World Applied Sciences Journal 32 (10): 2037-2041, 2014
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2014.32.10.21562

Confirmation of Salt Tolerant Rhizobia Through Nodule Soluble Protein Profiling by SDS-PAGE

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Abstract: Four chickpea (21cp, 22cp, 26cp and 27cp) and five green peas (3gp, 12gp, 24gp, 26gp and 27gp) were evaluated for gene expression under 300mM salt stress through nodule soluble protein profiling by SDS-PAGE on 12.25% gel. Protein profile revealed expression of 6 proteins in chickpea rhizobia (approximately 10, 15, 27, 35, 56 and 65 kDa) and 11 in green pea rhizobia (approximately 12, 35, 40, 55, 57, 65, 70, 95, 115, 130 and 150 kDa) under salt stress. Three unique bands (10, 27 and 56 kDa) were expressed by chickpea rhizobia and five unique bands (12, 55, 65, 115, 150 kDa) were observed in green pea rhizobia under salt stress. On the basis of salt stress protein expresionin persent work Therefore, all the tested isolates tested through SDS-PAGE of for chickpea and green pea are suitable candidate inoculants as biofertilizer in saline soils.

Key words: Salt resistance · Rhizobia, protein · SDS-PAGE · Chickpea · Green pea

INTRODUCTION

Salinity in the soil and irrigation water is an environmental problem and a major constraint for crop production. Currently, 20% of the world's cultivated land is affected by salinity, which results in the loss of 50% of agricultural yield [1, 2]. At present, there are nearly 954 million hectares of saline soils on the earth's surface. All these salt affected soils are distributed throughout the world. A large bulk of about 320 million hectares and of lands in South and South East Asia is under the grip of salinity.

Soil salinity is also a serious problem of agriculture in Pakistan. In Pakistan, about 6.30 million hectares of lands are salt-affected and of which 1.89 hectare is saline, 1.85 million hectare is permeable saline-sodic, 1.02 million hectare is impermeable saline-sodic and 0.028 million hectare is sodic in nature. It is estimated that out of 1.89 million hectares saline patches, 0.45 million hectares present in Punjab, 0.94 million hectares in Sindh and 0.5 million hectares in NWFP. The substantial rise in the water table has caused salinity and water logging in large areas of Sindh, Punjab, NWFP and Balochistan. The problem is more severe in Sindh and Southern Punjab than other parts of the country [3]. These areas are now subjected to severe degradation and desertification. Now a day efforts have been made to learn to live with salinity and make profitable use of saline land and water resources. Recently a safe alternative approach has been employed to combat salinity known as "Bio-saline agriculture technology", which involves the cultivation of salt tolerant species/cultivars with genetic traits to utilize salt affected soils. This technology gives economic return and provides vegetative covers to soil which reduces evaporation and hence the rate of salinization. This biological approach involves screening and selection of highly salt-tolerant plant species/varieties from the naturally existing germplasm or from these developed through breeding, hybridization and other techniques, and then introducing the selected plants for increased plant establishment and productivity in saline areas [4]. Many halophytes are reported to grow efficiently in saline

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soils [4]. Although chickpea (*Cicer arietinum* L.) and green pea (*Pisum sativum* L.) have not been reported to grow in saline soils but as the problem of salinity is growing there is need to test both of the crops for salt tolerance along with their symbiotic partner. Variation among strains of *Rhizobium* spp. in the symbiotic performance under saline conditions has been reported by many researchers [5-7]. Salinity tolerance among rhizobia varies species to species. *R. meliloti* strains tolerate 100 mM NaCl [8], *R. leguminosarum* have been reported to be tolerant to NaCl concentrations up to 350 mM NaCl in broth culture [9]. It is evident from several investigations that symbiotic effectiveness is positively correlated with high-tolerance under saline condition [10]. Therefore, there may be scope for selecting a *Rhizobium-legume* symbiosis that is better adapted to saline conditions.

Present work is undertaken to study effect of saline conditions on protein expression of rhizobial isolates by (SDS-PAGE) analysis and screening of salt tolerant strains on the basis of unique bands produced under salt stress.

MATERIALS AND METHODS

Rhizobia Used: All the rhizobia were isolated from root nodules of chichpea and green pea seedlings grown in 27 different soils collected from different cities of Pakistan. The root nodule isolate of chickpea are given code (cp) and green pea as pea (gp). Out of 19 chickpea and 11 green pea rhizobia, Ffour Chickpea (26cp, 27cp, 21cp and 13cp) and five green pea (27gp, 26gp, 3gp, 23gp and 24gp) root nodule rhizobia that performed better for salt stress in *in-vitro* test and *in-vivo* in pot experiment (Data shown Table 1, 2, & 3) were further evaluated at 300mM NaCl stress for salt tolerant gene expression.

Nodular SDS-PAGE Analysis: Nodular protein profile of selected rhizobia was carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [11] using 12.25% (w/v) and stacking gel [12].

Protein Extraction: 50 mg nodules were ground in 1ml of Phosphate buffer saline ph pH 7.4. After 24 hours samples were centrifuged at 10000 rpm for 5 minutes. Supernatant was collected in other tube and stored at 10°C. 200ml samples were suspended in equal volume of extraction buffer (100 mmol Tris (pH 6.8), 0.8% SDS, 25% glycerol, 4% mercapethanol & 02% bromophenole blue). The cells were disrupted by temperature shock (freezing (-70°C) followed by 25 minute boiling (90ºC). After lyses samples were centrifuged at 13,000 rpm for 20 min and stored at-20°C before analysis.

The electrophoretic procedure was carried out using slab type SDS-PAGE ModelÓAE-6530M, ATTA Japan. 12.25% resolving gel (3.0 M Tris-HCL HCl (Sigma) pH 9.0, 0.4% SDS (Wako) and 4.5% stacking gel (0.4 mol Tris-HCL HCl pH 7.0, 0.45 SDS) were prepared and polymerized chemically by addition of 17 µl by volume of N,N',N'N' tetramethylenediamine (TEMED) and 10% ammonium persulphate (Cica reagent).

Electrode buffer (0.05 M Tris, 0.192 M Glycine and 0.125% SDS) solution was put into the top and bottom pools of the apparatus. Gel plates were placed in the apparatus carefully so as to prevent bubbles formation at the bottom of gel plates. The samples (50 µl each) and molecular weight marker (PageRuler™ Plus Pre-stained Protein Ladder, Fermentas) (20 µl) were loaded. The gel was run at initial voltage of 70 V ev followed by 100 V ev for three h at 15°C.

Table 1:Percent survival range of chickpea and green pea rhizobia against NaCl

Chickpea Rhizobia			Greenpea Rhizobia		
Survival range	Isolates	Percentage	Survival Range	Isolates	Percentage
0 - 3.5	2cp, 5cp, 6cp, 7cp, 8cp, 9cp, 11cp, 12cp, 25cp, 26cp, 27cp	55	0 - 3.5	3gp,12gp	24
0 - 2	21cp	5	.1 - 3.5	7gp, 8gp, 27gp, BPgp	48
0 - 1.5	22cp, 24cp	10	.1 - 2	9gp, 23gp,	24
.1 - 3.5	3cp, 16cp	10	.1-1	24gp	12
.1 - 1.5	14cp	5	.3 - 2	19gp, 26gp	24
.3 - 2	13cp	5	.3-3.5	18gp	12
.3 - .8	19cp	5			
.5 - 3.5	BPcp	5			

Table 2: Effect of 300mM NaCl on mean values for different symbiotic parameters in chickpea inoculated with rhizobia (n=3)

Isolates	Nodule number	Nodule size (mm)	Nodule DW (mg)	Shoot length (cm)	Shoot DW (mg)	Root length (cm)	Root DW (mg)
2cp	6.2	8	65	20.5	11.2	27.2	64.4
3cp	100	100	100	27.0	32.9	19.3	85.1
5cp	100	100	100	18.9	24	6.01	20.2
6cp	100	100	100	28.8	19.3	21.2	33.0
7cp	82.2	35	87.5	23.5	36.0	76.8	94.2
8cp	27.8	38.7	20.4	37.8	21.4	1.7	92.2
9cp	65.2	46.8	82.9	24.5	19.2	97.8	88.4
11cp	100	100	100	18.1	37.9	3.2	81.0
12cp	54.2	21.3	60.5	33.0	13.8	34.1	61.0
13cp	100	100	100	25.0	16.3	67.4	84.4
14cp	100	100	100	18.8	32.5	41.3	73.5
16cp	100	100	100	13.9	49.8	36.7	51.8
19cp	29.4	50	72.6	39.6	30.6	13.8	83.2
21cp	22.8	49.0	94.6	19.7	22.4	9.0	79.3
22cp	16.5	27.5	71.2	40.9	34.0	5.0	87.1
24cp	37.8	32.2	85.1	35.4	30.6	20	35
25cp	28.1	7.4	87.5	22.8	28.8	15.3	1.7
26cp	19.0	31.2	41.6	12.3	30.4	3.3	41.3
27cp	32.3	29.2	62.2	31.0	35.2	3.01	7.8
BPcp	10.7	11.2	25	29.6	17.2	17.8	70.6
T(N)	0	0	0	42.1	11.3	38	3.3

Table 3: Effect of 300mM NaCl on mean values for different symbiotic parameters in green pea inoculated with rhizobia (n=3)

Isolates	Nodule number	Nodule size (mm)	Nodule DW (mg)	Shoot length (cm)	Shoot DW (mg)	Root length (cm)	Root DW (mg)
3gp	57.1	21.5	82.7	36.5	1.8	11.5	36.2
7gp	80	18.1	66.2	12.8	11.4	11.0	52.0
8gp	46.1	13.6	25.7	3.4	38.7	2.9	77.5
9gp	54.8	3.1	57.6	16.3	28.2	27.8	21.5
12gp	100	100	100	10.3	43.6	62.5	6.02
18gp	100	100	100	6.8	15.7	16.1	22.7
19gp	100	100	100	8	55.2	11.4	62.3
23gp	59	30	34.5	6.5	40.2	33.3	64.9
24gp	68.1	21.9	68.8	9.0	52.2	13.5	22.0
26gp	76.4	14.9	69.5	23.1	72.8	21.4	85.9
27gp	60	14.5	36.7	20.1	70.5	14.9	67.6
BPgp	26.6	13.0	25.8	14.9	46.0	2.6	42.3
T(N)	-	-	-	23.6	9.9	1.9	6.2

Staining and Destaining: After electrophoresis the gels were stained with solution 0.1 % (W/V) Commassie Brilliant Blue (CBB) R 250 dissolved in 10% (V/V) acetic acid (Cica), 40% (V/V) methanol and water in the ratio of 10Ó40Ó50 (V/V/V) for about an hour. Gels were destained in destain I (methanol 50 ml; glacial acetic acid 10 ml; volume made to one liter with distilled water) and destain II (methanol 50 ml; glacial acetic acid, 75 ml; volume made to one liter). Gels were shacked (Double shaker mixer DH-10) gently until the background of the gel became clear and polypeptide bands were clearly visible. The excess CBB was removed by addition of piece of tissue paper Kim wipe in the destaining solutisolute+on. After destaining the gels were read and Rf value of each protein was measured and there by molecular weight in kDa was estimated.

RESULTS AND DISCUSSION

Affect of salt stress on nodular protein expression of chickpea seedling inoculated by isolated rhizobia is shown in Figure. 1. Over all 6 proteins (approximately 10, 15, 27, 35, 56 and 65 kDa) are produced under salt stress among studied chickpea rhizobia. Among four chickpea soil isolate studied three proteins (15, 35 and 65 kDa) were over expressed. 15 kDa proteins were expressed

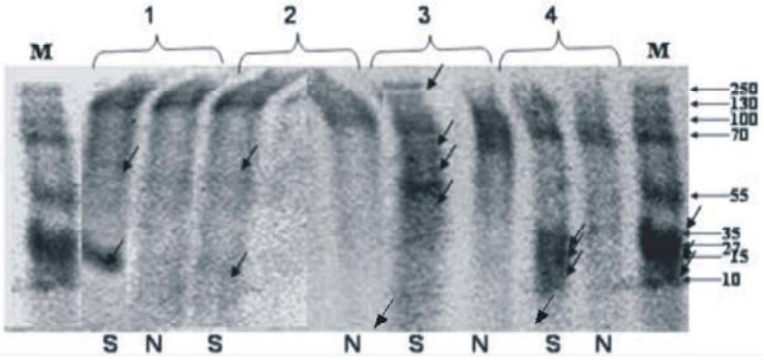

Fig 1: Comparison between normal and salt stressed whole cell protein profiles of chickpea nodule (M=Molecular weight marker, 1=26cp, 2=27cp, 3=21cp,4 =13cp) stained with (CBB)); S=300mM NaCl, N= 0mM NaCl; unique protein bands expressed under salt stress are highlighted by arrows.

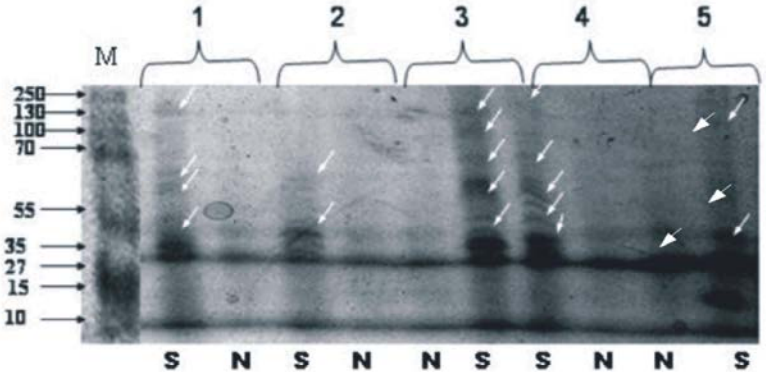

Fig 2: Comparison between normal and salt stressed whole cell protein profiles of green pea nodule (M=Molecular weight marker, 1=27gp, 2=26gp, 3=3gp, 4=23gp, 5=24gp) stained with (CBB); S=300mM NaCl, N= 0mM NaCl; unique protein bands expressed under salt stress are highlighted by arrows.

by 13cp, 26cp and 27cp while 65 kDa proteins in seedling inoculated by 21cp and 26cp. Three unique bands (10, 27 and 56 kDa) were expressed only under salt stress. Minimum number of stress proteins (2) was observed for 26cp and maximum (4) for 21cp. Similar results were reported in by [13] who screened seven strains of Rhizobium loti against acid tolerance and reported protein expression of 49.5 kDa and three soluble proteins of 66.0, 58.0 and 44.0kDa were observed [13].

Affect of salt stress on nodular protein expression of green pea seedling inoculated by isolated rhizobia is presented in Figure. 2. Over all 11 proteins rhizobia (approximately 12, 38, 40, 55, 57, 65, 70, 95, 115, 130 and 150 kDa) are produced under salt stress by green pea isolates. Among five green pea soil isolate studied, two proteins (40, 57 kDa) were over expressed. 40 kDa proteins were expressed by 3gp, 23gp and 26gp while 57 kDa protein in seedling inoculated by 23gp. 8 proteins (12, 38, 65, 70, 95, 115, 130 and 150 kDa) were detected after submitting the isolates to salt stress. Five unique bands (12, 55, 65, 115, 150 kDa) were observed only in stressed nodules. Six bands (38, 40, 57, 70, 95, 130 kDa) were shared by 4 seedlings. Minimum number of stress proteins (2) was observed for 26gp and maximum (7) for 23gp. Present results are consistent with the protein profiles of [14] who detected an over expression of six proteins approximately of 22, 25, 40, 65, 70, 95 kDa. They describe these proteins as salt-induced proteins. Previously describes overexpression over expression in four proteins of about 22, 38,68 and 97 kDa in *Rhizobium* sp. (STI) due to growth under salt stress have also been reported ~~by~~ [15]. A protein of 65 kDa is also reported to be expressed under salt stress in a *Rhizobium* strain [16]. [17] proposed that a A protein of about 40 kDa is involved in salt tolerance in *R. leguminosarium* bv *viciae* [17] . The resulting collection of salt tolerant isolates of chickpea and green pea will certainly provide a valuable resource for further evaluation by 2D gel analysis in order to identify and classify salt stress genes

CONCLUSION

All the isolates of chickpea and green pea that perform better in broth media as well as pot experiment under NaCl stress and expressed salt stress tolerant genes. Therefore, four chickpea (21cp, 22cp, 26cp and 27cp) and five green pea (3gp, 12gp, 24gp, 26gp and 27gp) salt tolerant isolates are most suitable candidate inoculants.

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