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ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF LOW MOLECULAR WEIGHT PEPTIDES FROM NONPRIMED AND HALOPRIMED SEEDLINGS of *Vigna mungo* L. AND *Cajanus cajan* L. AND THEIR IMPACT ON PHYSIOLOGICAL ASPECTS UNDER NaCl EXPOSURE

Sabarni Biswas¹, Amitava Ghosh² Alivia Paul¹, Asok K. Biswas^{1*}

¹Plant Physiology and Biochemistry Laboratory, Centre for Advanced Studies, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata-700019.

²Department of Botany, Ashutosh College, 92, Shyama Prasad Mukherjee Road, Kolkata-700026.

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ABSTRACT

Plants confront an array of environmental stresses by tightly regulating their signalling pathways involving low molecular weight peptide synthesis. In the conducted study, initially growth parameters and ion contents were measured from salt stressed and haloprimed seedlings of blackgram (*Vigna mungo* L.) and pigeonpea (*Cajanus cajan* L.). Seedlings raised from nonprimed and haloprimed seeds were grown in hydroponic solution supplemented with desired concentration of NaCl for 3 weeks under controlled physiological conditions. Attempts were made to isolate low molecular weight peptide(s) from non-stressed, NaCl stressed and haloprimed seedlings of blackgram and pigeonpea, to quantify their variations and detect their molecular weight using HPLC and MALDI-TOF analysis respectively. Free radical scavenging activities of these peptides were studied under NaCl exposure. Subsequent bioassays were performed to determine the effect of these peptides on their respective physiological parameters. Peptide abundance was maximum in control seedlings of both the cultivars, which under NaCl stress became scanty. CD spectroscopic analysis confirmed reduced secondary conformations and more unordered peptides under NaCl stress. Haloprimed seedlings recovered such adversities to variable extents, resulting in improved germination and growth of test seedlings.

* Corresponding author

E-mail: asokkbiswas16@gmail.com (Asok K. Biswas)

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1 Introduction

Salt stress is an increasing environmental challenge that limits plant productivity in arid and semi-arid regions of the world (Hussain et al., 2009; Etesami & Beattie, 2018). To confront under salinity, plants alter their physiological and biochemical mechanisms to withstand resulting stress (Parida & Das, 2005). Biologically active low molecular weight (LMW) peptides play regulatory role in determining several physio-biochemical processes in plants under abiotically stressed conditions. Some of them help in amplification of signals (Lindsey et al., 2002), some act as regulators of signalling events and cell-to-cell communication in plants (Murphy et al., 2012; Albert, 2013; Czyzewicz et al., 2013; Matsubayashi, 2014; Oh et al., 2018), whereas some play essential role in nitrogen fixation (Mylona et al., 1995; Muñoz-García & Ares, 2016). Small peptides play critical roles in cell proliferation, differentiation and mediate biotic and abiotic stress stimulation with metabolic intermediates (Mandal et al., 2008). Small signalling cysteine rich peptides have also been reported to play essential roles in stomatal patterning and density, symbiosis and a wide range of reproductive events viz., pollen tube formation, guidance and burst, activation of gametes and seed development (Hara et al., 2007; Sugano et al., 2010; Maróti et al., 2015; Bircheneder & Dresselhaus, 2016). Few of these have been isolated and characterized in details. Systemin is probably the first sequenced plant peptide hormone (18 amino acid in length) which was isolated from Solanaceae species. Till now, peptides have been isolated and characterized in plants like bitter melon (Leung et al., 1987), broad bean (Zhang & Lewis 1997), wheat (Ghosh et al., 2010) and mulberry (Jha et al., 2014). However, it has not been isolated from pulses like blackgram (*V. mungo*) and pigeon pea (*C. cajan*) exposed to high NaCl concentrations, to the best of our knowledge. Variations in levels of oxidative stress markers, activities of enzymatic and non-enzymatic antioxidants in *V. mungo* and *C. cajan* has been studied under salinity (Chatterjee et al., 2018) but, the variations in levels of the isolated LMW peptides from the same stands undone, so far as halopriming is involved. These pulses have been selected to work with since they are essential pulses often consumed as the cheapest source of protein. The prime purpose of the study lies not only in isolation and partial characterization of LMW peptides (having molecular weight between 0.3-5.0 kDa) induced by NaCl stress but also detection of antioxidative properties and conformational changes of these peptides in the chosen materials. The study also detects the efficacy of these LMW peptides isolated from haloprimered seedlings that favoured stress alleviation imparting better seedling performance. The worth of the study lies in the fact that since pulse production is extensively hampered in the saline regions, adopting cost effective, farmer friendly and environment friendly halopriming technique may help in improving pulse vigour and stand due to endogenous production

of LMW peptides in abundance that could improve legume germination and growth of test cultivars under saline conditions.

2 Materials and methods:

Fresh and viable seeds of *V. mungo* cv. Sulata and *C. cajan* cv. rabi were sourced from Pulse and Oilseed Research Institute, Behrampore, West Bengal. Seeds surface sterilized with 5% sodium hypochlorite solution for 15 minutes were divided into two batches. First batch was directly plated onto sterilized glass plates containing moistened blotting papers followed by their insertion into transparent plastic packets containing hydroponic solution (Widodo et al., 2009) which constituted the control set. For seeds to grow under salt stress, hydroponic solution (pH 6.8) was supplemented with 150mM NaCl. This constituted the nonprimed sets. The second batch of seeds were allowed to soak in sublethal dose of NaCl (50mM NaCl) for 2 hours for the purpose of halopriming, prior to their germination in hydroponic solution containing 150mM NaCl. The set ups were exposed to 16 hours of photoperiod at 27-30 °C and 200 $\mu\text{molm}^{-2}\text{s}^{-1}$ photon irradiance (Biswas et al., 2018). Previous report (Chatterjee et al., 2018) revealed that at 150mM NaCl, significant effect of salt toxicity was recorded in the test cultivars. Therefore, this dose was chosen for conducting the present study. After 21days of growth, seedlings of each cultivar were harvested for peptide isolation. Extractions were carried out in cold room at 4°C.

2.1 Determination of morphological parameters

After 21 days of growth, 10 seedlings were randomly harvested from control sets, salt stressed sets (150mM NaCl) and haloprimered sets (50mM NaCl primed followed by their exposure to 150mM NaCl) of *V. mungo* and *C. cajan* seedlings for determination of salt induced damage in root and shoot length (Saha et al., 2010).

2.2 Estimation of Na⁺, K⁺ and Cl⁻ contents

1g of dried root and shoot samples from each set were utilized for Na⁺ and K⁺ ion estimation (Saha et al., 2012). Samples were placed inside muffle furnace at 450 °C for 24 hours in weighed porcelain crucibles to generate ash. The collected ashes were dissolved in 10 ml of 2 (N) HCl. The ash-acid extracts were filtered through Whatman filter paper (No. 42) into a 50ml volumetric flask. The volume was then made upto 50ml with 2 (N) HCl. This filtrate was used for Na⁺ and K⁺ ion estimation using a Systronics Flame photometer Model No. 130.

Chloride (Cl⁻) contents were estimated from fresh shoot and root samples by titration (Sheen & Kahler 1938). 1g root and shoot samples from each treatment was used for Cl⁻ estimation. Samples were crushed in 10ml of Milli-Q water to which a pinch of

charcoal was added and left undisturbed for 10 mins. Plant extracts were made charcoal free by repeated filtration and centrifugation. Cl^- contents were estimated from filtrates by silver nitrate titration method.

Data obtained for the two cations (Na^+ , K^+) were expressed in mg g^{-1} dry weight and Cl^- contents were expressed in mg g^{-1} fresh weight.

2.3 Extraction and isolation of LMW peptides

For isolation and purification of LMW peptides, freshly harvested samples were pulverized in mortar and pestle using liquid nitrogen followed by its extraction in pre-chilled autoclaved distilled water. Extracts were centrifuged at 10,000 rpm with protease inhibitor PMSF for half an hour to remove interfering materials. Collected supernatant was again centrifuged to purify it. Repeated ether washes of the extracts helped to remove fats, lipids and oils (Jha et al., 2016). The extracts were thereafter preserved at -20°C for future experiments.

2.4 Ion exchange Chromatography

The peptide extracts were allowed to pass through cation and anion exchange resin columns (Dowex 50 and Dowex 1, Sigma Chemical Co., USA) (Azzoni et al., 2005). 3N ammonia and 1N HCl was used for cation and anion exchange column respectively during peptide elution procedure. Extracted solution was made ammonia and HCl free with a Lyophilizer (Lyolab BII). Thus, the extracts were freeze dried to smaller volumes.

2.5 Ultrafiltration

Lyophilized peptide extracts obtained from control seedlings, NaCl treated seedlings and haloprime seedlings were ultrafiltered through millipore stirred cell fitted with 5 kDa (YM 5) and 0.3 (YC 03) ultrafilter membrane (Amicon made). Thus, the filtrate collected had peptides having molecular weight between 0.3-5.0kDa (Zehadi et al., 2015). During ultrafiltration, maximum pressure of 1.5kg/cm^2 was maintained. The process was carried out thrice and the ultrafiltered samples were lyophilized and dissolved in 10ml distilled water (for each set) and cryopreserved.

2.6 Purification and analysis of peptides through HPLC analysis

High performance liquid chromatography (HPLC), WatersTM was performed with the extracted peptides. C18 column was used in 50% methanol (running solvent) fitted with 515 HPLC pump. A run time of 20 minutes and absorbance at 250nm was recorded. Column length was of 3.9mm X 150mm, injection volume 20 μl , flow rate of 0.5-1.0ml/min (Jha et al., 2014) with pump pressure

4000psi was maintained. Peptide(s) obtained at retention time around 5min was purified, concentrated and kept at -20°C . The obtained peak was isolated and re-injected into column to check its repetitive occurrence. Thus, these peaks were concentrated and collected to study their effect on various physiological aspects.

2.7 MALDI-TOF analysis

Extracts purified through HPLC analysis were subjected to MALDI-TOF mass spectra measurements carried out on Ultraflex extreme MALDI-TOF/TOF spectrometer, Bruker Corporation, Massachusetts, US, equipped with nitrogen laser wavelength. Dihydroxybenzoic acid (DHBA) was used as matrix. 30 mg mL^{-1} DHBA prepared in water: acetonitrile (1:1). Individual peptide extracts were mixed thoroughly in 1 μl of DHBA matrix solution and applied on sample plate in volume of 1 μl . Mixtures on sample plate were dried in an air stream followed by plate insertion into instrument for acquiring their respective spectra (Castañeda-Ovando et al., 2012).

2.8 Circular Dichroism Spectroscopy

Conformational changes in secondary structure of the isolated peptides was determined using CD spectropolarimeter (Model J-715, Jasco, Tokyo, Japan) at 25°C equipped with a temperature control unit using a 0.1cm path length quartz cell (Senthilkumar et al., 2008). Spectral scans were undertaken within wavelength range of 280-195nm. The percentages of secondary structures were deconvoluted using BESTSEL program.

2.9 Bioassays

Isolated LMW peptides from 21 days old *V. mungo* and *C. cajan* seedlings were used to test the following physiological traits:

2.9.1 Germination test

Surface sterilized seeds each of *V. mungo* and *C. cajan* were plated in petridishes to test their germination ability under salt exposure in presence of the peptides isolated respectively from their control seedlings, 150mM NaCl treated seedlings and 150mM NaCl treated haloprime seedlings. Three replicates were made for each set of seeds. The set ups were kept in plant growth chamber at 21°C , 68% humidity for one week under controlled condition of 16 hours of photoperiod followed by 8 hours of darkness.

2.9.2 Stomatal bioassays

Peels were made from abaxial surface of *Colocasia esculentus* L. leaves at 11am. Initially peels were placed in water for two hours to prevent shock. Thereafter, peels were soaked in peptides isolated from control sets, NaCl treated sets and haloprime sets

of *V. mungo* and *C. cajan* for 60mins and mounted on glass slides to study the effect of LMW peptides on opening and closure of stomatal aperture.

2.9.3 DPPH radical scavenging assay

Free radical scavenging activities of the isolated peptides was assayed (Chrysargyris et al., 2016). Reaction mixture contained 1ml of 0.3mM DPPH (1,1-diphenyl-2-picrylhydrazyl) and 0.02ml of aqueous peptide extracts. The reaction mixtures were vortexed and incubated in dark for 30minutes at 25°C. Absorbance was measured at 517nm. Reaction mixture without test sample served as blank. The free radical scavenging activity was expressed as percentage of inhibition and calculated by the following equation:

$$\% \text{ inhibition of DPPH activity} = [(A_s - A_b) / A_c] \times 100\%$$

where, A_b is the absorbance of blank, A_s is the absorbance of tested samples and A_c is the absorbance of control samples.

2.9.4 NO scavenging activity

Nitric oxide (NO) scavenging assay was performed for the isolated LMW peptides (Jha et al., 2016). Nitric oxide generated from sodium nitroprusside was measured from Greiss reaction (Maccocci et al., 1994). 300µl samples were mixed with 216 µl Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% NEDH [N-(1-naphthyl) ethylenediamine hydrochloride] and 300 µl 5mM sodium nitroprusside prepared in PBS (Phosphate buffer saline) solution. The reaction mixture was incubated for 60 minutes at 25 °C in a water bath followed by the addition of 2ml water. The absorbance of the samples was taken at 546nm. Radical scavenging activity was calculated as percentage inhibition of NO radical by the following formula:

$$\% \text{ inhibition of NO radical} = [(A_s - A_b) / A_c] \times 100\%$$

where, A_b is the absorbance of blank, A_s is the absorbance of tested samples and A_c is the absorbance of control samples.

2.10 Statistical analysis

Experiments were carried out in completely randomized design (CRD) having three replicates. Data were mean values of three experimental sets and significant values were detected using one way ANOVA (Analysis of Variance) followed by Dunnett's Multiple Comparison Test which represented values that were significant at $p < 0.05$.

3 Results

3.1 Effect of NaCl on growth

NaCl exposure resulted in retarded growth of *V. mungo* and *C. cajan* seedlings. Root and shoot lengths were decreased by 22% and 21% in *V. mungo* and by 46% and 40% in *C. cajan* seedlings respectively over their control plants. Halopriming of seeds prior to germination narrowed down the rate of inhibition by 18% and 36% in root of *V. mungo* and *C. cajan* respectively whereas in shoot the inhibition was reduced to 17% in *V. mungo* seedlings and by 34% in *C. cajan*, on an average, over nonprimed control (Figure 1).

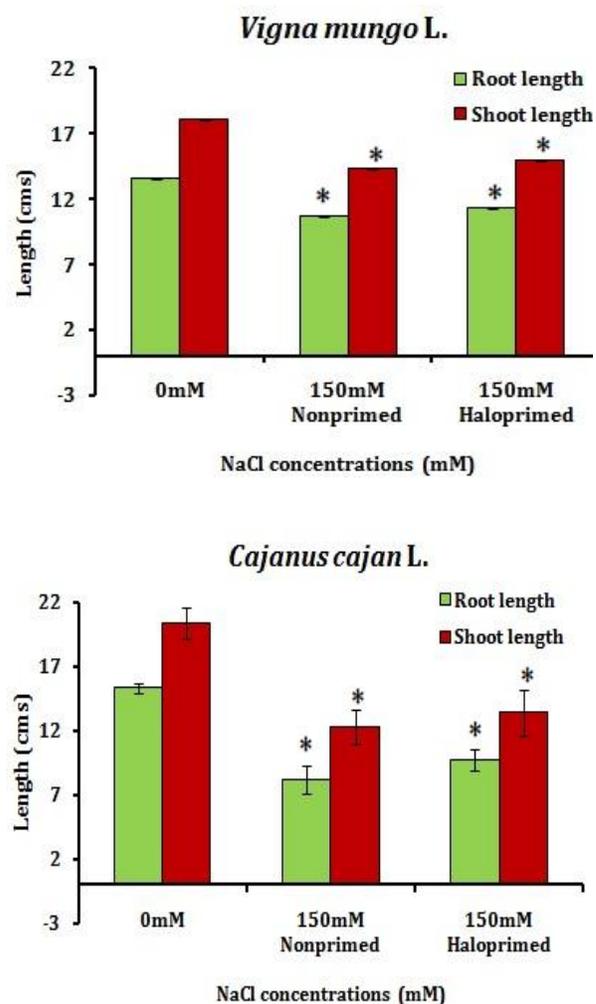


Figure 1: Effect of NaCl on root and shoot growth of 21 days old nonprimed and haloprimed seedlings of *Vigna mungo* and *Cajanus cajan*. Values are mean \pm SE with three replicates. * indicates statistically significant at $p \leq 0.05$ compared to nonprimed control.

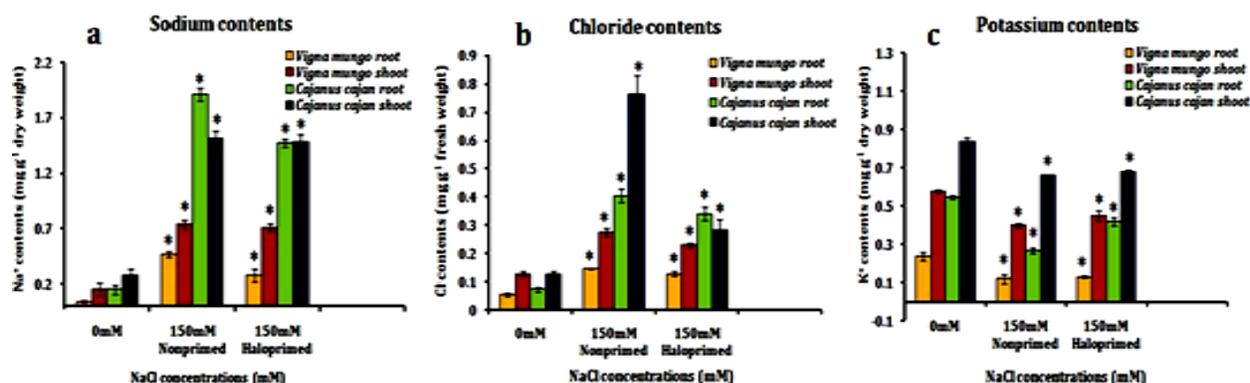


Figure 2 Effect of NaCl on a. Na⁺ contents, b. Cl⁻ contents and c. K⁺ contents of root and shoot of 21 days old nonprimed and haloprimered seedlings of *Vigna mungo* and *Cajanus cajan*. Values are mean \pm SE with three replicates. * indicates statistically significant at $p \leq 0.05$ compared to nonprimed control.

3.2 Effect of NaCl on Na⁺, K⁺ and Cl⁻ contents

Na⁺ and Cl⁻ contents increased while K⁺ contents decreased in root and shoot of both the legume cultivars on NaCl application (**Figure 2a, b, c**). The accumulation of Na⁺ and Cl⁻ was more in *C. cajan* than *V. mungo*. On an average, the uptake of Na⁺ in non-primed *V. mungo* and *C. cajan* increased on an average by 11 and 13-fold respectively in root whereas in shoot, the said increment was by 5-fold in *V. mungo* and by 8-fold in *C. cajan* over nonprimed control. Similar trend also was observed for chloride uptake. The chloride uptake, on an average, increased by 168% and 450% in root of *V. mungo* and *C. cajan* seedlings respectively whereas in shoot, the uptake increased by 114% and 493% in *V. mungo* and *C. cajan* respectively over non-primed control. Potassium content was found to decrease in root by about 50% in *V. mungo* and in *C. cajan* by 51% in nonprimed seedlings over control. In shoot of non-primed seedlings, the said content decreased by 31% in *V. mungo* whereas the contents were scaled to 21% in *C. cajan*.

Haloprimering of both the cultivars with 50mM NaCl, before their germination in different concentrations of NaCl, altered concentrations of ions in the test cultivars variably. Haloprimered seedlings showed lower uptake of Na⁺ and Cl⁻ ions along with increased accumulation of K⁺ ions. Sodium uptake in haloprimered *V. mungo* seedlings was found to decrease 7 fold in root and by 4 fold in shoot, on an average, whereas chloride uptake was lowered down on an average to 133% in root and 79% in shoot over nonprimed control. The decrease in potassium contents was lowered to 46% in root and the decrement was further diminished to 22% in shoot of haloprimered *V. mungo* seedlings, over non-primed control.

In haloprimered *C. cajan* seedlings, Na⁺ uptake was lowered down, on an average, by 10-fold in root and by 5-fold in shoot over

nonprimed control. Cl⁻ also accumulated in the test seedlings by 363% in root and 121% in shoot on an average over non-primed control. The inhibition in potassium uptake in NaCl primed seedlings of *V. mungo* diminished to 24% in root and 19% in shoot over nonprimed control attributing better seedling performance.

3.3 HPLC chromatogram

The HPLC chromatogram of the peptide(s) isolated from 21 days old *V. mungo* and *C. cajan* seedlings unambiguously discriminated the variation in presence of different content of LMW peptides (0.3 - 5kDa) indicated by varying peak area and peak heights.

From the HPLC chromatograms of the peptides obtained from control seedlings, 150mM salt treated seedlings and haloprimered seedlings, it is evident that in control seedlings, there was maximum expression of peptide at retention time (RT) 5.14 mins in *V. mungo* which indicates its abundance during seedling growth especially when LMW peptide is concerned. This expression was followed by several other peptides upto RT= 8.5 mins. But, in NaCl treated seedlings the authors speculated that though the peak at RT=5.18 retained, but its abundance was found to decrease by 82% over peptides isolated from control sets. Only two low abundance peaks till before 8.0 minutes were observed in NaCl treated seedlings. This indicated that under salt stress several peptides that usually take part in the growth process of the plant were degraded. In the haloprimered seedlings, the peaks that were found to get degraded in salt treated sets revived at RT's 7.2 and 8.5 respectively with the highest peak obtained at RT=5.222. The peak at RT=5.222 in haloprimered seedlings, provided an area which was about 4% greater than the area of the same peak obtained from 150mM salt treated seedlings and the inhibition in peptide content was reduced to 80% over control peptides (**Figure 3a**).

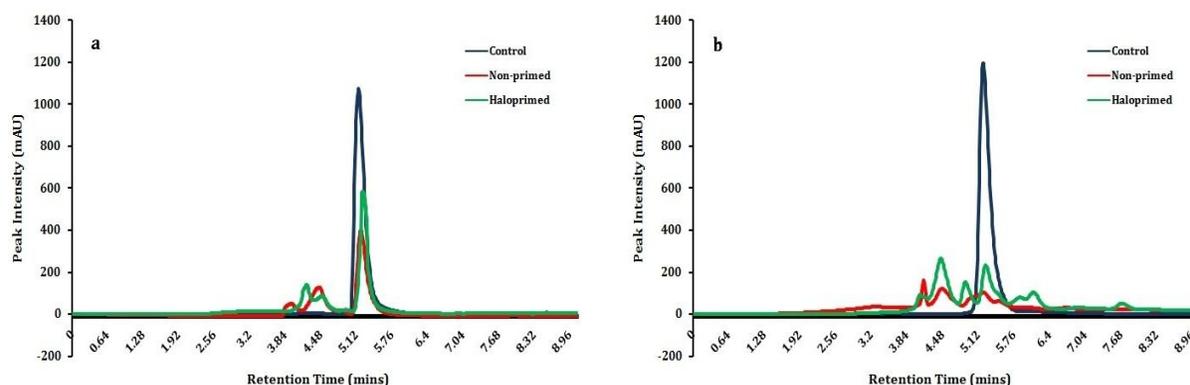


Figure 3 HPLC chromatogram indicating variation in the intensity and content of LMW peptides from control, non-primed salt stressed (150mM) and haloprimered salt stressed (150mM) sets in **a.** *Vigna mungo* **b.** *Cajanus cajan*.

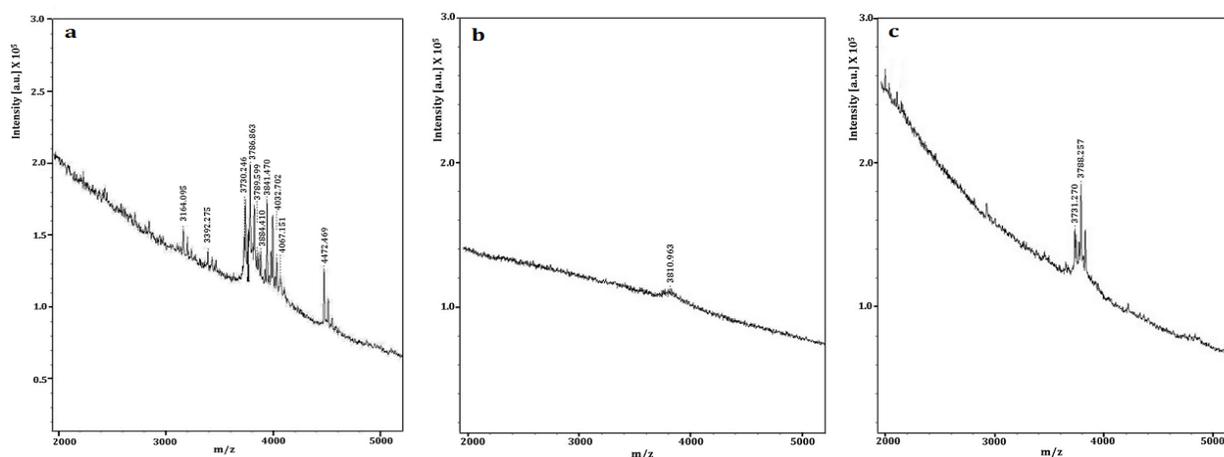


Figure 4 MALDI-TOF analysis of peptides isolated from 21 days old **a.** control seedlings **b.** nonprimed seedlings and **c.** haloprimered seedlings of *Vigna mungo*.

HPLC chromatograms of peptides isolated from control seedlings of *C. cajan* depicted maximal expression and abundance of peptides at RT=5.196. In 150mM NaCl treated seedlings, the abundance of the same peak was found to get reduced by 84% over peptides of control seedlings. This lowering of peptide abundance under NaCl stress probably resulted in the generation of other disintegrated peptide peaks till before RT=8.0. However, peptides isolated from haloprimered seedlings of *C. cajan* exhibited peak area which was 52% more than that of peak area of peptides isolated from 150mM NaCl treated seedlings and also the deterioration in peptide content was reduced to about 78% over control peptides (**Figure 3b**).

3.4 MALDI-TOF analysis

By MALDI-TOF analysis, the spectrum obtained from peptides isolated from 21days old non-treated or control seedlings of *V. mungo* depicted that several LMW peptides were variably responsible for germination and seedling growth under non-

stressed conditions. Noteworthy signals were recorded at m/z values of 3164.095, 3392.275, 3730.246, 3786.693, 3849.599, 3884.410, 3941.740, 4032.702, 4067.151 and 4472.469 representing molecular weight of peptides in Dalton (Da). The peptide having molecular weight of 3786.693Da obtained from control seedlings were prominently expressed (**Figure 4a**).

For peptides obtained from 150mM NaCl treated seedlings, only one signal was found at m/z value 3810.963Da which represented the molecular weight of the only peptide that could express itself and that too in very low concentration probably due to induced NaCl exposure. The molecular weight of this peptide is very close to the abundant peptide expressed in control seedlings (**Figure 4b**).

However, spectrum obtained for peptides isolated from haloprimered seedlings of *V. mungo* showed that the abundance of the peptide obtained at m/z 3788.257 (indicative of the molecular weight of the peptide in Dalton) was expressed in abundance

conferring to better growth of the seedlings under NaCl stress. The molecular weight obtained for the peptide from haloprimered seedlings was also very close to the peptide obtained from control seedlings. Another signal was also obtained at m/z 3731.270 indicating presence of a peptide that was also present in control seedlings having molecular weight of 3730.246Da (**Figure 4c**).

Similarly, spectrum obtained for peptides isolated from non-treated seedlings of *C. cajan* exhibited a single m/z signal revealing presence of peptide having molecular weight of 3702.078Da (**Figure 5a**). The abundance was also satisfactory indicating abundance of peptide during seedling germination and growth. The obtained results could be corroborated with the HPLC generated peaks of same peptide. In NaCl treated seedlings, the abundance of peptide was appreciably reduced as obtained from the m/z signal spectrum indicating molecular weight of the peptide to be 3499.527Da (**Figure 5b**). Peptides isolated from haloprimered seedlings of *C. cajan* seedlings again indicated that peptide abundance obtained at m/z signal of

3848.845Da probably responsible for better growth in haloprimered *C. cajan* seedlings (**Figure 5c**).

3.5 CD analysis of peptide structures

The LMW peptides isolated from NaCl challenged seedlings acquired lower percentages of secondary structure conformations (alpha helices + beta sheet/strand/turns) over peptides isolated from control seedlings (**Table 1**). The content of secondary structure decreased to about 51.6% in *Vigna* and by 24.1% in peptides isolated in *Cajanus* seedlings. However, peptides isolated from haloprimered test seedlings generated higher percentage of ordered secondary structure, 59.8% in *Vigna* and 44.5% in *Cajanus* indicative of stress release under salt stressed conditions. Disordered structure of peptides were found to be higher in salt stressed *Cajanus* by 57.4% seedlings and less in *Vigna* by 48.4%. These percentages of disordered conformation decreased to 35.6% in peptides isolated from haloprimered seedlings of *Cajanus* and to 35.4% in *Vigna*.

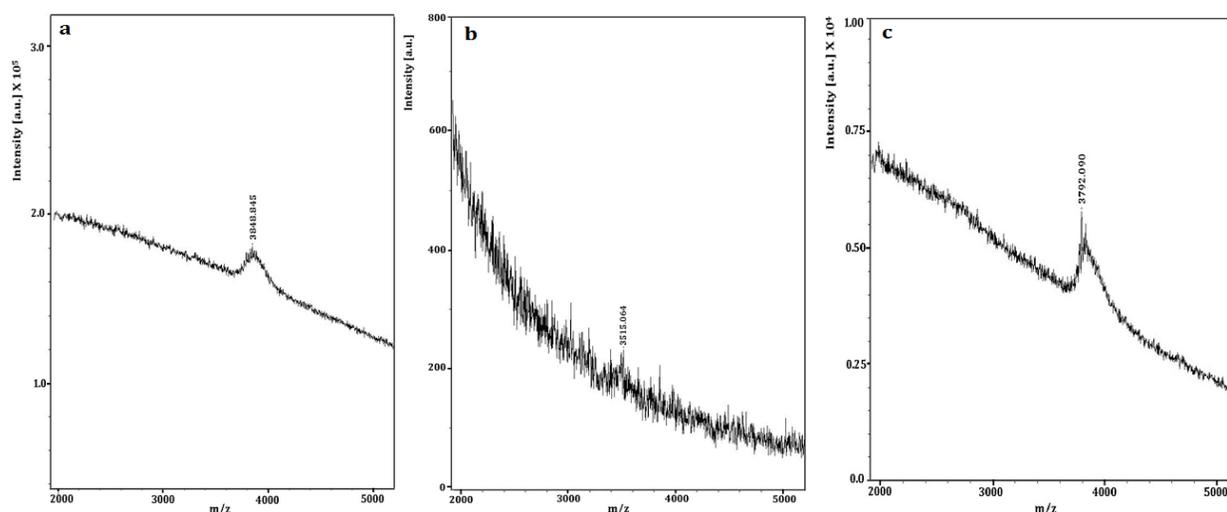


Figure 5 MALDI-TOF analysis of peptides isolated from 21 days old a. control seedlings b. nonprimed seedlings and c. haloprimered seedlings of *Cajanus cajan*.

Table 1 Estimates of Secondary structures in percentages from analysis of Circular Dichroism spectra.

Conformations	<i>Vigna mungo</i>			<i>Cajanus cajan</i>		
	Control	150mM Nonprimed	150mM Haloprimered	Control	150mM Nonprimed	150mM Haloprimered
α -Helix (Regular + distorted)	0.0	0.0	4.8	0.0	18.4	19.8
Antiparallel β -sheet (Left twisted+ Relaxed+ Right twisted)	38.0	0.0	31.2	37.7	0.0	0.0
Parallel β -strand	0.0	0.0	0.0	0.0	14.4	26.9
Turn	62.0	51.6	28.6	12.8	9.7	17.6
Others	0.0	48.4	35.4	49.4	57.4	35.6

3.6 Bioassay Results

3.6.1 Germination test

Surface sterilized blackgram and pigeon pea seeds were incubated in peptides isolated from their respective seedlings and treatments for 7 days. Of a total of 45 plated seeds, 38 germinated in case of blackgram (Figure 6a) and for pigeon pea it was 32 (Figure 6d). Peptides isolated from nonprimed NaCl stressed seedlings exhibited inhibition in rate of their respective seed germination to about 63% in blackgram (Figure 6b) and by 66% in pigeon pea (Figure 6e) in comparison to the effect of peptides that were isolated from their respective control seedlings. However on exposing seeds of both the cultivars to peptides isolated from their respective haloprimered seedlings depicted better

seed germination rate, where the rate of inhibition was appreciably narrowed down to 32% in blackgram (Figure 6c) and 25% in pigeon pea (Figure 6f) respectively over non-primed control.

3.6.2 Stomatal bioassay

Both the legume cultivars revealed that when epidermal peels were exposed to peptides isolated from control seedlings, stomata remained open (Figure 7a, 8a) but in peels exposed to peptides isolated from NaCl stressed seedlings, the guard cells were found to remain flaccid that caused stomatal closure (Figure 7b, 8b). However, when peels were subjected to peptides isolated from haloprimered seedlings, stomatal guard cells gained turgidity that resulted in stomatal opening (Figure 7c, 8c).

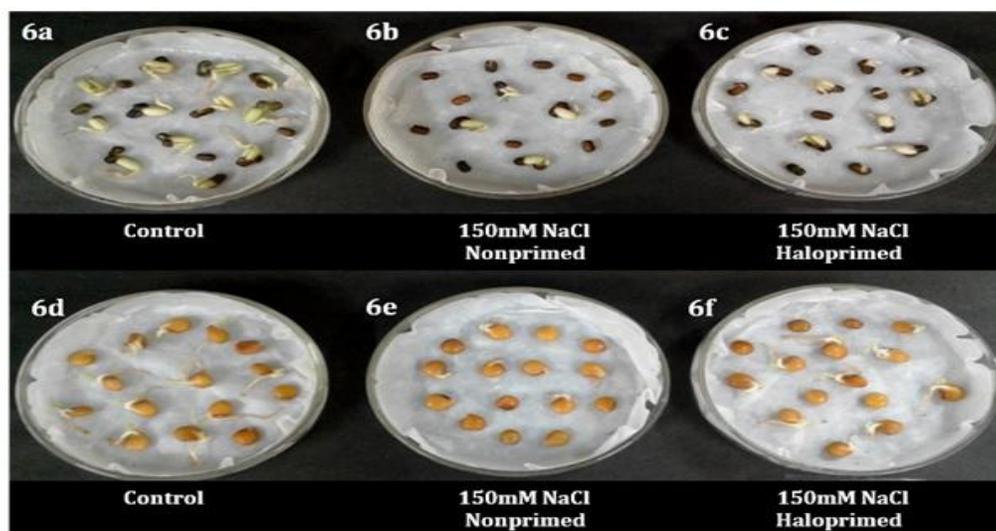


Figure 6: Effect of isolated LMW peptides (0.3-5.0kDa) on seed germination of nonprimed and haloprimered seeds of *Vigna mungo*. (a. control; b. 150mM nonprimed; c. 150mM haloprimered) and *Cajanus cajan* (d. control; e. 150mM nonprimed; f. 150mM haloprimered).



Figure 7 Effect of LMW peptides (0.3-5.0 kDa) on stomatal regulation in *Vigna mungo* a: Peptides isolated from control seedlings depicted opening of stomata b. Peptides isolated from salt stressed seedlings revealed complete stomatal closure c. Application of peptides isolated from haloprimered seedlings exhibited partial opening of stomata.

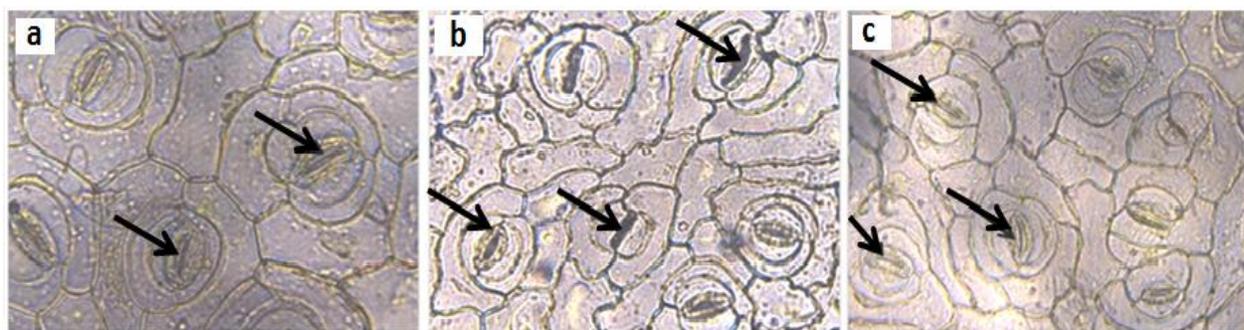


Figure 8 Effect of LMW (0.3- 5kDa) on stomatal regulation in *Cajanus cajan* a: Peptides isolated from control seedlings depicted opening of stomata b. Peptides isolated from salt stressed seedlings revealed complete stomatal closure c. Application of peptides isolated from haloprimered seedlings exhibited partial opening of stomata.

3.6.3 DPPH radical scavenging activity

DPPH radical scavenging activity was performed to detect the free radical scavenging activities of the peptides isolated from blackgram and pigeon pea (**Figure 9**). Peptides isolated from control seedlings exhibited radical scavenging activity of about 48% in blackgram and 34% in pigeon pea. In blackgram, the radical scavenging activity of peptide was reduced to about 28% whereas in pigeon pea it decreased to 8%, indicating higher radical scavenging in blackgram than pigeon pea over peptides isolated from their respective control seedlings. However, peptides isolated from haloprimered seedlings exhibited higher radical scavenging activity of about 43% in blackgram and 21% in pigeon pea over its control peptides.

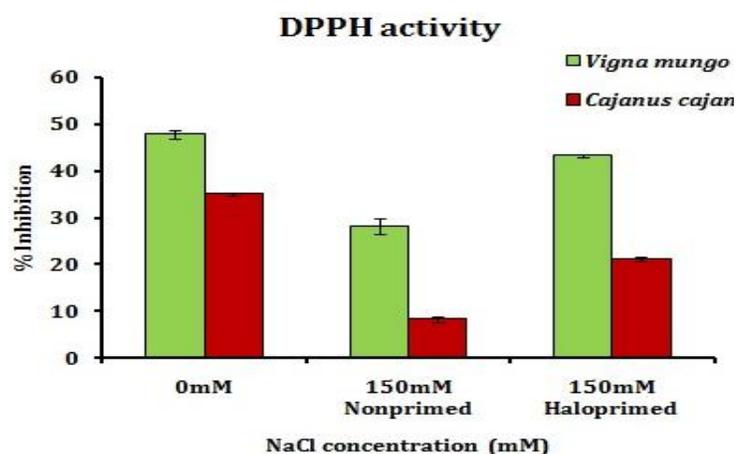


Figure 9 DPPH activity of LMW peptides isolated from 21days old control, nonprimed and haloprimered seedlings of *Vigna mungo* and *Cajanus cajan*. Values are mean \pm SE with three replicates. * indicates statistically significant at $p \leq 0.05$ compared to nonprimed control.

3.6.4 Nitric oxide scavenging activity

It has been demonstrated that under NaCl stress, nitric oxide scavenging activity of peptides obtained from both the cultivars decreased, the inhibition being more in *Vigna* than *Cajanus* (**Figure 10**). In *Vigna*, peptides isolated from the control seedlings exhibited 25% inhibition in their nitric oxide scavenging activity which was reduced to about 22% in peptides obtained from NaCl stressed seedlings indicating less quenching of radicals. Haloprimered seedlings of *Vigna mungo* produced peptides that showed higher inhibition of about 26% over the peptides isolated from control plants indicating improved nitric oxide scavenging activity. The peptides obtained from seedlings of *Cajanus* raised in water exhibited 24% inhibition in nitric oxide scavenging activity. The said inhibition was decreased to 20% in salt stressed *Cajanus* seedlings indicating lesser scavenging of nitric oxide

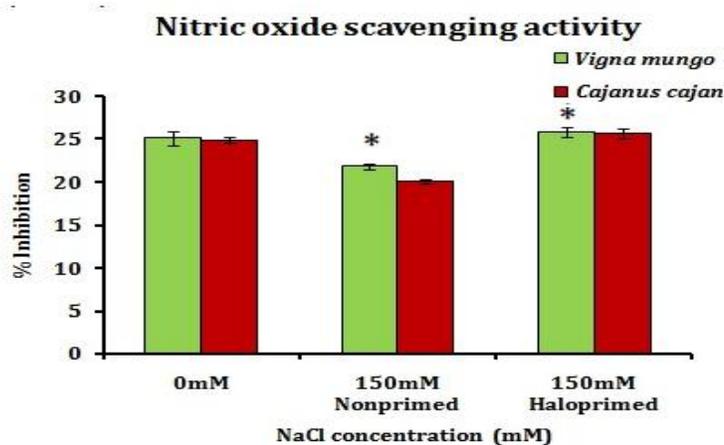


Figure 10: NO scavenging activity of LMW peptides isolated from 21days old control, nonprimed and haloprimered seedlings of *Vigna mungo* and *Cajanus cajan*. Values are mean \pm SE with three replicates. * indicates statistically significant at $p \leq 0.05$ compared to nonprimed control.

over the activity of its non-primed control peptides. However, peptides from haloprimered seedlings showed 25% inhibition over control peptides indicating greater radical scavenging property of haloprimered peptides.

4 Discussion

After 3 weeks of growth, NaCl induced toxicity resulted in stunted growth of *V. mungo* and *C. cajan* seedlings. This indicated salt induced metabolic perturbation was responsible for retarded growth of seedlings. However, when seeds of both the legume cultivars were primed with mild or sublethal dose of NaCl (50mM), increase in root and shoot lengths were recorded, probably an indication of their stress release due to haloprimering. Similar report has been published in salt stressed *V. radiata* (Saha et al., 2010), *N. sativa* (Gholami et al., 2015) and *Lolium perene* (Tilaki & Behtari, 2017).

Under NaCl stress, Na⁺ and Cl⁻ contents increased in the seedlings of salt stressed *V. mungo* and *C. cajan*. Accumulation of Na⁺ and Cl⁻ ions were more in *C. cajan* than *V. mungo*. Uptake of Na⁺ ions facilitated Cl⁻ ions to enter the roots down the chemical gradient (Moussa, 2004). Similar results were reported in salt-stressed *V. radiata* and *Broussonetia papyrifera* (Saha et al., 2012; Zhang et al., 2013). Under optimum physiological conditions, K⁺ ions are known to contribute to 6 -10% of the plant dry weight (Raven et al., 1976; Shin, 2014) and serves as a major inorganic osmolyte (Shabala & Cuin, 2008). It plays essential role in regulating several physio-biochemical processes of which stomatal opening and closing is crucial for maintenance of undisturbed synthesis of photoassimilates within plants (Shabala, 2003; Hasanuzzaman et al., 2018). NaCl exposure in the test seedlings resulted in reduced K⁺ contents and increased Na⁺ contents thereby causing stomatal regulation to get hampered significantly in both the legume cultivars as Na⁺ ions failed to replace the beneficial aspect of K⁺ ions. This happened because sodium ions competed with Na⁺-K⁺ co-transporter under salinity (Zhu, 2003; Almeida et al., 2017).

Haloprimering of seeds of *V. mungo* and *C. cajan* with 50mM NaCl resulted in decreased uptake of Na⁺ and Cl⁻ ions and increased accumulation of K⁺ ions in both the cultivars under 150mM NaCl exposure compared to non-primed control. Present outcomes are in agreement with the results obtained by seed preconditioning in salt stressed mungbean (Saha et al., 2012). This occurred due to preferential uptake of K⁺ ions over Na⁺ ions into the xylem element (Carden et al., 2003). Moreover, haloprimering of seeds stimulated Na⁺/H⁺ antiporter exchange activity of SOS1 protein pump as in *Hordeum vulgare* which promoted exclusion of Na⁺ from the root cells of haloprimered seedlings.

Small peptides play beneficial roles in dictating intercellular and intracellular signalling cascades related to growth and metabolism

in plants (Matsubayashi & Sakagami, 2006). Therefore, current research was aimed at isolation of ultra-filtered LMW peptides from both the legume cultivars which were subjected to HPLC and MALDI-TOF analysis. This aided not only in determining the peptide occurrences but also to compare their variations in content during their normal seedling germination and under salt stressed conditions. Further role of haloprimering in alleviating the salt induced adversities could also be deciphered. HPLC chromatograms of peptides isolated from both the test seedlings portrayed that in non-primed control seedlings, maximum peptide expression and occurrence took place during normal seedling growth. However, peptides obtained from NaCl stressed seedlings generated chromatogram that showed lesser peptide abundance compared to non-primed control seedlings. This probably indicated that several LMW peptides which usually participate in growth processes of plants were degraded under salinity. Haloprimering of seeds helped to overcome such adversities to considerable extent in salt stressed conditions as indicated by increased peak area and reappearance of a few peaks that was noted in control seedlings. This reveal that ameliorative efficacy of haloprimering lead to better growth and development of both the pulses under NaCl stressed environments. Matrix-assisted laser desorption ionization (MALDI) being a soft source of molecule(s) ionisation was utilized to generate fragmentation of isolated peptide(s) thereby helping to determine their molecular weight (Castañeda-Ovando et al., 2012). For both the tested materials, molecular weight of the most prominent peptide obtained from the m/z signals of MALDI spectrum was found to be close to each other. Intensities of this prominent peak showed a trend that was similar to its occurrence in HPLC spectra for both cultivars. The appearance of signal having m/z 3731.270 in MALDI spectra of peptides from haloprimered blackgram seedlings was similar to that of peptide signal at m/z 3720.246 derived from its control seedlings. This indicated that haloprimering resulted in reappearance of that LMW peptide facilitating better germination and growth of haloprimered seeds than directly salt treated seeds. Although no such peptide reappearances were noted in MALDI spectrum of *C. cajan* but, role of haloprimering was evident from the increased peak intensity of haloprimered peptides over direct salt treated ones. Thus, seed haloprimering in both the cultivars imparted better growth of primed seeds under salt stressed conditions due to prior salt acclimation.

Circular dichroism spectroscopy helped to determine percentages of secondary structure adopted by the peptides of both the pulses under salt stress and how these conformations altered in case of haloprimered peptides under salt exposure. Secondary conformations were found to decrease under NaCl induced desiccation manifesting deformation and distortion in peptide structure usually involved in normal plant growth and developmental processes. This reflected salt induced denaturation

of peptides, formation of more flexible, unfolded, unordered structures which affected the equilibrium between the contents of α -helical and β -sheet structures. Moreover, unordered structures increased in peptides obtained from salt stressed seedlings probably due to protein aggregation as it was observed in whey protein isolates under temperature and pH variations (Topmczynska-Mleko et al., 2014). But, in haloprimered peptides the percentages of secondary conformations of peptides were much more regularized over peptides of salt stressed seedlings. Higher percentages of secondary conformations and lower percentages of disorder probably helped the primed seeds to impart efficient metabolism under salinity stress in both the pulses.

The above obtained results were validated by bioassays performed with the isolated LMW peptides from both the cultivars. Bioassays helped to demonstrate the putative effects of respective peptides on seed germination and stomatal regulation and thereby seedling growth from which the authors could get some notion regarding their role on growth regulation in the test seedlings. Abundance of LMW peptide from control seedlings of both the varieties, promoted seed germination rate and kept stomatal pore open for CO₂ assimilation. This could be correlated with the higher K⁺ ion accumulation in shoots and satisfactory antioxidative activities of LMW peptides favouring luxuriant growth of both cultivars. Similar antioxidative properties have been reported in LMW isolated from mulberry plants (Jha et al., 2014). But, the peptides isolated from NaCl challenged seedlings of both varieties narrowed down the germination rate, failed to arouse turgidity in guard cells due to decreased uptake of K⁺ ions in shoot thereby resulting in stomatal shut down and hindered CO₂ fixation. Decrease in free radical scavenging activities of these peptides derived from NaCl stressed seedlings is also in agreement with the above physiochemical events causing stunted seedling growth in both the varieties. Similar effect has been reported in *Schizonepeta tenuifolia* when exposed to high salt doses at and above 75mM NaCl (Zhou et al., 2018). But, peptides obtained from haloprimered seedlings exhibited improved seed germination, increased turgidity of stomatal guard cells causing stomatal opening and carbon assimilation under NaCl stress. This could be corroborated with increased accumulation of K⁺ ions in shoots of haloprimered cultivars and antioxidative activity of LMW peptides that resulted in improved growth in both the varieties on salt exposure. However, the scavenging activity of peptides was found to be more efficient in *V. mungo* than *C. cajan*. This indicated more stress tolerance capability of blackgram over pigeon pea under NaCl stress, when seeds were haloprimered.

Therefore, adoption of cost effective haloprimering technique by poor farmers may help to boost legume production by improving crop stand in saline prone agricultural lands to partially meet up with the food crisis of the flourishing world population.

Conclusion

To conclude authors relate that under NaCl stress, growth was inhibited in both blackgram and pigeonpea seedlings due to lower abundance of LMW peptides. The isolated peptides exhibited antioxidant properties that effectively scavenged reactive oxygen species when present in plenty. The peptides were stress-responsive and increased on seed haloprimering when compared to that of salt stressed sets. Peptides isolated from haloprimered *V. mungo* had better antioxidant properties than those isolated from haloprimered *C. cajan* which could be correlated with improved growth in *V. mungo* as recorded by the authors. Secondary structural conformations were highly hampered under salt stress. But haloprimered *V. mungo* and *C. cajan* seeds showed improved physiological performances indicating that adopting this cost effective haloprimering technique helped to overcome salt induced adversities under salt exposure. Moreover, this technique may help to increase legume production in saline prone agricultural lands to meet up with the food demands of increasing population.

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Conflicts of Interest and financial disclosure

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References

- Albert M (2013) Peptides as trigger of plant defence. *Journal of Experimental Biology* 64: 5269-5279.
- Almeida DM, Oliveira MM, Saibo NJM (2017) Regulation of Na⁺ and K⁺ homeostasis in plants: towards improved salt tolerance in crop plants. *Genetics and Molecular Biology* 40: 326-345.
- Azzoni AR, Takahashi K, Woodard SL, Miranda EA, Nikolov ZL (2005) Purification of recombinant apotinin produced in transgenic corn seed: separation from CTI utilizing Ion-exchange chromatography. *Brazilian Journal of Chemical Engineering* 22: 323-330.
- Bircheneder S, Dresselhaus T (2016) Why cellular communication during plant reproduction is particularly mediated by CRP signalling. *Journal of Experimental Botany* 67: 4849-4861.

- Biswas S, Biswas AK, De B (2018) Metabolomics analysis of *Cajanus cajan* L. seedlings unravelled amelioration of stress induced responses to salinity after halopriming of seeds. *Plant Signaling and Behavior* 13: e1489670.
- Carden DE, Walker DJ, Flowers TJ, Miller AJ (2003) Single-cell measurements of the contributions of cytosolic Na⁺ and K⁺ to salt tolerance. *Plant Physiology* 131: 676-683.
- Castañeda-Ovando A, Sedo O, Havel J, Pacheco L, Galán-Vidal CA, Lopez EC (2012) Identification of anthocyanins in red grape, plum and capulin by MALDI-ToF MS. *Journal of the Mexican Chemical Society* 56: 378-383.
- Chatterjee P, Biswas S, Biswas AK (2018) Sodium chloride primed seeds modulate glutathione metabolism in legume cultivars under NaCl stress. *American Journal of Plant Physiology* 13: 8-22.
- Chrysargyris A, Panayiotou C, Tzortzakis N (2016) Nitrogen and phosphorus levels affected plant growth, essential oil composition and antioxidant status of lavender plant (*Lavandula angustifolia* Mill.). *Industrial Crops and Products* 83: 577-586.
- Czyzewicz N, Yue K, Beeckman T, De Smet I (2013) Message in a bottle: small signalling peptide outputs during growth and development. *Journal of Experimental Botany* 64: 5281-5296.
- Etesami H, Beattie GA (2018) Mining halophytes for plant growth-promoting halotolerant bacteria to enhance the salinity tolerance of non-halophytic crops. *Frontiers in Microbiology* 9: 1-20.
- Gholami M, Mokhtarian F, Baninasab B (2015) Seed halopriming improves the germination performance of black seed (*Nigella sativa* L.) under salinity stress conditions. *Journal of Crop Science and Biotechnology* 18: 21-26.
- Ghosh A, Mandal P, Sircar PK (2010) Wheat (*Triticum aestivum*) peptide(s) mimic gibberellin action and regulate stomatal opening. *Indian Journal of Experimental Biology* 48: 77-82.
- Hara K, Kajita R, Torii KU, Bergmann DC, Kakimoto T (2007) The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. *Genes and Development* 21: 1720-1725.
- Hasanuzzaman M, Bhuyan MHM, Nahar K, Hossain MS, Mahmud JA, Hossen MS, Masud AAC, Moumita OC, Fujita M (2018) Potassium: A vital regulator of plant responses and tolerance to abiotic stresses. *Agronomy* 8: 31-59.
- Hussain K, Majeed A, Nawaz K, Bhatti KH, Nisar FK (2009) Effect of different levels of salinity on growth and ion contents of black seeds (*Nigella sativa* L.). *Current Research Journal of Biological Sciences* 1: 135-138.
- Jha S, Ghosal M, Gupta SK, Ghosh A, Mandal P (2016) In-vitro free radical scavenging potential of oligopeptides derived from wheat and mungbean. *International Journal of Pharmacy and Pharmaceutical Sciences* 8: 428-432.
- Jha S, Mandal P, Bhattacharya P, Ghosh A (2014) Free-radical scavenging properties of low molecular weight peptide(s) isolated from S1 cultivar of mulberry leaves and their impact on *Bombyx mori* (L.) (Bombycidae). *Journal of Animal Science and Biotechnology* 5: 16-24.
- Leung SO, Yeung HW, Leung KN (1987) The immunosuppressive activities of two abortifacient proteins isolated from seeds of bittermelon (*Momordica charantia*). *Immunopharmacology* 13: 159-171.
- Lindsey K, Casson S, Chilly P (2002) Peptides: new signalling molecules in plants. *Trends in Plant Science* 7: 78-83.
- Mandal P, Misra TK, Sarkar A, Ghosh A, Sircar PK (2008) Dynamic peptide profiles of germinating mungbean: In relation to their nature and separation pattern. *Indian Journal of Plant Physiology* 13: 111-117.
- Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Gardes-Albert M (1994) Antioxidant action of *Ginkgo biloba* extracts EGb 761. *Methods in Enzymology* 234: 462-475.
- Maróti G, Downie JA, Kondorosi É (2015) Plant cysteine-rich peptides that inhibit pathogen growth and control rhizobial differentiation in legume nodules. *Current Opinion in Plant Biology* 26: 57-63.
- Matsubayashi Y (2014) Posttranslationally modified small-peptide signals in plants. *Annual Review of Plant Biology* 65: 385-413.
- Matsubayashi Y, Sakagami Y (2006) Peptide hormones in plants. *Annual Review of Plant Biology* 57: 649-674.
- Moussa HR (2004) Amelioration of salinity-induced metabolic changes in soyabean by weed exudates. *International Journal of Agriculture and Biology* 6: 499-503.
- Muñoz-García J, Ares S (2016) Formation and maintenance of nitrogen-fixing cell patterns in filamentous cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America* 113: 6218-6223.
- Murphy E, Smith S, De Smet I (2012) Small signalling peptides in *Arabidopsis* development: How cells communicate over short distance. *Plant Cell* 24: 3198-3217.
- Mylona P, Pawlowski K, Bisseling T (1995) Symbiotic nitrogen fixation. *Plant Cell* 7: 869-885.

- Oh E, Seo PJ, Kim J (2018) Signaling peptides and receptors coordinating plant root development. *Trends in Plant Science* 23: 337-351.
- Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety* 60: 324-349.
- Raven PH, Evert RF, Curtis H (1976) *Biology of plants*. Worth Publishers, New York.
- Saha P, Chatterjee P, Biswas AK (2010) NaCl pretreatment alleviates salt stress by enhancement of antioxidant defense and osmolyte accumulation in mungbean (*Vigna radiata* L. Wilczek). *Indian Journal of Experimental Biology* 48: 593-600.
- Saha P, Kunda P, Biswas AK (2012) Influence of sodium chloride on regulation of Krebs cycle intermediates and enzymes of respiratory chain in mungbean. *Plant Physiology and Biochemistry* 60: 214-222.
- Senthilkumar S, Chang E, Jayakumar R (2008) Diffusible amyloid oligomers trigger systemic amyloidosis in mice. *Biochemistry Journal* 415: 207-215.
- Shabala S (2003) Regulation of potassium transport in leaves: from molecular to tissue level. *Annals of Botany* 92: 627-634.
- Shabala S, Cuin TA (2008) Potassium transport and plant salt tolerance. *Physiologia Plantarum* 133: 651-669.
- Sheen RT, Kahler HL (1938) Effect of ions on mohl method for chloride determination. *Industrial and Engineering Chemistry Analytical Edition* 10: 628-629.
- Shin R (2014) Strategies for improving potassium use efficiency in plants. *Molecular Cell* 37: 575-584.
- Sugano SS, Shimada T, Imai Y, Okawa K, Tamai A, Mori M, Hara-Nishimura I (2010) Stomagen positively regulates stomatal density in *Arabidopsis*. *Nature* 463: 241-244.
- Tilaki GAD, Behtari B (2017) Alleviation of ionic and osmotic stress of salinity in seedling emergence of *Lolium perenne* L. with halopriming treatments growing in an hydroponic system. *Journal of Plant Nutrition* 40: 219-226.
- Topmczynska-Mleko M, Kamysz E, Sikorska E, Puchalski C, Mleko S, Ozimek L, Kowaluk G, Gustaw W, Wesolowska-Trojanowska M (2014) Changes of secondary structure and surface tension of whey protein isolate dispersions upon pH and temperature. *Czech Journal of Food Sciences* 32: 82-89.
- Widodo PJH, Newbiggin E, Tester M, Bacic A, Roessner U (2009) Metabolic responses of salt stress in barley (*Hordeum vulgare* L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. *Journal of Experimental Botany* 60: 4089-4103.
- Zehadi MJA, Masamba K, Li Y, Chen M, Chen X, Sharif HR, Zhong F (2015) Identification and purification of antioxidant peptides from Lentils (*Lens culinaris*) hydrolysates. *Journal of Plant Sciences* 3: 123-132.
- Zhang M, Fang Y, Ji Y, Jiang Z, Wang L (2013) Effects of salt stress on ion content, antioxidant enzymes and protein profiles in different tissues of *Broussonetia papyrifera*. *South African Journal of Botany* 85: 1-9.
- Zhang Y, Lewis K (1997) F abatins: new antimicrobial plant peptides. *FEMS Microbiology Letters* 149: 59-64.
- Zhou Y, Tang N, Huang L, Zhao Y, Tang X, Wang K (2018) Effect of salt stress on plant growth, antioxidant capacity, glandular trichome density, and volatile exudates of *Schizonepeta tenuifolia* Briq. *International Journal of Molecular Sciences* 19: 252-266.
- Zhu JK (2003) Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology* 6: 676-683.