandher and Kahlon (1978), where they have observed that the growth of *R. leguminosarum* was increased with increase in NaCl stress[16]. For the formation of root nodules the Rhizobia grow and proliferate in the cells of roots thereby creating nodules. Thus the accumulated osmolytes in nodules provide a optimal environment for survival of rhizobia in nodules even in condition of stress.

The nodules Ng, H and Hg were isolated from the stressed plant. These nodules thus accumulated more osmolytes in general than the N nodules. Similar studies were also carried out by Pandher and Kohlon (1978) whereas they observed that the growth of some strains of *R. leguminosarum* decreased and that of some strains increased in presence of NaCl stress[16].

Sucrose and Trehalose also enhanced growth of N and H *Rhizobium* even in presence of glyphosate stress. Le Rudulier (1983) also reported the same observation for some Rhizobia[17]. Breedveld (1991) also observed Trehalose accumulation in larger quantity in peanut rhizobia under the increasing osmotic stress in presence of hypersalinity[9].

Sucrose and Trehalose were accumulated at a higher level in the stress nodules than the N nodules. These osmoprotective substances are suggested to play a significant role in maintaining the nitrogenase activity of bacteriods under salt stress and also in triggering the synthesis of endogenous osmolytes[18]. Sucrose and ecotine were also shown to be osmoprotectants for *Sinorhizobium meliloti*[19].

Proline has always been reported to be most potent osmotic effector[20]. Accumulation of proline in root nodules in stress conditions has

Figure - 1

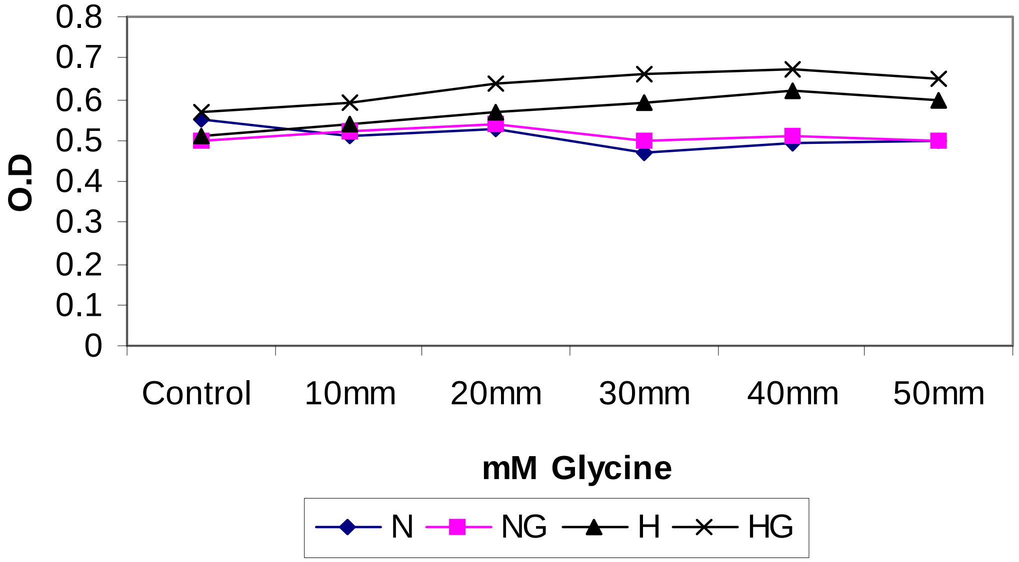

Figure - 2

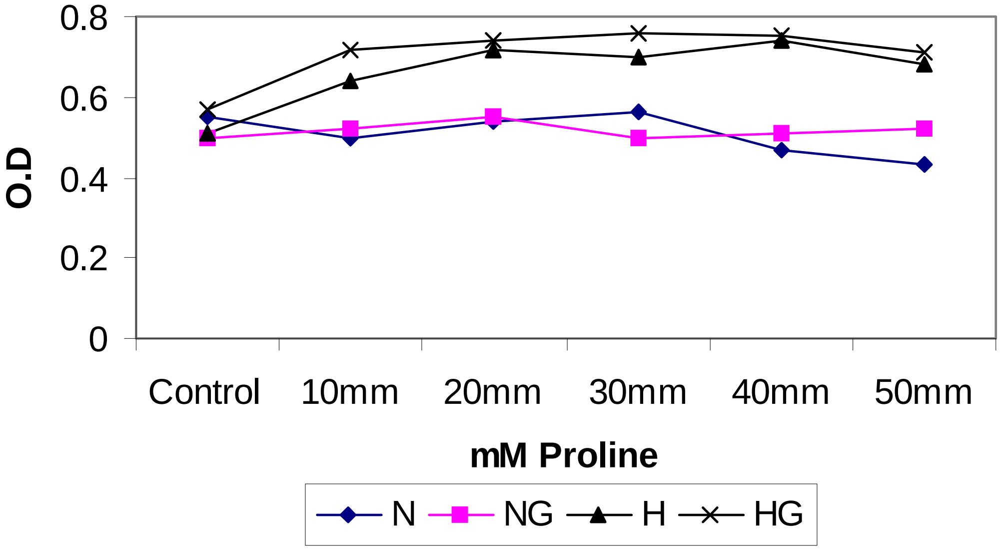

Fig. - 1 & 2 : Effect of osmolytes on the growth of *Rhizobium* strains N, H, Ng, and H_g.

Figure - 3

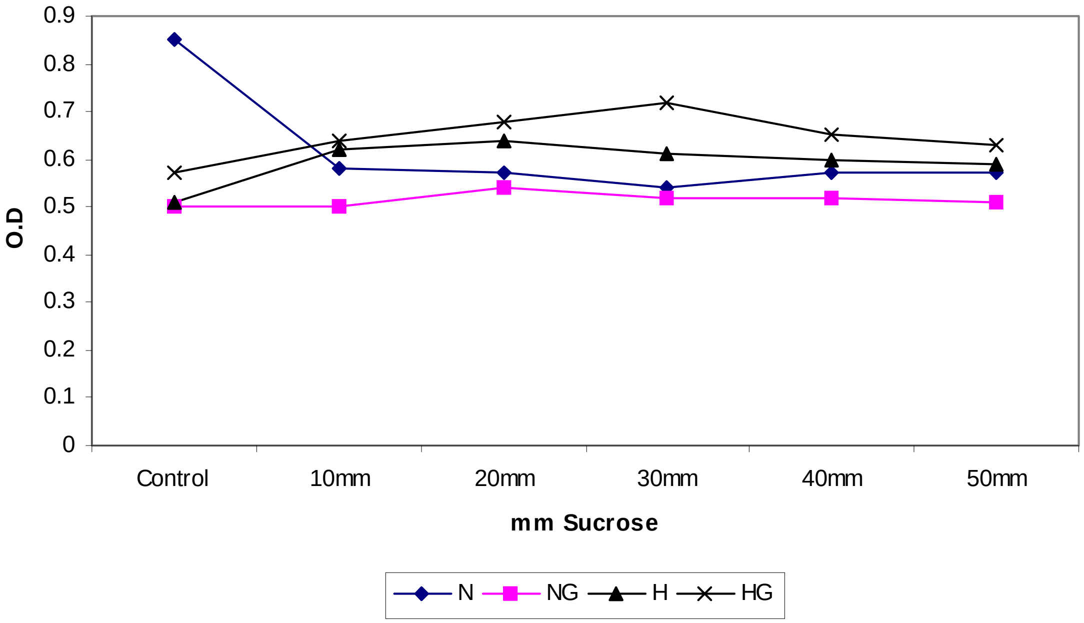

Figure - 4

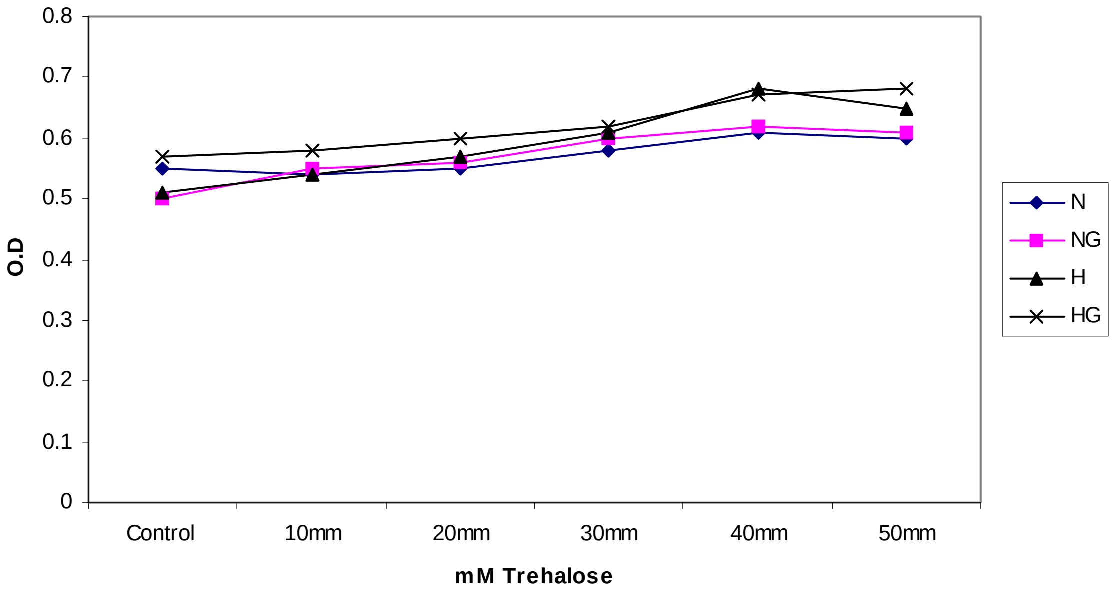

Fig. -3 & 4 : Effect of osmolytes on the growth of *Rhizobium* strains N, H, Ng, and H$_g$.

been observed by Bauillard (1983)[17] and Chiddarwar (1997)[12]. It was also found that proline was accumulated up to 80 % of amino acid pool under variety of stress conditions (Delauney, 1993[21] and Bohnert, 1996[22]). Proline seems to have a diverse role under osmotic stress, such as stabilization of proteins, membranes and sub cellular organelles[23].

In the present investigation growth of N *Rhizobium* in presence of glycine in normal (N) and stress conditions (Ng) was not much affected, however for H and Hg cases there appear to be a increase in growth, where glycine has served as a good carbon and nitrogen source. Similar results were also reported by Landfold (1986) for one *Rhizobium* strain, where glycine was not a good osmoprotectant for non-salt tolerating rhizobia, but in presence of NaCl it supported growth[24]. The role of glycine in the family Rhizobiaceae appears to be of an energy source, while its contribution to osmoprotectants is restricted to certain strains thus glycine acts as a genuine osmolyte. It was found that the concentration of glycine is more in salt tolerant strain of *R. meliloti* than in sensitive strain[25]. Thus osmoprotectants like glycine, betaine, proline, trehalose, enhance the growth of rhizobia, when they are present in culture media[26].

Accumulation of these compatible solutes in the root nodules shows the adaptability of these plants and the specific nodulating *Rhizobium* to stress conditions. Salinity and weedicide stress being the serious threat to agriculture in arid and semi arid regions, introduction of plants and the rhizobia capable of surviving and thriving under these stress conditions is worth investigating[27]. Thus selection of salt tolerant genotypes and stress tolerant rhizobia should be selected to improve the crop productivity in stress conditions.

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