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ANALYSIS OF PROLINE AND MDA AND PROTEIN PROFILE IN SEEDLINGS OF COWPEA EXPOSED TO NACL SALINITY – INFLUENCE OF RHIZOBACTERIUM ON SALT TOLERANCE

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ABSTRACT

This research was carried out in order to test the influence of the Rhizobacterial culture on NaCl salinity tolerance in Cowpea plants at seedling stage. Rhizobacterial strains BR2 and BR3 were used to inoculate seeds of Cowpea (*Vigna-unguiculata*) varietiy (Pusa Sukomal). Seed germination percentage, quantitative analysis of proline, MDA and protein and protein profile was carried out in 7 and 15 days old seedlings exposed to 0, 25, 50 and 75mM NaCl salinity in both Rhizobacterium inoculated and uninoculated (control) seedlings. Seed germination percentage is reduced to the level of NaCl. This effect was reduced in the presence of Rhizobacterium. At 25mM NaCl, the germination percentage was 100% in seeds inoculated with BR2 and BR3 and the germination was up to 94 percent. Similarly, the proline content increased in all groups of seedlings to increase in NaCl concentration, irrespective of Rhizobacterium presence. However, the seedlings with Rhizobacterium inoculation had significantly high levels of proline as compared to control, in absence of Rhizobacterium. While MDA levels were decreased in seedlings inoculated with BR2 and BR3 strains of Rhizobacterium, compared to seeds without Rhizobcterium, indicating the reduction in lipid peroxidation and the increase in tolerance on increased NaCl salinity. The protein content, in general, increased up to 50mM NaCl concentration and reduced at 75mM level. Our studies in SDS –PAGE analysis suggests that some proteins (approximate 50, 48, 32 and 27 kDa) were found to be expressed due to salt stress, whereas a few proteins (~65kDa and ~40kDa) found suppressed due to high salt concentrations. Rhizobacterium strains BR2 and The present study suggest that the inoculation of Rhizobacterium could reduce the stress induced due to saline condition.

KEYWORDS: Cowpea, MDA, Proline, Rhiozobacteria, Salt Stress

INTRODUCTION

Salt stress is an important abiotic environmental factor which retards the growth of plants and induces several physiological and morphological alterations in the plant (Gama *et al.*, 2007). At present, out of 1.5 billion hectares of cultivated land around the world, about 77 million hectares (5 %) are affected by excess salt content (Sheng *et al.*, 2008). It has been estimated that an approximate area of 7 million hectares of land is covered by saline soil in India (Patel *et al.*, 2011). Salinity adversely affects plant growth and development, hindering seed germination, seedling growth, enzyme activity (Seckin *et al.*, 2009), DNA, RNA, protein synthesis and mitosis (Tabur and Demir, 2010; Javid *et al.*, 2011). Germination is the most sensitive stage among the various stages of plant growth. Poor germination is the most common and primary effect of salinity. Malondialdehyde (MDA), a product of lipid peroxidation, and Proline content has been considered an indicator of oxidative damage and osmotic adjustment under abiotic stress conditions (Hyun *et al.*, 2003).

Salinity has been reported to show inhibitory effects on protein quality and quantity (Sultana *et al.*, 2000, Mishra *et al.*, 2006, Taffouo *et al.*, 2010).

The Rhizobacteria are known to ameliorate the negative effects of salinity on a number of crops such as Tomato, Pepper, Canola, Bean and Lettuce (Barassi *et al.* 2009; Kang *et al* 2009; Egamberdieva 2009; Steel 1972; Singh *et al.*, 1997). The salinity stress problem is especially acute in arid and semiarid regions where Cowpea is a widely cultivated species (Souza *et al.*, 2004). Cowpea with high level of protein content (23-35%) and its capability to associate with symbiotic Rhizobacteria to fix atmospheric nitrogen to ammonia in the soil and increase its fertility makes it important in sustaining the nutritional balance of low income population (Singh *et al.*, 1997).

The present study was carried out to analyze the role of Rhizobacterium salinity tolerance in Cowpea plants at seedling stage when they are exposed to different levels of NaCl salinity.

MATERIALS AND METHODS

In the present work effect Rhizobacterium on cowpea plant growth grown under saline conditions at seedling stage was carried out using Cowpea (*Vigna-unguiculata*) variety- Pusa Sukomal and two strains of Rhizobacterial - BR2 and BR3 (all Obtained from IARI, New Delhi). For salinity studies, we used 0mM (Control), 25mM, 50mM and 75mM concentration NaCl saline water.

Two sets of seeds each with 25 seeds were taken - one as non-inoculated and another as inoculated. The Rhizobacterium strains BR2 and BR3 cultures (14 days old) were harvested by centrifugation and the pellet was coated on seeds in one set (With Rhizobacterium) just before germination. Both the sets– without and with Rhizobacterium inoculation were left for germination on filter boats dipped in 0, 25, 50 and 75Mm NaCl solutions at room temperature for 15 days . Each experiment was carried out in three replicates and was repeated twice.

Germination percentage was recorded on the seventh day. Biochemical studies were carried out in 7 and 15 days old seedlings.

Proline content was estimated spectrophotometrically according to the method described by Lattanzio *et al.*, (2009). The measurement of lipid peroxidation as the amount of MDA was determined using the methods by Madhava Rao *et al.*, (2000) and Baryla *et al.*, (2000). The data collected from each replicate was pooled and statistical analysis was done. Mean and standard error were calculated for each experimental data.

Qualitative analysis of proteins was done in 7 and 15 day old seedling samples. Protein in the samples was initially precipitated with 0.5% TCA and dissolved the precipitate in (0.1M pH 6.8) tris –HCl buffer and quantified by Bradford (1976) assay method. Samples were then run on 10% SDS-PAGE gel along with low molecular weight markers and stained with compulsive blue stain. The gel was photographed and the banding pattern was evaluated.

RESULTS AND DISCUSSIONS

Germination Percentage

The seed germination process of plants is affected by salt stress (Taffouo *et al.*, 2009). This may be due to the creation of an osmotic potential that prevent water uptake or the conditions that are created for entry of ions that may be toxic for embryo or developing seedlings (Almodares *et al.*, 2007).

Further, salinity known to increase the osmotic potential of the growth medium resulted more energy required to absorb water, that decreased the germination (Jamil *et al*., 2004).

Results on germination percentage, given in Table 1, shows that the percentage germination has reduced from 100% in control to 96.67, 93.33 and 89.33%, in absence of Rhizobacterium, when exposed to 25, 50 and 75mM of NaCl. The seeds inoculated with Rhizobacterium showed 100% germination in control as well as an exposure to 25mM NaCl water, in the presence of either BR2 or BR3 respectively (Table 1). There after percentage was reduced to 96.67% and 90.67% (in presence of BR2) and 98.67 and 94.67 % (in the presence of BR3), on exposure to 50mM and 75mM NaCl salinity respectively (Table 1 and Figure 1).

Table 1: Effect of NaCl Salinity on the Seed Germination in Cowpea Var. Pusa Sukomal

NaCl concentration	Percentage (%) Germination		
	Without Rhizbacterium	With Rhizobacterium BR2	With Rhizobacterium BR3
0mM	100±0.00	100±0.00	100±0.00
25mM	96.67±0.67	100±0.00	100±0.00
50mM	93.33±1.33	94.67±1.33	98.67±1.33
75mM	89.33±1.33	90.67±2.67	94.67±1.33

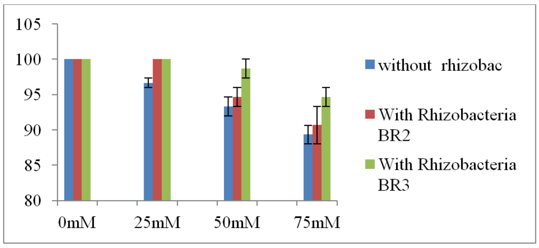

Figure 1: Percentage Germination of Pusa Sukomal Seeds Grown in Different NaCl Concentrations Solutions with or without Rhizobacterium (BR2 & BR3) Application

Proline Content

Halotolerant and halophilic microorganism having beneficial effects such as IAA production, nitrogen fixation, phosphate solubilization and production of ammonia can accumulate osmolytes in stress conditions (Zhou et al, 2015).

The mean proline content in 7 and 15 days old seedlings was 177.495µg/g and 168.380µg/g fresh weight (g FW) in 7 and 15 days old seedlings. At low concentration of salinity (25mM) the proline content increased to 345.683µg/g FW and 231.847µg/g FW in 7 and 15 days seedlings of Pusa Sukomal. Further, the level of proline has risen to 475.974µg/g FW (50mM) and 599.358µg/gFW (75mM) in 7 days and to 362.665µg/g FW (50mM) and 566.305µg/g FW (75mM) in the 15 days old seedlings (Table 1).

With inoculation of BR2 and BR3 strains the proline content in the control (0mM) has increased to 286.007µg/g FW and 294.066µg/g FW in 7 day old seedlings and in 15 days old seedlings the proline content was 204.503µg/g FW and 209.828µg/g FW respectively (Table 1).

The levels of proline in presence of Rhizobacterium recorded as 553.209µg/g FW (BR2) and 564.722µg/g FW of plant (BR3) in 7 days seedlings and 562.563µg/g FW (BR2) and 566.305µg/g FW (BR3) in 15 days seedlings growing in 50mM NaCl salinity stress conditions. Similarly, at 75mM NaCl salinity stress the concentration of proline was 613.365µg/g FW (BR2) 613.557µg/g FW (BR3) in 7 days seedlings and 581.848µg/g FW (BR2), 609.336µg/g FW (BR3) in 15 days old seedling (Table 2).

Our results are in accordance with the earlier reports of Silveira *et al* (2001) and Girija *et al* (2002) that showed increases in proline content with increase salinity levels in Cowpea and peanut plants. Tawfik *et al* 2008 also reported that the proline content in cowpea leaves has increased with increasing NaCl levels and increased further more in Rhizobacterium inoculated plants.

Lipid Peroxidation and MDA Content

The Malondialdehyde produced during peroxidation of membrane lipids is often used as an indicator of oxidative damage. In our study, the MDA content, given in the Table 2, in 7 and 15 days seedlings of un-inoculated control under non saline conditions was 117.887 µg/g FW and 115.675µg/g FW respectively, showing no significant difference in different stages of growth. The level of MDA increased up to 159.606/ 200.377µg/g FW and 184.574/306.887 µg/g FW on exposure to NaCl at 50 and 75mM concentrations at 7/15 days of seed germination (Table 2)

Similar patterns of increase in MDA levels was noticed in seedlings inoculated with BR2 recorded 106.509 to 195.636µg/g FW while 15 day seedlings recorded 113.146 to 298.985µg/g WF MDA, which is almost equivalent to the control without Rhizobacterial inoculation.

Cowpea seeds inoculatedBR3 showed 136.00 and 95.11µg/g FW MDA content at 0mM and 117.56 and 141.18µg/g FW MDA at 50 and 75mM NaCl stress in 7 day old seedlings. Whereas the 15 day old seedlings recorded 176.989 and 220.604µg/g FW MDA at 50 and 75mM NaCl stress respectively, which is less than the control as well as seeds inoculated with BR2 (Table 2).

Malondialdehyde (MDA) is a product of decomposition of polyunsaturated fatty acid and their increase in plant have been widely used as an indicator of lipid peroxidation in salt stress serving as discriminating the sensitive and tolerant species to salinity (Meloni *et al* 2003 Stepien and Klobus, 2005). Ahmad et al 2008 reported that the lipid peroxidation level was higher in salt sensitive compared to tolerant cultivar of *Pisum sativum.* Lipid peroxidation increased in salt stressed plants is in agreement with our study. Similar results were found in Ahmad 2010 in mustard also Rasool *et al* 2013 in chickpea and Valdineia Soares Freitas *et al* 2011 in Cowpea.

Table 2: Mean Value of Biochemical Parameters- Proline, MDA and Protein Content Cowpea Var. Pusa Sukomal with or Without Rhizobacteral Culture under Different (0, 25, 50 And 75mm NaCl) Salinity Stress Conditions

NaCl Concentrations	Cowpea Var. Pusa Sukomal					
	Proline Content (µg/g FW)		MDA Content (µg/g FW)		Protein Content(µg/g FW)	
Control	**7th day**	**15th day**	**7th day**	**15th day**	**7th day**	**15th day**
0mM	177.495±0.253	168.380±0.830	117.887±0.316	115.675±4.275	116.22 ±2.14	149.78±0.8
25mM	345.683±0.166	231.847±8.059	124.524±0.316	137.482±4.275	152.00±5.88	127.11±4.26
50mM	475.974±0.253	362.665±0.997	159.606±1.139	184.574±3.014	168.22 ±5.67	174.22±11.55
75mM	599.358±0.253	566.305±0.249	200.377±1.672	306.887±5.086	122.89 ±6.68	131.56±2.34
With Rhizobacteria BR2						
0mM	286.007±0.253	204.503±8.059	106.509±0.316	113.1468±2.468	126.22±6.81	122.67±1.82
25mM	384.924±0.253	240.913±8.807	113.146±3.644	127.369±3.160	136.67±5.83	137.11±3.45
50mM	553.209±0.287	562.563±1.080	130.529±3.014	187.103±2.212	153.33±3.93	165.11±7.02
75mM	613.365±0.166	581.848±5.733	195.636±0.632	298.985±0.632	139.78±8.86	107.56±4.73
With Rhizobacteria BR3						
0mM	294.066±0.253	209.828±7.644	91.339±1.759	104.929±6.152	136.00±5.58	95.11±6.91
25mM	467.627±0.583	323.233±0.830	107.141±0.547	118.835±6.344	112.00±1.82	126.00±2.12
50mM	564.722±6.312	566.305±0.249	117.571±0.547	176.989±0.836	117.56±3.53	150.67±11.43
75mM	613.557 ±0.253	609.336±8.475	192.16±0.836	220.604±03.014	141.18 ±7.47	118.89±2.4

Protein Content

Numerous biochemical processes are known to be influenced by salt stress, which includes both protein content as well as its quality, either negatively or positively (Sultana *et al.*, 2000, Mishra et al., 2006, Taffouo *et al.*, 2010).

The mean protein content in 7 and 15 days old seedlings of Cowpea var. Pusa Sukomal under saline conditions without or with Rhizobacterium inoculation was given in Table 2. Cowpea seedlings grown under control conditions, without rhizobacterium inoculation, recorded 116.22µg/g FW of protein on 7 days and 149.78µg/g FW of protein on 15th days. When the germinating seeds were exposed to NaCl salinity, protein content was increased initially to 152.00µg/g FW (25mM), 168.22µg/g FW (50mM), thereafter it reduced at 75mM concentration to 122.89 µg/g FW in 7 days old seedlings (Table 2). Similarly, in 15 day old seedlings, content of protein increased to 127.11µg/g FW at 25mM NaCl concentration and 174.22µg/g FW at 50mM NaCl salinity. The protein level reduced to 131.56µg/g FW in 15 days old seedlings in 75mM salinity conditions (Table 2).

With inoculation of BR2 strain the protein content in the control (0mM) seedling was 126.22µg/g FW and 122.67µg/g FW in 7 days and 15 days seedlings respectively. On exposure to salinity at 25, 50and 75mM concentrations, seedlings at 7 days age recorded 136.67µg/g FW, 153.33µg/g FW, and 139.78µg/g of protein and 15 day seedlings recorded 137.11µg/g FW, 165.11µg/g and 107.56µg/g FW of protein BR2 (Table 2).

Similarly with inoculation of BR3 strain the 7 & 15 days seedlings recorded 136.00µg/g FW and 95.11µg/g FW of protein in control (0mM). While on exposure to 25, 50 and 75mM salinity these seedlings showed 112.00µg/g FW, 117.56µg/g FW and 141.18µg/g FW protein in 7 days old seedlings and 126.00µg/g FW, 150.67µg/g FW and 118.89µg/g FW protein in 15 days old seedlings (Table 2).

Plants under stress conditions accumulate proteins that protect cells from harmful stress effects. Increase in protein quantity may be because of plant tolerance to the NaCl concentration that it was exposed to and similarly decrease in protein content may be due to increased NaCl stress at higher concentrations (Omar *et al.*, 1993). Prolonged salt stress could affect protein synthesis and provokes its decline (Caplan *et al.*, 1990). In the present study also demonstrates an increase in protein content up to 50mM NaCl salinity and thereafter it decreased at 75mM (Table 2). The levels of protein are high in seedlings inoculated with BR2 followed by control and BR3 (Table 2)

SDS-PAGE Protein Profile at Seedling Stage 7 and 15 Days

SDS-PAGE gel pictures showing the protein profile in 7 and 15 days old Cowpea seedlings exposed to salinity, without and with inoculation of Rhizobacterial strains BR2 and BR3 is given in the Figures 2 and 3 respectively.

The 7th day of seed germination in uninoculated seedlings, 4 proteins (26-68 kDa) at 0mM, 7 proteins (22-55 kDa) at 25mM, 11 proteins (17-100 kDa) at 50mM, and 9 proteins (16-100 kDa) at 75mM NaCl concentration were found to express. However, 6 proteins (26-100 kDa) at 0mM, 7 proteins (25-100 kDa) at 25mM, 10 proteins (17-96 kDa) at 50mM and 10 proteins (17-100 kDa) at a 75mM NaCl concentration were found expressed in BR2 inoculated Pusa Sukomal seedlings. While in BR3 inoculated Pusa Sukomal 8 proteins (17-96 kDa) at 0mM, 2 proteins (43-67 kDa) at 25mM, 3 proteins (26-67 kDa) at 50mM and 7 proteins (17-67 kDa) at 75mM NaCl concentration were found expressed on the 7th day germination.

Similar patterns of protein expression was recorded in the 15th day of the treatment. At 15th day, 2 proteins (42- 68 kDa) at 0mM, 2 proteins (42- 68 kDa) at 25mM, 9 proteins (17- 96 kDa) at 50mM, and 5 proteins (28- 68 kDa) at 75mM NaCl concentration were found expressed in un-inoculated seedlings. Whereas, 7 proteins (33- 96 kDa) at 0mM, 2 proteins (45- 65 kDa) at 25mM, 9 proteins (17- 96 kDa) at 50mM and 4 proteins (27- 65 kDa) at a 75mM NaCl concentration were found expressed in BR2 inoculated seedlings. In BR3 inoculated Pusa Sukomal seedlings, 6 proteins (22-100 kDa) at 0mM, 4 proteins (33- 100 kDa) at 25mM, 8 proteins (16- 100 kDa) at 50mM and 5 proteins (28- 100 kDa) at 75mM NaCl concentration were found to express (Figure 3).

Induction and suppression of specific proteins under saline conditions to salt stress has been demonstrated earlier by various researchers (Sultana *et al.*, 2000, Tort and Turkyilmaz, 2004, Beltagi *et al.*, 2006, Chen *et al.*, 2007 and Kapoor and Shrivastava, 2010). Induction of 127 and 52kDa proteins and repression of 260 and 38kDa proteins was observed by Hussanein, (1999) in peanut plants grown under NaCl salinity stress conditions. Similarly, Elshintinaway and Elshourbagy (2001) observed that the protein content of 26kDa increased, while the protein content of 13 and 20kDa decreased and 24kDa proteins disappeared under NaCl stress in wheat plants. Similar synthesis of specific proteins under salt stress was reported in *the Hordeum Vulgaris L.* (Popava *et al.,* 1995), with tomato (Elennany, 1997) and also in rice (Radha Rani *et al.*, 1994; Pareek 1999; Djanaguiraman, 2000). Our study suggests that some proteins (approximate 50, 48, 32 and 27 kDa) were found to be highly expressed due to salt stress, whereas a few proteins (~65kDa and ~40kDa) found suppressed at high salt (75mM) concentration. Rhizobacterium strains BR2 and BR3 significantly decreased the level of these salts induced proteins when compared to control. Strain BR3 lowered the expression of the salt induced proteins much efficiently when compared to control or BR2. Furthermore, expression of a protein (~65kDa and ~40kDa) was highly expressed by the both BR2 and BR3 strains in comparison to control. However, the level of this protein was lowered in the presence of salt in un-inoculated groups. BR2 and BR3 inoculation maintained the expression of this protein under salt stress.

CONCLUSIONS

In the present study, Cowpea variety Pusa Sukomal inoculated with BR3 showed better germination under highest concentration of NaCl used (75mM). The Proline and protein content increased with salinity and a reduction in MDA content in this combination compared to control and BR2, indicate the combination of host plant variety and BR3 was best. Present study results clearly suggest that the inoculation of Rhizobacterial strains of seeds could reduce the stress induced by NaCl salinity at seedling stage, the most vulnerable stage in plant growth, and improves salinity tolerance in Cowpea. However, the selection of the correct variety of Cowpea and Rhizobacterial strains to significantly increase the tolerance to saline conditions.

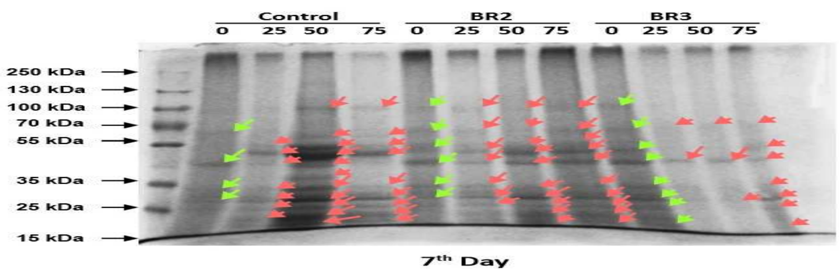

Figure 2: SDS-PAGE Protein Profile of Cowpea Variety Pusa Sukomal with and without Rhizobacterial Inoculation under Different 0, 25, 50 and 75mm NaCl Salinity Conditions at 7 Days

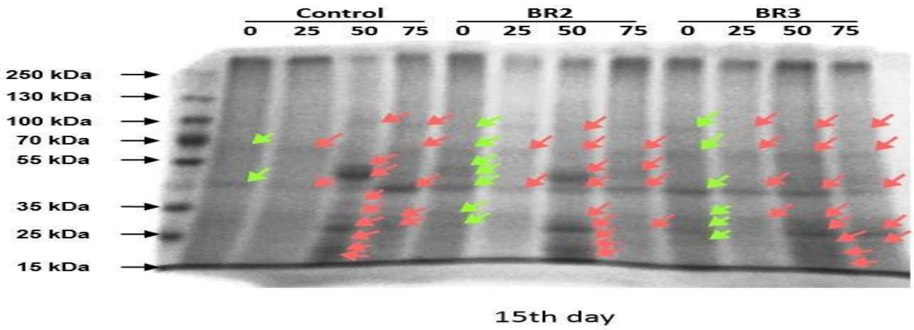

Figure 3: SDS-PAGE Protein Profile of Cowpea Variety Pusa Sukomal with and without Rhizobacterial Inoculation under Different 0, 25, 50 and 75mm NaCl Salinity Conditions at 15 Days

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