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Anti-stress phytohormones impact on proteome profile of green gram (*Vigna radiata*) under salt toxicity

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Authors' Contribution	**Farheen, J.** & **S. Mansoor** developed the concepts the study; **J. Farheen** designed, conducted and statistical analysed the experiment and as well as drafted the research findings; **S. Mansoor** critically reviewed the research findings.
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ABSTRACT

Green gram (*Vigna radiata*) is considered the chief legume in Pakistan. Thus, current study was conducted to examine the ameliorating effect of phytohormones pre-treatments under salt stress on proteome profile of green gram by sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The soluble green gram seedlings proteins were resolved on 4% stacking and 12% resolving gels. The SDS-PAGE resolved 24 polypeptide bands ranging from 200 to 17kDa. Among these, 12 out of 24 bands of proteins were essentials house-keeping or growth proteins of green grams. While, 120, 114.6, 51.8, 29.1, and 22.8 kDa bands were over-expressed under 50 to 350mM salt with phytohormones treatments. The others 104.5 kDa, 99.8 kDa, 95.3 kDa, 91.0 kDa, 55 kDa, 46 kDa, and 17kDa bands were related to the GA3, IAA, and SA induced tolerance. Overall 120 kDa, 114.6 kDa, 104.5 kDa, 99.8, 95.3 kDa, 51.8 kDa, 29.1 kDa and 22.8kDa bands were first time identified in the current study. The information retrieved from NCBI protein database, the resolved peptides were principally belonging to 7S and 8S vicilin, 2S, 8S, 11S, and 16.5S globulins. It is determined that seed priming with SA enhanced tolerance in green gram by rapidly synthesizing stress alleviating peptides.

Keywords: Cluster analysis, dendrogram, mungbean, salt stress, SDS-PAGE

INTRODUCTION: Various world-wide health concerning organization recommended the use of high graded plant protein such as legumes to prevent the risk of metabolic disorder (Hou *et al.*, 2019). Legumes are most important protein crop on the earth. Among the legumes, the green gram is the major pulses. Its seeds are rich in superior quality storage protein, which account 85% of the total protein while, another 15% have not been broadly studied (Yi-Shen *et al.*, 2018). The soluble storage protein comprises of 60% globulins, 25% albumin and 15% prolamins. Globulins are further divided into 3.4% basic-type (7S), 7.6% legumin-type (11S), and 89% vicilin-type (8S) (Mendoza *et al.*, 2001; Itoh *et al.*, 2006). Other than proteins, the green gram seeds also contain starch, fiber, phenolic compound, saponins, vitamins, calcium zinc, potassium, folate, magnesium, manganese and very low in fat that made it meager man's meat (Hou *et al.*, 2019). It is also a good source of green manure and fodder (El-Kafafi et al., 2015). Its root has ability to fix 30 to 50 Kg/ha atmospheric nitrogen in the soil which is essential for maintaining soil fertility (Chadha, 2010). The green gram is the valuable and the major Rabi pulse crop of Pakistan. Its cultivation area in 2016-2017 was about 179,000 hectares with seed yield of 130,000 tones. In comparison during 2017-2018, it was cultivated on 161,800 hectares land with 118,800 tones seed yield (GOP, 2018). One of the reasons of this 9% decrease in both land and productivity is the shortage of irrigated land due to soil salinity. The salinity induce oxidative bust in the mungbean cells, caused by responsive oxygen species (ROS) such as hydrogen peroxide, singlet oxygen, hydroxyl radical and superoxide radical. The ROS create hindrance in various metabolic processes of plant via interacting with macromolecules like proteins (Alharby *et al.*, 2016). However, phytohormones like gibberellic acid (GA3), indole acetic acid (IAA), and salicylic acid (SA) take part in the biosynthesis of salt tolerance proteins under salinity. These salt tolerance proteins acclimate plants under salinity stress. Application of biotechnology plays a significant role in agriculture (Khan *et al.*, 2017). Therefore, production of particular proteins under salt stress is a specific response of cell which can be analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is the simple, valid, and cost-effective biochemical marker (Mushtaq *et al.*, 2018). This marker has been widely used to determine the extent of evolutionary variations in crops (El-Kafafi et al., 2015).

OBJECTIVES: The present study was directed first time with the aim to investigate the toxic effect of sodium chloride (0-350 mM) and stress acclimation by pre-treatment of GA3, IAA, and SA on the proteome profile of NM-92 cultivar of a Pakistani green gram.

MATERIALS AND METHODS: The present study was replicated thrice in the plant laboratory of Department of Genetics, Faculty of Science, and University of Karachi. The seeds of mung bean cultivar NM-92 were acquired from National Agricultural Research Centre (NARC), Islamabad. These freshly collected 15 seeds^{-1} treatment / replication were divided into two sets. The first was named as sodium chloride (SC) stress treatments were imbibed in sterile distilled water (DW) whereas, second set soaked in gibberellic acid (GA3) (BDH Chemicals, England), indole acetic acid (IAA) (Fluka, Switzerland), and salicylic acid (SA) (J.T. Baker, Holland) in the separate beaker for 24 hours under dark condition. After 24 hours, given ample time to both the sets at room temperature. After recovery, all 20 treatments were sown in the 150 X 30 mm sized petri-dishes containing 0, 50, 150, 250, and 350 millimolar (mM) sodium chloride solution (Fisher Scientific, UK) for 72 hours.

Protein extraction: Protein extraction was done by taking 0.3g of seedlings in an ice chilled mortar and crushed by adding

600µL 0.2 M Tris-HCl buffer having pH 7.5 contained 5% SDS ($^{w}/_{v}$) and 5% 2-mercaptoethanol ($^{v}/_{v}$). The homogenate was incubated at 0°C for 30 min., boiled in the water bath for 3 min. at 100°C. Samples were centrifuged in Heraeus Biofuge D-37520, Germany for 30 min. at 8000 rpm. The protein supernatant was saved at below 0°C for quantitative and qualitative determination with minor modifications. The total soluble protein content of the samples was estimated via "Bovine Serum Albumin (BSA) standard curve" and explicit in µg protein milligram^{-1} fresh weight of mung seedlings.

Bovine serum albumin standard curve (2000 µg/mL): Total protein standard curve was made by dissolving 0.05g of Bovine Serum Albumin (BSA) in 25mL of distilled water. Ten serial dilutions were made from 0.1 mL to 1mL by BSA solution then performed Lowry. A standard curve of total proteins was plotted by taking BSA absorbance at Y-axis and 2000 µg BSA / mL at X-axis

Sample preparation for SDS-PAGE: For qualitative assessment of total proteins; the 35µL of saved protein supernatant was combined with 15µL of sample diluting buffer (SDB). The SDB was made up of 0.0625 M Tris-HCl pH 6.8 with 2% of SDS, 10% of glycerol, 0.003% of bromophenol blue dye and 5% of 2-mercaptoethanol. Boil the 50µL protein SDB supernatant at 100°C in water bath for 3 min., centrifuged at 6000 rpm for 4 min. The supernatant was loaded on SDS-PAGE gel with the given formulae.

$$\text{Protein loaded (µL) in the SDS–PAGE} = \frac{10\ \mu L\ (\text{sample used in Lowry})}{\mu g\ \text{protein (from BSA standard curve)}} \times 500\ \mu g/mL$$

The SDS- PAGE: Total proteins were fractionated via SDS-PAGE with 4% stacking and 12% resolving gel. The resolving gel of 12% was made by taking 6mL solution A, 1.8 mL 3 M Tris 1 M HCl buffer pH 8.8, 144µL 10% SDS, 5.74 mL sterile distilled water, 720µL 1.5% ammonium persulphate (APS) in deionized water and 10µL TEMED. While, stacking was composed of 1.25mL of solution A, 2.5mL of 0.5M Tris 1M HCl buffer pH 6.8, 100µL 10% SDS, 1.8 mL of distilled water, 500µL 1.5% APS and 12µL TEMED. Solution A was prepared by conjoining 30% acrylamide and 0.8% *N, N'*-methylene-bisacrylamide in deionized water. To avoid polymerization in the beaker; the prepared solution was quickly poured into the 3 mm thick gel plates after adding TEMED. The stacking was lined over resolving gel, then combs were inserted between the gel plates of SCIE-PLAS TV-100 separation system, UK, and allowed to polymerize for ½ an hour. After polymerization gel was placed in the tank which were filled with Tris-Glycine buffer (electrode buffer) pH 8.4 then combs were removed. The electrode buffer contained 0.3% Tris, 1.41% Glycine and 0.1% SDS in 2000mL d/w. The gel was pre-run for 15 min. at 60 volts and 120 mA currents. The prepared SDS-PAGE samples were loaded in wells with BlueStep™ Broad Range Protein Marker, AMRESCO, USA as standard and run at 60 volts & 120 mA for about 45 min. When samples entered in resolving gel, and then gave 100 volts and 200 mA currents for around 2.5 hours. Furthermore, electrophoresis was carried out at a constant watt.

The Gel was washed with 30% ethanol on Uni Thermo Shaker NTS-1300 EYELA, Japan at the constant shaking for 30 min. Then gels were placed in 10% glacial acetic acid in 50% methanol solution (Fixative) for 24 hours. SDS Gel was stained until protein bands were visible thereat placed as 5% of Methanol in 7.5% acetic acid glacial solution to destain the bands background. SDS-PAGE stain composed of 0.125% coomassie brilliant blue R-250 dissolved in 40% of Methanol and 7% acetic acid glacial solution. The stain was stirred on Magnetic stirrer & hot plate M6/1, Germany for 6-10 hours before used. Photographs were taken by Sanyo digital camera VPC-T1284BL and bands were scored through numbering pattern. Gels preserved in 10% acetic acid solution at 4°C.

Interpretation of bands and data analysis: The total soluble protein bands relative mobility calculated by below formulae and Dendrogram was constructed via SPSS v. 20

$$Y = (\text{Slop} \times RF) + \text{Intercept}$$

Where,

$$RF = \frac{\text{Migrated distance of protein band}}{\text{Migrated distance of dye front}}$$

$$\text{Slop} = \frac{\text{Log MW of protein marker lower limit band} - \text{log MW of protein marker upper limit band}}{RF\ \text{protein marker lower limit band} - RF\ \text{of protein marker upper limit band}}$$

Molecular weight of unknown protein = 10^Y

RESULTS: The total soluble proteins extracted from green gram were perceived by SDS-PAGE Blue Step™ broad range biochemical markers. The protein-based marker was used to evaluate the toxic effect of sodium chloride along with pre-treatments of GA3, IAA, and SA on proteome assay. In the current work, seedlings total soluble proteome resolved 24 polypeptide bands ranging from 200 to 17.1 kDa were recognized by using SDS-PAGE. The figure 1 showed Dendrogram assay, which classified the 20 treatments of SC, GA3, IAA and SA into two major clusters where, the cluster I was the largest one (figure 1).

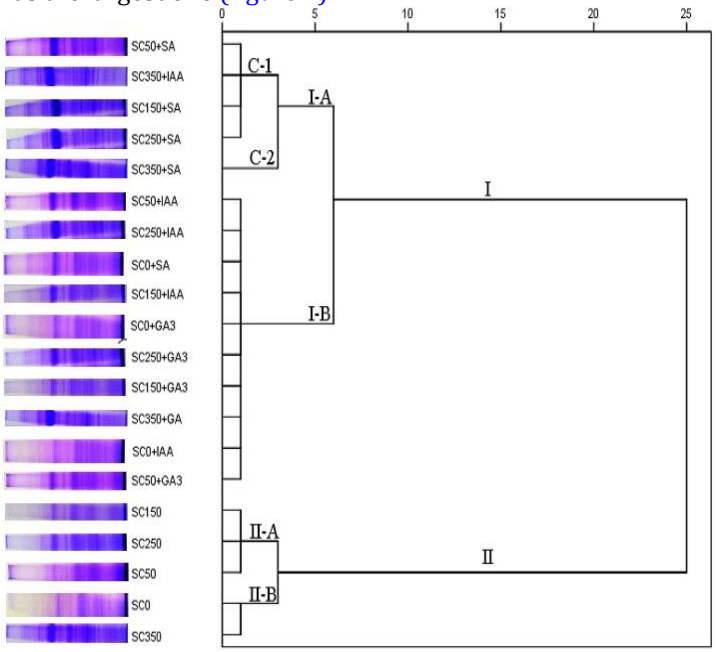

Figure 1: The Dendrogram of HS, GA3, IAA and SA with and without salt stress grouped into two major clusters based on mungbean total soluble proteins similarity index which was constructed after SDS-PAGE analysis of five mungbean varieties.

Cluster I consisted of 15 treatments that further divided into I-A, and I-B. The pre-treatments of SC50+SA, SC150+SA, SC250+SA, and SC350+IAA were grouped together into C-1 of sub-cluster I-A. The C-2 of sub-cluster I-A, pre-treatment SC350+SA was most diverse among 20 treatments. The C-1 treatments showed 99% homology when compared with each other while, it was 97% similar with C-2. The sub-cluster I-B comprised another 10 treatments, SC0+GA3, SC50+GA3, SC150+GA3, SC250+GA3, SC350+GA3, SC0+IAA, SC50+IAA,

SC150+IAA, SC250+IAA, and SC0+SA that were also 99% similar for total proteins. Sub-cluster I-B pre-treatments was exhibiting 94% homology with the sub-cluster I-A. The second cluster was the smallest one that was divided into two sub-clusters, II-A and II-B. The II-A was comprised of SC50, SC150, and SC250 while, sub-cluster II-B consisted of SC0 and SC350. Within each sub-cluster, pre-treatments expressed 99% homology whereas, II-A was 97 different from II-B. Furthermore, cluster I showed 75% similarities with cluster II (figure 1). The seedlings storage proteome profile of green gram was shown in table 1.

No.	Rf	kDa	SC0 mM				SC50 mM				SC150 mM				SC250 mM				SC350 Mm			
			SC	G	I	S	SC	G	I	S	SC	G	I	S	SC	G	I	S	SC	G	I	S
01	0.02	200.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
02	0.12	120.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
03	0.14	114.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
04	0.16	109.4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
05	0.18	104.5	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1
06	0.20	099.8	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1
07	0.22	095.3	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1
08	0.24	091.0	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1
09	0.28	077.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
10	0.31	068.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
11	0.37	055.0	1	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1
12	0.39	051.8	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
13	0.41	049.0	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0.43	046.0	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1
15	0.49	038.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
16	0.53	033.0	1	1	1	1	0	1	1	1	0	1	1	1	0	0	0	1	0	0	0	1
17	0.56	029.1	0	0	0	0	0	1	1	1	0	1	1	1	0	1	1	1	0	0	1	1
18	0.59	026.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
19	0.65	024.0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	0	0	1	1
20	0.67	022.8	0	0	0	0	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1	1
21	0.69	022.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
22	0.73	021.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
23	0.77	019.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
24	0.80	017.0	0	0	1	1	0	0	1	1	0	0	0	1	0	0	0	1	0	0	0	1

Table 1: Proteome assay of mungbean at 0, 50, 150, 250, and 350 mM sodium chloride (SC) along with gibberellic acid (G), indole acetic acid (I), and salicylic acid (S) application via 12 % SDS-PAGE gel. The 1 showed the presence of protein bands and 0 showed the absence of band.

The results showed that 120kDa, 114.6 kDa, 51.8 kDa, 29.1 kDa and 22.8 kDa proteins bands were not induced at 0 mM SC, GA3, IAA, and SA. The table 1 depicted the presence of 120 kDa and 114.6 kDa bands only at 350 mM SC level with all phytohormones treatments. Similarly, 51.8 kDa protein bands were appearing at 150SC, 250SC and 350SC stress with phytohormones. Based on the information collected from the NCBI protein database, this peptide was related to the 8S globulin alpha subunits. The two other, 7S globulins sub-units having 29.1kDa and 22.8 kDa molecular weights bands were synthesized under 50mM, 150mM, 250mM, 350mM SC stress with phytohormones. Concerning protein polypeptide of molecular weight 104.5 kDa, 99.8 kDa, 91.0 kDa, 55.0 kDa, and 46.0 kDa, those were induced by GA3, IAA and SA at 0 to 350 mM SC. While, 17kDa protein band was appearing in SA, and IAA treated samples and 95.3kDa band was only present in SA treatment. Other 12 protein bands were present in all treatments proved as house-keeping proteins of green gram (table 1).

DISCUSSION: The SDS-PAGE profiling for proteome is the reliable and applied biochemical approach that has been used as biochemical marker in various crop differentiation, and characterization. In the current study, first time SDS-PAGE was utilized to investigate the impact of GA3, IAA, and SA pre-soaking on green gram under salt toxicity. The salt toxicity adversely affects all seed, seedling, and plant metabolic process (Parveen *et al.*, 2016). At salt toxicity, the endogenous GA3, IAA, and SA levels markedly decrease (El-Khallal *et al.*, 2009). In such condition, exogenous application of GA3, IAA, and SA enhance seedlings survival rate by increasing synthesis of seed storage proteins. Likewise, our Dendrogram characterization based on 20 treatments showed significant diversity under 0 to 350 mM SC stress. The salicylic acid treatments were grouped together except SC0+SA treatment, exhibiting a close relationship, which proved its acclimating role under salt stress. These findings will help plant breeder toward enhancing food quality and quantity of green gram in future breeding programme on saline sodic land.

The SDS-PAGE assay revealed 200. kDa, 109.4 kDa, 77 kDa, 68 kDa, 49 kDa, 38 kDa, 33 kDa, 26 kDa, 24 kDa, 22 kDa, 21 kDa and 19 kDa fractions as essential green gram proteins. Among these, 68 kDa, 49 kDa, 33 kDa, 26 kDa, 24 kDa and 21 kDa peptides were seed biotinylated isoform protein (Riascos *et al.*, 2009), putative NADH-ubiquinone oxidoreductase subunit H (Gostinčar *et al.*, 2019), heat shock protein 33 (Hamidian *et al.*, 2015), globulin protein, seed coat / maturation protein (Dhaubhadel *et al.*, 2005), and protein for dimerization. While, 22 kDa proteins belonged to the class of prolamin alpha zein

Z1C1_2, Z1C1_4, and Z1C1_8 precursors, and 19kDa peptide was related with Z1A1_2, Z1A2_2, and Z1B_6 precursors (Miclaus *et al.*, 2011). Further, the 91 kDa peptide is sucrose synthase SS1 protein, and 77kDa protein is the NADPH-cytochrome P450 reductase (Wang *et al.*, 2004). Also, the phosphatase-associated two other proteins having 46 and 55 kDa molecular weight were reported earlier in *Mucuna pruriens.* Hameed *et al.* (2012) and Malviya *et al.* (2008) found 55 and 46kDa peptides as 7S vicilin small sub-units and 17kDa as 11S globulins sub-unit in the studied *Vigna radiata.* Some other molecular weight proteome such as 68 kDa and 49kDa are 7S vicilin, 33kDa is 8S vicilin, 38 and 26kDa 8S globulins, 24kDa 11S globulins, and 22kDa 16.5S globulins. These proteins required for germination and seed establishment of green gram plant (Hameed *et al.*, 2012).

The vast accumulation of 23kDa and 22kDa peptides under salt stress by salicylic acid, were reported previously in the mangrove *Bruguiera parviffora* and *Zea mays* (El-Khallal *et al.*, 2009). Correspondingly, El-Kafafi *et al.* (2015) reported the presence of 115kDa, 23kDa, and 22kDa bands in the salt tolerant lines of green gram. These proteomes induced under salt stress may play a pivotal part in the stress acclimation and osmotic adjustment. Similarly, the induction of 104 kDa and 100kDa MW polypeptide by SC stress in the salt tolerant genotypes of green gram indicated the functional role of phytohormones in various metabolic and defense response El-Kafafi *et al.* (2015); Alharby *et al.* (2016), El-Khallal *et al.* (2009), Qados (2010). Ali *et al.* (2007), Alharby *et al.* (2016), and El-Kafafi *et al.* (2015) observed 17kDa, 26kDa, 33kDa and 77kDa bands involving in salt tolerance and can be considered as a positive biochemical marker for salt stress. Further, 26 kDa MW peptide also functions as osmotin under the salt stress that involved in enhancing the accumulation of glycine betaine and proline in the cells. Hence, proteome assay of green gram showed that GA3, IAA, and SA could regulate the expression of salt stress proteins that are anticipated to play a crucial part in the salt tolerance mechanism. Likewise, the involvement of phytohormones in the induction of changes in the proteome profile pattern was attributed to their part in managing cell division by regulating some genes of apical meristems.

CONCLUSION: Finally, the results revealed the presence of the ten new bands with MW of 200kDa, 120 kDa, 114.6 kDa, 109.4kDa, 104.5kDa, 99.8kDa, 95.3kDa, 51.8kDa, 29.1kDa and 22.8kDa have not reported previously under salt stress with phytohormones treatments in green gram. Furthermore, it was observed that phytohormones alleviate the negative impact of salt stress on green gram by enhancing synthesis of salt defense polypeptides. Hence, higher accumulation of proteins was observed in salicylic acid treated seedlings. Thus, present work recommended the pre-soaking of phytohormones to overcome the toxic impact of sodium chloride on green gram. Further research is needed on a biomolecular level to reveal the mechanism of signalling pathways under sever salt stress.

CONFLICT OF INTEREST: Both authors have declared that no disagreement of interest regarding this research.

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