Pak. J. Bot., 54(6): 2069-2079, 2022. DOI: http://dx.doi.org/10.30848/PJB2022-6(36)

INVESTIGATION OF DEFENSE MECHANISM ALTERATIONS INDUCED BY PHYTOHORMONES, HEAT-SHOCK AND SALT TOXICITY IN GREEN GRAM [*VIGNA RADIATA* (L.) WILCZEK]

JABEEN FARHEEN[1,2*] AND SIMEEN MANSOOR[1]

[1]Department of Genetics, University of Karachi, Karachi-75270, Pakistan
[2]School of Material Science and Engineering, Zhejiang Sci-Tech University, China. Hangzhou, 310018 P.R. China
**Corresponding author's email: jabeenfarheen@hotmail.com*

Abstract

Salt toxicity cause a drastic reduction in green gram yield which plays a pivotal role in the human diet especially in vegetable-based society. With this respect, the destructive action from 50 to 350 mM NaCl and its recovery through phytohormones and heat-shock were evaluated on growth, biomolecules, and quality and quantity of DNA using advanced cost-effective and quick plant DNA extraction method. The salicylic acid (SA) 50 µM, indoleacetic acid (IAA) 100 µM, and gibberellic acid (GA3) 100 µM were used as seed soaking (24 h) treatment before 40ºC heat-shock (1 h) then salt stress (144 h) applied on *Vigna radiata* (green gram) variety NM-92. The growth restraining impact of salt along with 40ºC was quite evident with enhancing NaCl concentrations. The utmost growth, and least malondialdehyde (8.76 µM g^{-1} FW), proline (16.31 µM g^{-1} FW), and hydrogen peroxide (7.01 µM g^{-1} FW) were related to SA+30°C+NaCl 50 mM. While, ascorbate peroxidase, catalase, and superoxide dismutase maximum activities were associated with SA+30°C+NaCl 350 mM. The modified extraction method-2 yields high-quality DNA without liquid nitrogen, CTAB, Proteinase K, and prolong heating (65ºC). This method is ideal for the extraction of a large number of samples and storage for a year. DNA extraction results showed a 1.8 ratio at $A260/A280$ from all treatments that is suitable for RAPD-PCR reaction. Conclusively, 50 µM SA presoaking enhanced DNA stability under extreme salt stress that can be beneficial to grow green gram in the highly saline-affected zone.

Key words: DNA extraction technique, Mung bean, Plant growth regulator, Salinity stress, Random amplified polymorphic DNA, Polymerase chain reaction analysis.

Introduction

Salt toxicity not only adversely affects 33% of irrigated and 21% of the cultivable area throughout the world that extents 10% of total fields of the globe (Machado & Serralheiro, 2017; Shahid *et al*., 2018), but also decrease the yield of crops. Additionally, the Food and Agriculture Organization (2010) reported that the indigent horticulture becomes the root cause of a lack of nutrition in 1 billion individuals out of 7.7 billion world population. In this context, the green gram is a highly nutritive, and eco-friendly crop that enhances the fertility of the soil zone. Although the excess salt accumulation in its rooting medium destroys the yield of green gram crop worldwide (Sehrawat *et al*., 2014). To this purpose, advancement in salt tolerance in green gram is an imperious objective for improving the yield and fulfil the food necessities of the world's population (Anon., 2010).

Several methodologies have been acquired to increase salinity tolerance in green gram (Jini & Joseph, 2017). Among them, seed priming with exogenous application of phytohormones like salicylic acid (SA), is an inexpensive and effective way to alleviate excess Sodium chloride (NaCl) induced toxicity in plants. Such as 10 µM SA enhanced germination rate of rye seedlings then 100, 500 and 1,000 µM SA under salt stress (Yanik *et al*., 2018). Another finding demonstrated that 5 µM Gibberellic acid (GA3) pre-treatment enhanced antioxidant enzymes that in turn alleviated salt stress in *Vigna radiata* plants (Ghosh *et al*., 2015). Similarly, the earlier research study on saline stressed *Zea mays* with 2,000 µM Indole acetic acid (IAA) showed a high level of antioxidant enzymes activities (Kaya *et al*., 2013). In mustard, heat-shock (42°C for 5 h) application prior to salt stress improved morpho-physiology of stressed seedlings (Hossain *et al*., 2013). Briefly, the seeds can be made more resistant with SA, IAA, GA3, and heat-shock (HS) pre-soaking, which quickly enhance the biosynthesis of antioxidant defense enzymes (Ghosh *et al*., 2015; Yanik *et al*., 2018), that in turn increase growth, relative water content (RWC) and photosynthetic pigments (PP).

Also, sodium chloride toxicity damage the genetic makeup of green gram. It breaks the strand of nucleic acid, induced various kinds of structural chromosomal aberrations and mutation in the sequence of DNA (Farheen & Mansoor, 2019). However, several molecular biology techniques like random amplification of polymorphic DNA (RAPD) (Kumar *et al*., 2016), and other gene detecting methods (Abbas *et al*., 2015) were introduced for the estimation of variation in green gram DNA damage caused by various eco-toxin (Kumar *et al*., 2016) such as salt stress (Sehrawat *et al*., 2014). These advance PCR-based techniques' efficacy depends on the DNA extraction method (Aboul-Maaty & Oraby, 2019). Therefore, a quick, easy, and cost-effective DNA extraction technique is the need of the day. Moreover, we have developed the advance and efficient technique for extracting complex plant DNA under salt toxicity that yields high-quality DNA in a brief time without costly chemicals such as liquid nitrogen and Proteinase K, no specific gadgets, prolong heating (60ºC) or incubation (65ºC) to denature DNA. In this regard, the current investigation was made to evaluate the pre-soaking of SA,

IAA or GA3 along with HS either 30ºC or 40ºC would ameliorate the contrasting impact of high NaCl toxicity on 10 morpho-biomolecular parameters of green gram seedlings and also to quantify the DNA destruction induced by salt toxicity and its alleviation via SA pre-soaking through simple cheap, quick, effective, and innovative DNA extraction technique. Additionally, Dellaporta *et al*., (1983), Doyle & Doyle (1987), Doyle & Doyle (1990), Saghai-Maroof *et al*., (1984) and modified CTAB-based method-1 were contrasted with a modified extraction method-2 for effective investigation of DNA destruction under salt toxicity.

Materials and Methodology

Experimental design for phytohormones treatment evaluation: The seven days experiment was conducted in 150 x 30 mm size Petri dishes with green gram variety NM-92 in the lab of the Department of Genetics, University of Karachi and Pakistan. In this experiment, the 30 uniform sized seeds/treatment (total 1,200 seeds/replication) were sterilized (Farheen *et al*., 2018) and divided into two major groups for distilled water (DW) and phytohormones treatment. The distilled water treatments seeds were imbibed in DW for 24 hours (h) and in phytohormones treatment seeds were soaked in SA 50 µM, IAA 100 µM, and GA3 100 µM solution separately for 24 h. The washed seeds were left for few hours in the dark. Then the seeds of both the groups were further divided into two sub-groups first was 30ºC control group and second was 40ºC HS group. The 30ºC control group seeds were kept at room temperature (30ºC) for 1 h while HS group seeds were subjected to 40ºC in DW filled large test tube for 1 h in water bath (Uni Thermo Shaker NTS-1300 EYELA, Japan). The HS seeds were kept for a few hours in the dark for recovery then both sub-groups' seeds were arranged on 0, 50, 150, 250, and 350 mM NaCl (Fisher Scientific, UK) moistened filter paper line Petri dishes for six days. The whole experiment was performed at optimum temperature i.e. 30ºC (evaluate during the pilot study) excluding HS treatment (Table 1).

After seven days, morphological estimation such as seedling length (SL), total fresh biomass (TFB), RWC, and PP were recorded as previously described method of Farheen *et al*., (2018). The Lipid peroxidation was estimated by Carmak & Horst (1991) with some alteration (Ashraf *et al*., 2013). While malondialdehyde (MDA) content was calculated through the 156 mM^{-1} cm^{-1} extinction coefficient (Ashraf *et al*., 2013) and expressed as µM MDA $milligram^{-1}$ protein $milliliter^{-1}$. The amount of free Proline (PRO) in salt-stressed and treated seedlings were assessed through 250 µg proline $milliliter^{-1}$ standard curve expressed as µM proline $gram^{-1}$ fresh weight (Bates, 1973). The absorbance of proline-ninhydrin chromophore phase was taken on Y-axis and µg proline on X-axis. The hydrogen peroxide (H_2O_2) estimation was performed in the dark (Velikova *et al*., 2000), while activity was calculated through 0.28 μM^{-1} cm^{-1} extinction coefficient (Ashraf *et al*., 2013).

$$\text{Hydrogen peroxide } \mu M = \frac{\text{Absorbance at 390 nm}}{0.28} DF \quad \text{……..……... (1)}$$

$$\text{Dilution factor (DF)} = \frac{\text{The final volume of the reaction solution}}{\text{Tissue weight}} \text{……..……. (2)}$$

The antioxidant defense enzymes like ascorbate peroxidase (APX) estimation and specific activity were computed by 2.8 mM^{-1} cm^{-1} (Mansoor & Naqvi, 2013), and catalase (CAT) were 40 mM^{-1} cm^{-1} extinction coefficient (Mansoor & Naqvi, 2013). While, superoxide dismutase (SOD) activity was (Mansoor & Naqvi, 2013) calculated using the following formula;

$$\text{Samle O.D.} = \frac{\text{Dark incubated tube O.D.} - \text{Light incubated tube O.D.}}{\text{Dark incubated tube O.D.}} \text{……...……. (3)}$$

$$\text{Specific activity} = \frac{\text{Sample O.D.}}{\text{µg protein in 150 µL sample supernatant}} \text{ X 1000 ……... (4)}$$

Where, O.D is the absorbance.

Experimental design of DNA extraction for PCR: The second experiment was planned after getting optimum results in 50 µM SA treatments among three evaluated phytohormones along with two temperature treatments. In this regard, an equal size, sterilized NM-92 seeds (100 seeds / replication) were imbibed in DW and 50 µM SA solution in a separate beaker for 24 h. After recovery, imbibed seeds were kept at 30ºC for 1 h then 100 x 15 mm Petri dishes were labelled and lined with filter paper. Petri dishes were moistened with 0, 50, 150, 250 and 350 mM concentration of NaCl thereat then 10 seeds / treatment were placed in each concentration of salt for 144 h. At 168 h of growth, seedlings were harvested for various DNA extraction techniques. Many DNA extraction method have been examined such as Dellaporta *et al*., (1983), Doyle & Doyle (1987), Doyle & Doyle (1990), and Saghai-Maroof *et al*., (1984). Although, mostly methods were Cetyl-trimethyl-ammonium-bromide (CTAB)-based method therefore CTAB method have been modified (Doyle & Doyle, 1987; Doyle & Doyle, 1990; Saghai- Maroof *et al*., 1984) for better results and ease of work.

Table 1. Treatments details of phytohormones and heat-shock application prior salinity stress on NM-92 green gram genotype.

Pre-soaking (24 h)	Heat-shock treatment (1 h)	Sodium chloride treatments in millimolar (144 h at 30°C)
DW	30 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM
	40 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM
GA3 100 µM	30 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM
	40 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM
IAA 100 µM	30 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM
	40 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM
SA 50 µM	30 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM
	40 °C	0 mM
		50 mM
		150 mM
		250 mM
		350 mM

DW: distilled water, GA3: 100 µM gibberellic acid, IAA: 100 µM indole acetic acid, SA: 50 µM salicylic acid, 30: 30°C, 40: 40°C heat-shock, and NaCl: sodium chloride

Modified CTAB-based Method-1: One gram seedlings were extracted in 1 mL of 2X CTAB buffer and mixed constantly on Uni Thermo Shaker NTS-1300 EYELA, Japan at 65ºC for 45 minutes. The 2X CTAB buffer was prepared by dissolving 1.21% Tris, 5.84% NaCl, 0.58% Ethylenediaminetetraacetic acid, 2% CTAB, 2% Polyvinylpyrrolidone-40 (PVP-40), 0.4% 2-mercaptoethanol. After cooling down the samples, 500 µL of Chloroform: Isoamyl alcohol (24:1) mixture were mixed vigorously for 10 minutes and then centrifuged (DLAB-D2012 *plus*, USA) the samples at 12,000 rpm for 15 minutes and 1 ml of upper aqueous phase was transferred in the fresh Eppendorf. Added 660 µL ice-chilled 2-propanol in the 1 mL samples supernatant and mixed gently. Centrifuged the sample supernatant at 12,000 rpm for 10 minutes then discard the supernatant and washed sample pellet with 500 µL of 75% ethanol and incubate for 5 minutes. Afterward, the sample pellet was centrifuged at 12,000 rpm for 5 minutes and discarded ethanol. Dry the sample pellet by inverting the eppendorf at 37ºC for 20-30 minutes thereat dissolved the pellet in sterile ddH_2O and stored at -20 ºC. After evaluation of previously described methods, the below listed improved, highly efficient and cost-effective extraction technique was introduced that was based on Dellaporta *et al*., (1983).

Modified extraction method-2: Ice-chilled (> -20ºC) 0.25 g seedlings were extracted with 1 mL of pH 8 buffer in pre-chilled (-20ºC) mortar and pestle. The buffer was composed of 0.02 M Ethylenediaminetetraacetic acid, 0.5 M NaCl, 0.1 M Tris, and 7 M Urea then autoclaved, introduced 2% Sodium-dodecyl sulfate and 0.15% 2-mercaptoethanol before use. After extraction, 1 mL homogenate was transferred into the Eppendorf and gently mixed with 1 mL of Phenol (pH 8): Chloroform: Isoamyl alcohol solution (25:24:1 ratio). The mixture was centrifuged in the DLAB-D2012 *plus* (USA) at 14,000 *x* g for 15 min at 37ºC and 900 µL upper aqueous phase was collected carefully in new Eppendorf and then 900 µL of Chloroform: Isoamyl alcohol (24:1 ratio) solution was added. The tubes were gently inverted several times to mix well then centrifuged at 14,000 *x* g for 10 min at 37ºC. Shift upper aqueous phase into the new eppendorf. Where, the supernatant was found dirty then repeated several times Chloroform: Isoamyl alcohol (24:1) step until the supernatant became clear.

Pellet down the extracted DNA by mixing 100 µL of chilled sodium acetate buffer (3 M sodium acetate pH 7 was set by pure glacial acetic acid) and 700 µL ice-chilled 2-propanol in the collected clear upper aqueous phase then left undisturbed for 10-20 min. After centrifugation at 14,000 *x* g for 15 min, the pellet was washed quickly with absolute ethanol and air-dried. The pellet of DNA was suspended in 100 µL of sterile ddH_2O.

If it is required to remove RNA then 1 µL of DNase free RNase A (20 µg/ µL) will be introduced at 37ºC for 30 min and then samples will be saved at -20ºC for further analysis. The total genomic DNA quality was determined by 0.8% agarose gel electrophoresis (HU6 SCIE-PLAS, UK) and quantity was evaluated via nanodrop 2000 spectroscopy (USA) at 230, 260, 280, and 320 nm against DW as blank. All samples were standardized at 50 ng μL^{-1} and computed through below formulae (Wilson & Walker, 2005);

$$\text{Purity of DNA from Protein} = \frac{\text{Absorbance at 260 nm}}{\text{Absorbance at 280 nm}} \quad \text{.............................. (5)}$$

$$\text{Purity of DNA from Polysaccharide} = \begin{matrix}\text{Absorbance at 260 nm}\\ \text{Absorbance at 280 nm}\end{matrix} \quad \text{.................... (6)}$$

ds DNA concentration = 50 *ng* μL^{-1} x O.D. 260 nm x dilution factor …… (7)

DNA concentration (ng μL^{-1}) = (O.D. 260 nm – O.D. 320 nm) x DF x 50 ng μL^{-1} …….. (8)

DNA yield in ng = DNA Conc. x Total purified sample volume in µL ……. (9)

The decamer RAPD primers (OPA-12: 5'– TGCCGAGCTC– 3'), having 70% GC content (IDT, USA) was selected from the set of OPA, OPG, and OPO due to their highest reproducibility and strong products amplification. The genomic DNA amplification was carried out in 200 µL PCR tubes using Gradient PCR (DLAB-TC1000-G, USA) having 25 µL volume of reaction mixture comprising 12.5 µL Nova *Taq*™ PCR Master mix, USA, 0.625 µL of 25 pmol RAPD primer, 1 µL genomic DNA of green gram, and nuclease-free PCR water provided with Nova *Taq*™ master mix, USA. The gradient PCR programmed for 35 cycles, in which initial denaturation was completed at 94ºC for 5 min. then denaturation for 1 min at 94ºC, RAPD primer annealing step for 1 min at 30-37ºC, and extension for 2 min at 72ºC. The final extension was carried out for 10 min at 72ºC (Kumar *et al*., 2016). For authentication, a complete reaction mixture of PCR without green gram template DNA was also included as negative control. The PCR products were separated on 1.5% and 1.8% agarose gel containing 10 ng/µL ethidium bromide in 1X TAE buffer with 2 µL of GeneRuler 1 kb DNA ladder (Thermo Scientific, USA) at 80 volts for 2 h. The amplified DNA bands were visualized, analyzed, and photographed under SCIE-PLAS SYGF/1785 UV-gel documentation system, UK.

Statistical design: The experimental design for phytohormones treatment evaluation was replicated four times in the factorial CRD and experimental design for DNA extraction for PCR analysis was triplicated in the CRD (Steel & Torrie, 1997). All experimental data represented in the form of tables and figures were computed on Excel 2016 and analyzed by MANOVA (IBM SPSS version 19, Inc., Chicago, IL, USA). The means of eleven parameters with three phytohormones and two heat-shock (3x2) treatments were analyzed at 0.05 level of significance via Duncan test (DMRt).

Results

In the current investigation the three phytohormones per-soaking treatment, two temperatures, and five different concentrations of sodium chloride were assessed on green gram seedlings. It was observed that the growth parameters of green gram seedlings were severely affected as salt concentration increased. Furthermore, the most adverse reduction in SL (79%), TFB (38%), RWC (23%), and PP (72%) was recorded when HS (40ºC) and 350 mM salt stress were jointly applied without phytohormones pre-soaking treatment (Table 2a). However, the pre-soaking of GAз, IAA, and SA showed improvement in the growth of seedlings. Among pre-treatments, SA imbibed seeds showed a significant promotion in growth with 30ºC HS at all concentration of NaCl when compared with GAз, and IAA pre-treatments. The SA+30ºC+50 mM NaCl expressed 14% enhancement in SL, 36% in TFB, 5% in RWC, and 43% in PP that was higher than control (Table 2a).

In the case of MDA, PRO, and H_2O_2, their concentration was significantly increased as salt stress progressed on a seedling. However, the drastic enhancement in MDA (80%), PRO (155%), and H_2O_2 (67%) was observed when 40ºC HS was applied with 350 mM sodium chloride. The SA pre-imbibition treatment showed the highest reduction in MDA, PRO, and H_2O_2 among 40 treatments. The combination of SA with 30ºC HS caused 21% reduction in MDA, 19% in PRO, and 38% reduction in H_2O_2 under 50 mM salt concentration respectively, which was lesser when compared with control (Table 2b).

The antioxidant enzymes activities were gradually increased as salt concentration progressed from 50 to 350 mM level. The maximum APX, CAT and SOD enzyme activities were recorded at 350 level of NaCl with 30ºC HS treatment (Table 2b). The highest APX (492%), CAT (955%), and SOD (606%) activities were found with SA pre-soaked seeds along with 30ºC HS application under 350 mM NaCl stress compared with control.

The impact of sodium chloride toxicity and salicylic acid prompted alleviation was tested by DNA quantification. The six extraction methods such as Dellaporta *et al*., (1983), Doyle and Doyle (1987), Doyle and Doyle (1990), and Saghai-Maroof *et al*., (1984), modified CTAB-based method-1 and modified extraction method-2 were compared to accurately quantify the DNA of green gram under salt toxicity (Table 3). Among six methods, the extracted DNA pellet quantity and quality was found optimum with advanced extraction method (Table 3, Fig. 1). Furthermore, the yield of extracted DNA of advanced extraction method ranged from 404 ng/ µL to 559 ng/µL. Other DNA extraction method like modified CTAB-based method produced somewhat similar range (411-368 ng/µL) of DNA yield. However, four classical methods exhibited less purity ($A260/A230 > 2$) and yield than modified methods (Table 3). Moreover, the extracted samples with other five methods had $A260/A280$ ratio below 1.819, while with the advanced extraction method the ratio of $A260/A280$ range between 1.823-1.887. Similarly, a comparison of each lane in Fig. 1 showed a significant difference in yield and quality of extracted green gram DNA. The clear and a single band was only found with the advanced extraction method (Fig. 1, lane 6^{th}). In the case of RAPD-PCR amplification, the advanced extraction method from the control sample (DW+ 30ºC+ N0) showed well-differentiated, clear, and stable DNA bands which revealed reliability for better PCR outcome (Fig. 2).

Table 2a. DMRt comparison of the three phytohormones pre-soaking influence along with two temperature treatment on growth parameters in NM-92 genotype exposed to different levels of sodium chloride.

Treatments	Seedling length (cm)	Total fresh Biomass (g)	Relative water content (%)	Photosynthetic pigments (mg^{-1} FW)
DW+30+N0	14.5 ± 0.101^{c}	4.5 ± 0.015^{c}	83 ± 0.387^{c}	1.89 ± 0.017^{c}
GA3+30+N0	16.3 ± 0.141^{b}	5.6 ± 0.051^{b}	86 ± 0.398^{b}	2.30 ± 0.030^{b}
IAA+30+N0	17.0 ± 0.171^{b}	6.1 ± 0.091^{a}	87 ± 0.371^{b}	2.66 ± 0.016^{b}
SA+30+N0	17.5 ± 0.182^{a}	6.5 ± 0.103^{a}	89 ± 0.374^{a}	2.87 ± 0.026^{a}
DW+40+N0	13.1 ± 0.134^{d}	4.3 ± 0.022^{c}	82 ± 0.248^{d}	1.39 ± 0.041^{e}
GA3+40+N0	15.0 ± 0.011^{c}	5.1 ± 0.035^{b}	83 ± 0.258^{c}	1.93 ± 0.015^{c}
IAA+40+N0	15.7 ± 0.225^{bc}	5.8 ± 0.012^{b}	85 ± 0.167^{b}	2.35 ± 0.031^{b}
SA+40+N0	16.4 ± 0.122^{b}	6.2 ± 0.013^{a}	86 ± 0.239^{b}	2.53 ± 0.004^{b}
DW+30+N50	11.3 ± 0.236^{d}	4.3 ± 0.010^{c}	82 ± 0.433^{d}	1.35 ± 0.021^{e}
GA3+30+N50	12.6 ± 0.027^{d}	5.1 ± 0.006^{b}	84 ± 0.263^{c}	1.75 ± 0.032^{d}
IAA+30+N50	14.1 ± 0.249^{c}	5.5 ± 0.003^{b}	85 ± 0.265^{b}	1.87 ± 0.025^{c}
SA+30+N50	14.5 ± 0.342^{c}	6.0 ± 0.107^{a}	87 ± 0.344^{b}	2.05 ± 0.033^{c}
DW+40+N50	10.8 ± 0.074^{e}	4.0 ± 0.106^{c}	80 ± 0.119^{e}	1.15 ± 0.051^{f}
GA3+40+N50	11.2 ± 0.201^{d}	4.8 ± 0.005^{c}	81 ± 0.129	1.29 ± 0.014^{e}
IAA+40+N50	11.8 ± 0.135^{d}	5.2 ± 0.014^{b}	83 ± 0.455^{c}	1.50 ± 0.022^{e}
SA+40+N50	12.2 ± 0.182^{d}	5.7 ± 0.103^{b}	85 ± 0.322^{b}	1.71 ± 0.034^{d}
DW+30+N150	10.2 ± 0.257^{e}	4.0 ± 0.002^{c}	80 ± 0.288^{e}	1.20 ± 0.046^{f}
GA3+30+N150	10.7 ± 0.118^{e}	4.6 ± 0.206^{c}	82 ± 0.377^{d}	1.42 ± 0.018^{e}
IAA+30+N150	11.0 ± 0.159^{d}	4.7 ± 0.015^{c}	84 ± 0.345^{c}	1.63 ± 0.023^{d}
SA+30+N150	11.5 ± 0.194^{d}	5.0 ± 0.201^{b}	86 ± 0.211^{b}	1.88 ± 0.047^{c}
DW+40+N150	09.2 ± 0.166^{e}	3.8 ± 0.019^{d}	76 ± 0.213^{f}	1.00 ± 0.035^{f}
GA3+40+N150	09.5 ± 0.217^{e}	3.9 ± 0.202^{d}	77 ± 0.114^{f}	1.15 ± 0.019^{f}
IAA+40+N150	09.9 ± 0.262^{e}	4.4 ± 0.101^{c}	80 ± 0.321^{e}	1.22 ± 0.013^{e}
SA+40+N150	10.1 ± 0.031^{e}	4.8 ± 0.016^{c}	83 ± 0.282^{c}	1.64 ± 0.042^{d}
DW+30+N250	05.9 ± 0.272^{f}	3.4 ± 0.205^{d}	77 ± 0.177^{f}	0.90 ± 0.036^{f}
GA3+30+N250	06.2 ± 0.282^{f}	3.8 ± 0.018^{d}	79 ± 0.245^{f}	1.22 ± 0.024^{e}
IAA+30+N250	07.2 ± 0.311^{f}	4.2 ± 0.001^{c}	81 ± 0.113^{d}	1.41 ± 0.028^{e}
SA+30+N250	08.5 ± 0.291^{f}	4.4 ± 0.204^{c}	83 ± 0.311^{c}	1.59 ± 0.002^{d}
DW+40+N250	04.9 ± 0.054^{g}	3.1 ± 0.121^{d}	73 ± 0.165^{g}	0.66 ± 0.037^{g}
GA3+40+N250	05.7 ± 0.336^{f}	3.7 ± 0.080^{d}	75 ± 0.200^{f}	0.86 ± 0.011^{g}
IAA+40+N250	06.8 ± 0.042^{f}	4.1 ± 0.203^{c}	77 ± 0.198^{f}	0.95 ± 0.003^{f}
SA+40+N250	07.9 ± 0.328^{f}	4.3 ± 0.017^{c}	80 ± 0.187^{e}	1.22 ± 0.020^{e}
DW+30+N350	04.1 ± 0.013^{g}	2.9 ± 0.012^{e}	73 ± 0.111^{g}	0.70 ± 0.038^{g}
GA3+30+N350	04.8 ± 0.068^{g}	3.5 ± 0.117^{d}	76 ± 0.127^{f}	0.90 ± 0.021^{f}
IAA+30+N350	05.1 ± 0.072^{g}	3.8 ± 0.116^{d}	80 ± 0.333^{e}	1.00 ± 0.004^{f}
SA+30+N350	05.9 ± 0.108^{g}	4.1 ± 0.075^{e}	81 ± 0.112^{d}	1.32 ± 0.039^{e}
DW+40+N350	03.0 ± 0.089^{h}	3.0 ± 0.104^{de}	64 ± 0.224	0.53 ± 0.044^{g}
GA3+40+N350	03.2 ± 0.114^{gh}	3.2 ± 0.013^{d}	69 ± 0.323^{g}	0.65 ± 0.027^{g}
IAA+40+N350	03.5 ± 0.015^{gh}	3.6 ± 0.062^{d}	72 ± 0.312^{g}	0.75 ± 0.022^{g}
SA+40+N350	03.9 ± 0.092^{gh}	3.9 ± 0.041^{d}	76 ± 0.110^{f}	1.00 ± 0.010^{f}

Shown data was taken as a mean of four replications. All DMRt values were significant at $p<0.05$. Where, D/W: distilled water, 30: 30°C, N0: 0mM NaCl, GA3: 100 µM gibberellic acid, IAA: 100 µM indole acetic acid, SA: 50 µM salicylic acid, 40: 40°C heat-shock, N50: 50mM NaCl, N150: 150mM NaCl, N250: 250mM NaCl, and N350: 350mM NaCl

Table 2b. DMRt comparison of the three phytohormones pre-soaking influence along with two temperature treatment on biomolecular parameters in NM-92 genotype exposed to different levels of sodium chloride.

Treatments	MDA (µM g^{-1} FW)	PRO (µM g^{-1} FW)	H_2O_2 (µM g^{-1} FW)	APX (µM oxidized ascorbate mg^{-1} protein 2 min^{-1})	CAT (µM H_2O_2 mg^{-1} protein g^{-1} FW min^{-1})	SOD (units mg^{-1} protein 30 min^{-1})
DW+30+N0	08.7±0.112^{e}	13.5±0.016^{e}	09.3±0.182^{d}	090±.0297^{f}	011±0.019^{fg}	017±0.034^{fg}
GA3+30+N0	07.9±0.229^{e}	12.1±0.023^{ef}	07.5±0.101^{e}	106±0.100^{ef}	023±0.010^{f}	026±0.011^{f}
IAA+30+N0	07.0±0.250^{e}	11.0±0.034^{ef}	06.6±0.001^{f}	134±0.109^{e}	028±0.050^{f}	030±0.021^{f}
SA +30+N0	06.6±0.321^{f}	10.7±0.068^{fg}	05.3±0.050^{fg}	176±0.211^{e}	030±0.011^{ef}	035±0.201^{e}
DW+40+N0	10.8±0.381^{d}	14.0±0.324^{e}	10.3±0.013^{d}	085±0.110^{f}	016±0.001^{f}	019±0.392^{f}
GA3+40+N0	10.1±0.213^{d}	13.0±0.341^{ef}	08.6±0.049^{e}	093±0.223^{f}	020±0.052^{f}	024±0.212^{f}
IAA+40+N0	08.0±0.234^{e}	12.0±0.388^{ef}	07.7±0.063^{e}	115±0.312^{e}	022±0.020^{f}	026±0.032^{f}
SA +40+N0	06.9±0.322^{ef}	11.7±0.087^{ef}	06.3±0.002^{f}	164±0.108^{e}	025±0.012^{f}	034±0.383^{ef}
DW+30+N50	11.0±0.236^{e}	15.6±0.355^{e}	09.5±0.041^{d}	144±0.231^{e}	034±0.040^{e}	041±0.192^{e}
GA3+30+N50	08.2±0.326^{e}	13.8±0.067^{e}	08.3±0.051^{e}	189±0.415^{e}	049±0.041^{e}	048±0.223^{e}
IAA+30+N50	07.7±0.254^{e}	13.4±0.041^{e}	07.9±0.003^{e}	231±0.513^{d}	054±0.065^{d}	056±0.047^{d}
SA+30+N50	07.4±0.382^{e}	12.7±0.081^{ef}	06.8±0.020^{f}	266±0.101^{d}	060±0.043^{cd}	065±0.234^{d}
DW+40+N50	11.3±0.214^{e}	17.9±0.543^{cd}	10.9±0.047^{cd}	125±0.242^{e}	031±0.050^{ef}	037±0.185^{e}
GA3+40+N50	10.5±0.238^{d}	15.3±0.748^{e}	09.4±0.010^{d}	140±0.616^{e}	046±0.066^{e}	042±0.376^{e}
IAA+40+N50	08.8±0.327^{e}	14.4±0.762^{e}	08.8±0.032^{de}	199±0.333^{e}	050±0.009^{d}	050±0.050^{d}
SA+40+N50	08.4±0.255^{e}	13.4±0.409^{e}	07.4±0.019^{f}	212±0.107^{d}	055±0.078^{d}	062±0.178^{d}
DW+30+N150	11.8±0.387^{e}	17.7±0.280^{d}	10.7±0.004^{d}	218±0.712^{d}	058±0.002^{d}	055±0.249^{d}
GA3+30+N150	08.9±0.198^{e}	15.8±0.721^{e}	09.4±0.052^{d}	277±0.861^{d}	065±0.057^{e}	064±0.061^{d}
IAA+30+N150	08.4±0.240^{e}	14.0±0.099^{e}	08.8±0.046^{e}	321±0.815^{e}	071±0.081^{e}	074±0.253^{e}
SA+30+N150	07.8±0.328^{e}	13.8±0.283^{e}	07.2±0.011^{ef}	374±0.913^{e}	075±0.412^{e}	088±0.362^{e}
DW+40+N150	11.7±0.256^{e}	19.5±0.632^{e}	12.8±0.033^{e}	164±0.982^{e}	051±0.521^{d}	049±0.072^{d}
GA3+40+N150	11.0±0.385^{e}	16.9±0.082^{d}	10.5±0.048^{d}	207±0.102^{de}	058±0.003^{d}	058±0.161^{d}
IAA+40+N150	10.0±0.113^{d}	16.1±0.341^{d}	09.8±0.005^{d}	268±0.225^{d}	062±0.111^{e}	066±0.263^{d}
SA+40+N150	09.4±0.241^{d}	16.0±0.022^{d}	08.4±0.014^{e}	313±0.343^{cd}	067±0.147^{e}	077±0.080^{e}
DW+30+N250	12.1±0.252^{e}	22.2±0.245^{bc}	11.5±0.035^{e}	325±0.357^{e}	068±0.551^{e}	074±0.159^{e}
GA3+30+N250	11.3±0.321^{e}	19.6±0.085^{e}	10.0±0.012^{d}	387±0.178^{e}	077±0.004^{e}	081±0.274^{e}
IAA+30+N250	09.1±0.118^{d}	16.6±0.021^{d}	09.5±0.006^{d}	412±0.187^{bc}	085±0.121^{b}	089±0.098^{e}
SA+30+N250	08.8±0.243^{e}	15.1±0.038^{d}	07.7±0.045^{e}	465±0.103^{b}	092±0.642^{b}	098±0.355^{b}
DW+40+N250	12.9±0.253^{b}	25.5±0.063^{b}	13.0±0.037^{bc}	250±0.116^{d}	061±0.132^{cd}	066±0.287^{e}
GA3+40+N250	11.6±0.354^{e}	22.2±0.622^{bc}	11.2±0.018^{cd}	312±0.135^{cd}	069±0.030^{e}	072±0.106^{e}
IAA+40+N250	10.5±0.246^{d}	19.7±0.001^{e}	10.1±0.009^{d}	354±0.873^{e}	072±0.643^{e}	083±0.296^{e}
SA+40+N250	10.1±0.259^{d}	17.1±0.023^{d}	09.3±0.053^{d}	396±0.446^{e}	083±0.005^{b}	090±0.117^{bc}
DW+30+N350	13.1±0.387^{b}	24.9±0.042^{b}	13.6±0.039^{b}	410±0.104^{bc}	071±0.142^{e}	084±0.305^{e}
GA3+30+N350	12.9±0.247^{b}	22.6±0.043^{b}	11.6±0.054^{e}	466±0.473^{b}	084±0.672^{b}	099±0.128^{b}
IAA+30+N350	11.9±0.369^{e}	18.4±0.011^{e}	10.4±0.016^{d}	498±0.567^{b}	096±0.158^{b}	108±0.314^{b}
SA+30+N350	11.4±0.367^{e}	17.1±0.661^{d}	09.9±0.008^{d}	533±0.868^{a}	116±0.006^{a}	120±0.349^{a}
DW+40+N350	15.7±0.248^{a}	34.5±0.712^{a}	15.5±0.043^{a}	328±0.105^{e}	065±0.185^{e}	077±0.130^{e}
GA3+40+N350	14.3±0.362^{b}	28.1±0.012^{b}	14.3±0.044^{b}	369±0.343^{e}	072±0.148^{e}	091±0.321^{cb}
IAA+40+N350	13.9±0.112^{b}	23.9±0.172^{b}	12.0±0.017^{e}	399±0.566^{e}	086±0.007^{b}	097±0.142^{b}
SA+40+N350	13.1±0.249^{b}	20.7±0.127^{bc}	10.3±0.055^{d}	420±0.106^{b}	090±0.196^{b}	110±0.332^{b}

Footnote is same as Table 2a except MDA: Malondialdehyde, PRO: Proline, H_2O_2: Hydrogen peroxide, APX: Ascorbate peroxidase, CAT: Catalase, and SOD: Superoxide dismutase

Table 3. Purity and yield of extracted DNA by six methods from distilled water (DW) and salicylic acid treated NM-92 green gram seedlings under salt toxicity.

Treatments	Purity of DNA		DNA yield (ng/µL)	Treatments	Purity of DNA		DNA yield (ng/µL)
	A260/280	A260/230			A260/280	A260/230	
	Dellaporta *et al*., (1983)				**Saghai-Maroof *et al*., (1984)**		
DW+30+N0	1.080	2.011	119	DW+30+N0	1.753	2.602	266
DW+30+N50	1.230	2.411	123	DW+30+N50	1.760	2.520	268
DW+30+N150	1.112	2.564	182	DW+30+N150	1.670	2.928	224
DW+30+N250	1.650	2.641	174	DW+30+N250	1.755	2.980	219
DW+30+N350	1.432	2.630	126	DW+30+N350	1.754	2.900	200
SA+30+N0	1.672	2.601	113	SA +30+N0	1.713	2.920	276
SA+30+N50	1.605	2.546	153	SA+30+N50	1.738	2.730	250
SA+30+N150	1.617	2.566	147	SA+30+N150	1.760	2.410	244
SA+30+N250	1.246	2.511	128	SA+30+N250	1.757	2.360	220
SA+30+N350	1.310	2.577	198	SA+30+N350	1.724	2.154	163
	Doyle & Doyle (1987)				**Modified CTAB-based method**		
DW+30+N0	1.601	2.610	200	DW+30+N0	1.804	1.964	407
DW+30+N50	1.625	2.520	199	DW+30+N50	1.780	1.999	395
DW+30+N150	1.662	3.581	187	DW+30+N150	1.770	2.986	393
DW+30+N250	1.690	3.001	165	DW+30+N250	1.710	2.095	375
DW+30+N350	1.619	2.443	294	DW+30+N350	1.719	2.090	368
SA +30+N0	1.607	2.553	206	SA+30+N0	1.809	1.580	411
SA +30+N50	1.603	2.557	203	SA+30+N50	1.819	2.147	400
SA +30+N150	1.611	2.588	164	SA+30+N150	1.792	1.925	394
SA +30+N250	1.507	2.532	158	SA+30+N250	1.807	1.976	390
SA +30+N350	1.599	2.591	156	SA+30+N350	1.747	2.298	374
	Doyle & Doyle (1990)				**Advanced extraction method**		
DW+30+N0	1.690	2.819	141	DW+30+N0	1.839	1.519	555
DW+30+N50	1.612	2.560	111	DW+30+N50	1.886	1.701	444
DW+30+N150	1.611	2.527	124	DW+30+N150	1.849	1.733	436
DW+30+N250	1.612	2.644	156	DW+30+N250	1.843	1.762	432
DW+30+N350	1.621	2.543	100	DW+30+N350	1.845	1.890	416
SA +30+N0	1.576	2.519	101	SA+30+N0	1.885	1.610	559
SA +30+N50	1.533	2.536	150	SA+30+N50	1.887	1.700	473
SA +30+N150	1.644	2.700	133	SA+30+N150	1.840	1.728	472
SA +30+N250	1.489	2.598	190	SA+30+N250	1.846	1.823	413
SA +30+N350	1.654	2.866	126	SA+30+N350	1.823	1.760	404

Shown data was taken as the mean of triplicate. All DMRt values were significant at $p<0.05$. Where, DW=distilled water, 30=30°C, N0=0mM NaCl, N50=50mM NaCl, N150=150mM NaCl, N250=250mM NaCl, and N350=350mM NaCl, and SA=50 µM salicylic acid

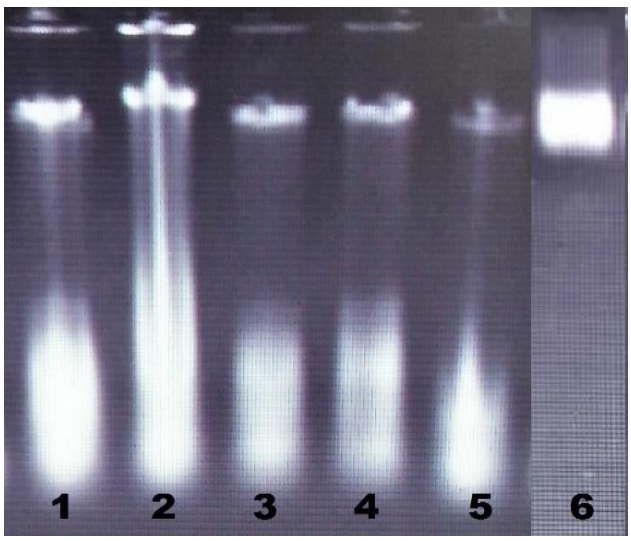

Fig. 1. Agarose gel electrophoresis 0.8% showed the quality of six used extraction methods from 24 h DW pre-soaked with 0 mM NaCl (144 h) treated NM-92 seedlings, where Lane 1=modified CTAB-based method-1; Lane 2=Dellaporta *et al*., (1983); Lane 3= Doyle & Doyle (1987); Lane 4= Doyle & Doyle (1990); Lane 5= Saghai-Maroof *et al*., (1984); and Lane 6= modified extraction method-2.

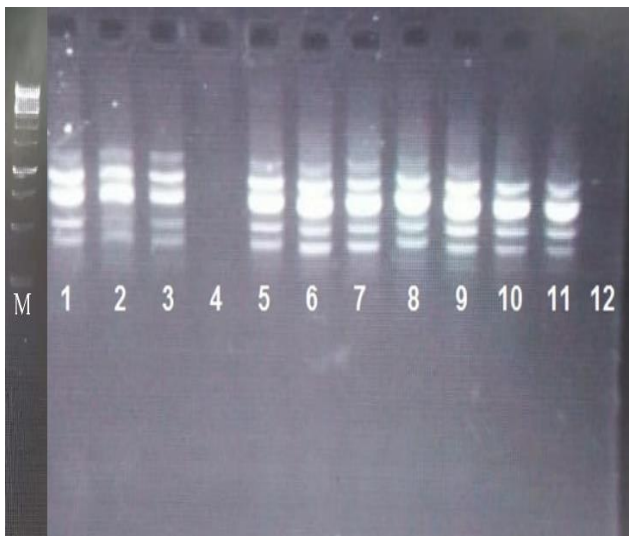

Fig. 2. RAPD Gradient PCR products resolved on 1.5% agarose gel from 24 h DW pre-soaked then 0 mM NaCl (144 h) treated NM-92 green gram seedlings generated by OPA-12: 5'–TGCCGAGCTC–3', where Lane M= GeneRuler 1 kb DNA ladder; Lane 1=30.0ºC; Lane 2=30.5ºC; Lane 3=30.9ºC; Lane 4=31.6ºC; Lane 5=32.3ºC; Lane 6=33.1ºC; Lane 7=33.9ºC; Lane 8=34.7ºC; Lane 9=35.4ºC; Lane 10=36.1ºC; Lane 11=36.6ºC; and Lane 12=37.0ºC.

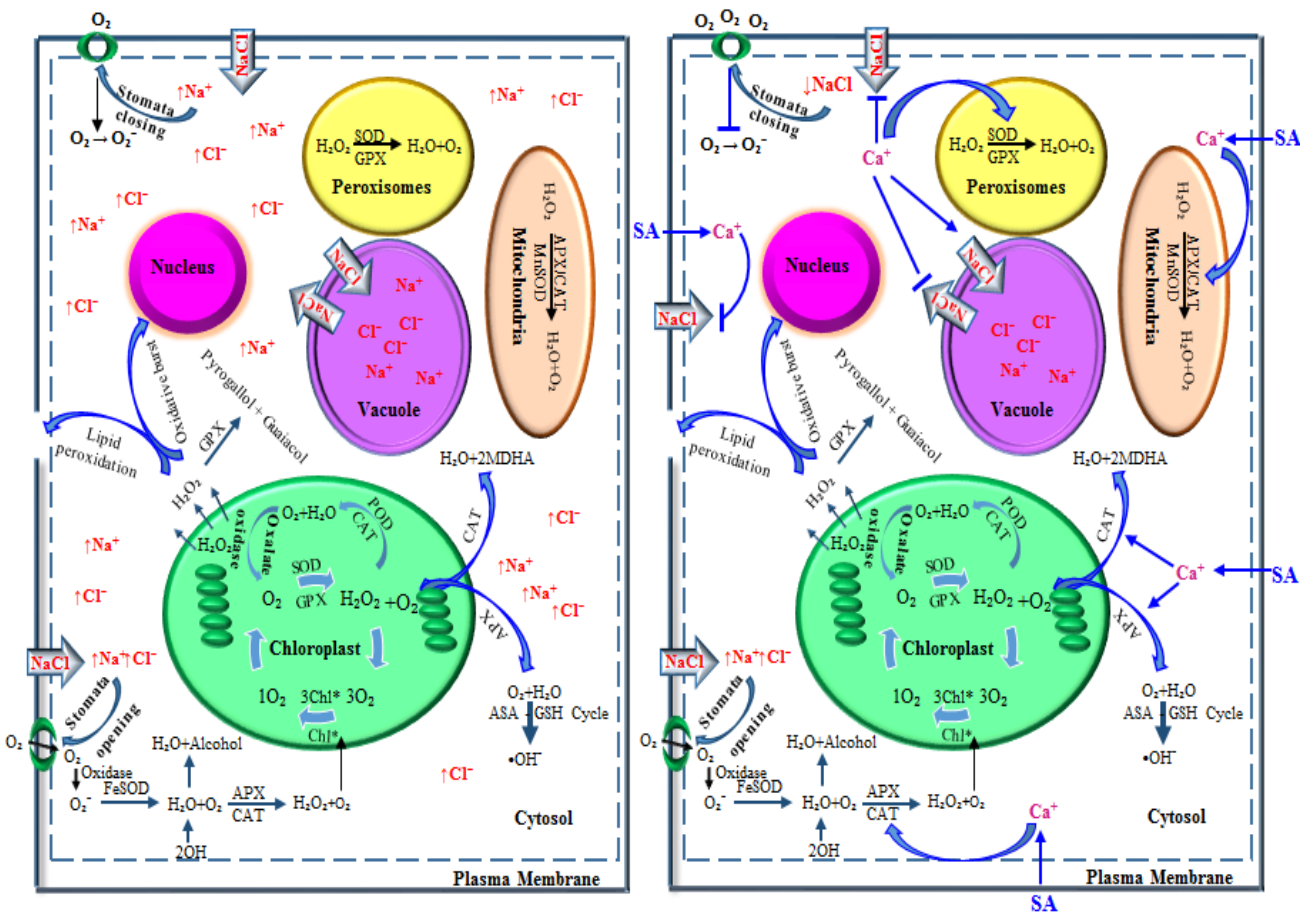

Fig. 3. Biosynthesis of hydrogen peroxide (left-hand side figure) and its degradation through SA signaling under salt stress (right-hand side figure). Exogenously applied SA activates calcium (Ca^{+}) molecules which deactivate the influx of Na^{+} & Cl^{-} ions in the cell. APX: ascorbate peroxidase; ASA-GSH: ascorbate-glutathione; CAT: catalase; Chl*: excited-state chlorophyll; 3Chl*: triplet excited-state chlorophyll; FeSOD: iron-containing superoxide dismutase; GPX: guaiacol peroxidase; H_2O: pure water; MDHA: Monodehydroascorbate; MnSOD: manganese-containing superoxide dismutase; NaCl: sodium chloride; O_2: oxygen; O_2^{-}: superoxide radical; $^1O_2$: singlet oxygen; $^3O_2$: triplet oxygen; $\bullet OH^{-}$: hydroxyl radical; POD: peroxidase; and SOD: superoxide dismutase.

Discussion

Sodium chloride toxicity induced a gradual reduction in green gram growth such as SL, TFB, RWC, and PP (Table 2a). These outcomes are in concurrence by Isik (2022), Farheen *et al*., (2018), and Yanik *et al*., (2018), who demonstrated that growth and photosynthetic machinery destruct by the application of sodium chloride. Qirat *et al*., (2018), reported that the wild barley growth and PP were decreased due to the application of excess sodium chloride. These adverse impacts might be credited to the non-accessibility of water, which concentrate Na^+ and Cl^- ions that causing ion toxicity in green gram seedlings. Whereas, reduced PP maybe related to the increasing activity of proteolytic enzyme Chlorophyllase which destroys PP as a result seedling lose its pigments (Kaya *et al*., 2013). Application of phytohormones or HS prior to NaCl treatment alleviated the toxic effect of sodium chloride, but among all, SA was found to show better improvement. Whilst, SA mitigated the hazardous effect of NaCl over *Vigna radiata* seedlings. Fayez & Bazaid (2014) also reported betterment in morpho-physiology of barley with 50 µM SA under various levels of NaCl.

The MDA, PRO, and H_2O_2 are frequently used as functional tool to measure the severity of salt toxicity caused by reactive oxygen species (ROS). Among these, MDA is accountable for lessening enzymes activities, the fluidity of lipid bilayer, leakiness of membrane proteins, and ion channel (Ghosh *et al*., 2015). Present results confirmed the prominent enhancement in MDA at all levels of NaCl which suggested cellular membrane destruction in green gram seedling (Table 2b). In contrast, reduction in MDA content seemed to be a SA, IAA, and GA3 pre-soaking impact caused acclimation against salt toxicity. Resembling outcomes have already been reported by Shereen *et al*., (2022) from rice, Ashraf *et al*., (2013) from green gram, Yanik *et al*., (2018) from rye, and Fayez & Bazaid (2014) from barley.

The PRO is an amino acid produced in ample quantity in the cells under salt stress (Ghosh *et al*., 2015; Chunthaburee *et al*., 2016). Thus, it has been recommended that PRO aggregation under salt toxicity is a consequence of a drop in osmotic potential (Abbaspour, 2012), which is the result of growth hindrance (Chunthaburee *et al*., 2016). It was likewise noted that the SA pre-soaking before inducing salt toxicity exhibited a reduction in PRO content compared to IAA, GA3, and sodium chloride stressed treatments at both HS levels (Table 2b). These observations are in conformity with the previous researchers who reported that SA-spray together with NaCl showed a decline in PRO level in the tested plant (Hussain *et al*., 2011). Furthermore, the excessive accumulation of PRO neither protect plants from salt stress nor acquire salt tolerance (Chunthaburee *et al*., 2016). Hence, PRO over-accumulation was related to a symptom of salt injury rather than an indicator of salt resistance (Lutts *et al*., 1999). Thus, it is suggested that the decrease in MDA, PRO, and H_2O_2 by SA+30ºC HS+N0 application indicates the alleviation of salt stress which decreases membrane damage in plant cells (Table 2b). Likewise, the APX, CAT, and SOD activities were increased as NaCl induce stress. That may explain the active participation of APX, CAT, and SOD enzymes in the detoxification process of major ROS like H_2O_2 in green gram seedlings under NaCl-induced toxicity. Related findings have been found in salt-treated green gram (Ghosh *et al*., 2015), carrot (Qirat *et al*., 2018), and rye (Yanik *et al*., 2018). Moreover, an exogenous application of SA overwhelmed the damaging impact of NaCl on the cell machinery especially double-strand break of DNA, protein misfolding and lipid bilayer degradation. That may be due to alteration in the endogenous SA level which increases the biosynthesis of antioxidant enzymes which protect DNA from its damage. Likewise, the enhanced expression of mRNA of APX, CAT and, SOD genes was also revealed under NaCl stress (Alharby *et al*., 2016; Arias-Moreno *et al*., 2017) that further vindicated current study. Thus, salicylic acid plays an essential role in regulating the cellular redox equilibrium and protect the development of green gram under oxidative hurts through improving resistance. Based on current analysis, the model for salt-induced toxicity signaling pathway is explained (Fig. 3).

The relatively fast, cost-effective, and high-quality DNA extraction technique is always the need of the day. Plants have several kinds of polyphenols, lipids, proteins, and polysaccharides which create deterrent during extraction and high-quality of DNA (Sahu *et al*., 2012; Abdel-Latif & Osman, 2017). The previous protocols were mainly based on toxic CTAB, expensive liquid nitrogen, and hectic prolong heating (65ºC) steps that yield low-quality DNA. For this purpose in the current study the advanced extraction method based on Dellaporta *et al.*, (1983) was used to determine the salt toxicity in green gram without any expensive chemical and least risky steps. The advanced extraction method utilized pre-chilled mortar, pestle and green gram samples, an ideal alternative to expensive liquid nitrogen (Abdel-Latif & Osman, 2017; Aboul-Maaty & Oraby, 2019). Also, the incorporation of 0.15% 2-mercaptoethanol successfully eliminated polyphenol or phenol (Sahu *et al*., 2012; Aboul-Maaty & Oraby, 2019), which occurred in the plant genome. While, the 0.5 M concentration of NaCl, 3 M Na acetate, and ice-chilled 2-propanol improved the purity and quantity of extracted pellet and successfully eliminate polysaccharides and secondary metabolites from DNA pellet (Sahu *et al*., 2012; Abdel-Latif & Osman, 2017; Aboul-Maaty & Oraby, 2019). Further, the presence of NaCl in the extraction buffer also elevated the solubility and co-precipitation of secondary metabolites during the precipitation of pellet with absolute ethanol (Abdel-Latif & Osman, 2017). Likewise, the high proportion of Urea, SDS and appropriate quantity of Tris-EDTA in extraction buffer helped cell lysis and deactivated the DNase and RNase activities which protect DNA from its degradation. Moreover, the lipids, proteins, and cellular impurities were removed from samples through Chloroform: Isoamyl alcohol (Aboul-Maaty & Oraby, 2019). Also, to enhance the storage duration of extracted DNA, the pellet was resuspended in the ddH_2O instead of TE buffer. The TE buffer contained a chelating agent which affected all the molecular analysis (Aboul-Maaty & Oraby, 2019).

The purity of extracted pellet which revealed the presence of polyphenols is a pivotal parameter for any DNA-based analysis (Aboul-Maaty & Oraby, 2019). Further, the presence of polyphenols declines the quality and quantity of DNA pellet. Moreover, the purity of extracted samples with Dellaporta *et al*., (1983), Doyle & Doyle (1987), Doyle & Doyle (1990), and Saghai-Maroof *et al*., (1984) protocols were not satisfactory because almost all samples showed less than 1.760 ratios at $A260/A280$, i.e. under the prime ratio of 1.8 (Sahu *et al*., 2012; Abdel-Latif & Osman, 2017; Aboul- Maaty & Oraby, 2019). A similar value of DNA purity (1.8) has been observed with advanced extraction methods followed by a modified CTAB-based method which indicated the protein and polyphenol free extraction (Table 3). Likewise, DNA samples purity was also evaluated at $260/230\ nm$ which is known as a secondary measure of DNA pellet purity (Wilson & Walker, 2005). The absorbance ration of $260/230\ nm$ revealed the contamination of salts residues and polysaccharides in the extracted pellet and recommended range of value is 1.5 to 1.8 (Aboul-Maaty & Oraby, 2019). Below 1.5 and above 1.8 shows the trace of contamination in the extracted DNA pellet. Therefore, in the present analysis, the advanced extraction method followed the recommended range of values at $260/230\ nm$ wavelength than other examined methods that showed more than 1.9 value (Table 3). Current outcomes are well-confirmatory with the findings of Sahu *et al*., (2012) and Abdel-Latif & Osman (2017).

Out of six extraction techniques, Dellaporta *et al*., (1983), Doyle & Doyle (1987), Doyle & Doyle (1990), and Saghai-Maroof *et al*., (1984) extraction methods showed shared DNA bands on agarose gel due to the presence of high level of proteins, polysaccharide, and polyphenols in the pellet (Fig. 1). These contaminants caused hindrance in the very first step of DNA analysis and extraction, and later on showed brown pellet of DNA. Also, these 4 classical methods gave low DNA yield. Similar observation have been reported with Doyle and Doyle methods (Sahu *et al*., 2012; Abdel-Latif & Osman, 2017). Sahu *et al*., (2012), indicated that the appearance of the brownish hue of extracted pellet showed contamination of polyphenols. Additionally, the ratio greater than 1.9 at $260/280\ nm$ wavelength indicated the presence of RNA contamination (Aboul-Maaty & Oraby, 2019). However, intact at the top and a single DNA band (Fig. 1, Lane 6^{th}) on 0.8% agarose gel showed a high level of purity or RNA-free extraction that was suitable for PCR amplification. The supported finding was reported by previous researchers, who documented that the contamination of RNA could be better identified through agarose gel electrophoresis, where the appearance of another band proved the presence of RNA in the samples (Aboul-Maaty & Oraby, 2019). Furthermore, smear at the bottom of the gel indicated the degradation or sharing of DNA structure that directly affected the quality of PCR analysis (Devi *et al*., 2013; Aboul-Maaty & Oraby, 2019). While green gram sample extracted with advanced extraction method showed successful RAPD-PCR (Fig. 2). The distinct, and clear RAPD bands' appearance reflected the efficacy of the advance method which made it suitable for PCR-based molecular examination (Devi *et al*., 2013).

Conclusions

Sodium chloride concentration from 50 mM to 350 mM considerably reduced SL, TFB, RWC, PP, and MDA content. However, it was found that 350 mM NaCl had the most adverse effect on the morpho-biochemistry and DNA profile of green gram seedlings. While, among the phytohormones pre-soaking treatments, the SA at 30°C prior to salt stress significantly reduced the perilous effect of salt toxicity in green gram and improve growth and antioxidant enzymes compared to efficiently control. Thus, it is recommended that the pre-imbibed seeds of 50 µM salicylic acid may be used to overcome the adverse impact of salt stress over a green gram. Further, the advanced DNA extraction method proved better than the previously described method for PCR-based amplification of complex plant genome due to its simple steps and without using toxic and expensive chemicals such as CTAB, Proteinase K, and liquid nitrogen that made it ideal for large sample extraction stored over a year. Furthermore, it is very useful for toxicity estimation, whole-genome sequencing, characterization of plant population, marker-assisted PCR-based analysis to identify the genetics tolerance, and diversity of plants.

Acknowledgments

The authors are very thankful to Dr M. Arsalan Ashraf (Assistant Professor, Department of Botany, Government College University, Faisalabad, Pakistan) for his very kind help in computing H_2O_2 and MDA contents.

References:

Abbas, G., A. Hameed, M. Rizwan, M. Ahsan, M.J. Asghar and N. Iqbal. 2015. Genetic Confirmation of mungbean (*Vigna radiata*) and mashbean (*Vigna mungo*) interspecific recombinants using molecular markers. *Front. Plant Sci.*, 6, Article ID 1107. https://doi.org/10.3389/fpls.2015.01107

Abbaspour, H. 2012. Effect of salt stress on lipid peroxidation, antioxidative enzymes, and proline accumulation in pistachio plants. *J. Med. Plants Res*., 6(3): 526-529. DOI: 10.5897/JMPR11.1449

Abdel-Latif, A. and G. Osman. 2017. Comparison of three genomic DNA extraction methods to obtain high DNA quality from maize. *Plant Methods*, 13, Article ID 1. https://doi.org/10.1186/s13007-016-0152-4

Aboul-Maaty, N.A.F. and H.A.S. Oraby. 2019. Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. *Bull. Natl. Res. Cent.*, 43, Article ID 25. https://doi.org/10.1186/s42269-019-0066-1.

Alharby, H.F., E.M.R. Metwali, M.P. Fuller and A.Y. Aldhebiani. 2016. The alteration of mRNA expression of SOD and GPX genes, and proteins in tomato (*Lycopersicon esculentum* Mill) under stress of NaCl and/or ZnO nanoparticles. *Saudi J. Biol. Sci*., 23: 773-781. DOI: 10.1016/j.sjbs.2016.04.012

Arias-Moreno, D.M., J.F. Jiménez-Bremont, I. Maruri-López and P. Delgado-Sánchez. 2017. Effects of catalase on chloroplast arrangement in *Opuntia streptacantha* chlorenchyma cells under salt stress. *Sci. Rep.,* 7(1): Article ID 8656. DOI: 10.1038/s41598-017-08744-x

Ashraf, M., A.M. Rasool, Q. Ali, M.Z. Haider, A. Noman and M. Azeem. 2013. Salt-induced perturbation in growth, physiological attributes, activities of antioxidant enzymes

and organic solutes in mung bean (*Vigna radiata* L.) cultivars differing in salinity tolerance. *Arch. Agron. Soil Sci*., 59(12): 1695-1712. https://doi.org/10.1080/03650340.2012.758840

Bates, L.S., R.P. Waldren and I.D. Teare. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil*, 39: 205-207. https://doi.org/10.1007/BF00018060

Carmak, I. and W.H. Horst. 1991. Effects of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (*Glycine max*). *Physiol. Plant.*, 83(3): 463-468. https://doi.org/10.1111/j.1399-3054.1991.tb00121.x

Chunthaburee, S., A. Dongsansuk, J. Sanitchon, W. Pattanagul and P. Theerakulpisut. 2016. Physiological and biochemical parameters for evaluation and clustering of rice cultivars differing in salt tolerance at seedling stage. *Saudi J. Biol. Sci.*, 23: 467-477. DOI: 10.1016/j.sjbs.2015.05.013

Dellaporta, S.L., J. Wood and J.B. Hicks. 1983. A plant DNA minipreparation; version II. *Plant Mol. Biol. Rep.*, 1(4): 19-21. https://doi.org/10.1007/BF02712670

Devi, K.D., K. Punyarani, S. Singh and H.S. Devi. 2013. An efficient protocol for total DNA extraction from the members of order Zingiberales - suitable for diverse PCR based downstream applications. *Springer Plus*, 2, Article ID 669. https://doi.org/10.1186/2193-180-2-669.

Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.*, 19: 11-15.

Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. *Focus*, 12: 13-15.

Anonymous. 2010. Food and agriculture organization land and plant nutrition management service. http://www.fao.org/ag/agl/agll/spush (accessed 21 October 2020)

Farheen, J. and S. Mansoor. 2019. Cytogenetic impact of sodium chloride stress on root cells of *Vigna radiata* L. seedlings. *Turk. J. Biochem.*, 45(2): Article ID 20180352. https://doi.org/10.1515/tjb-2018-0352.

Farheen, J., S. Mansoor and Z. Abideen. 2018. Application of salicylic acid improved growth, photosynthesis pigments and oxidative stability in mungbean seedlings at salt stress. *Pak. J. Bot*., 50(3): 901-912.

Fayez, K.A. and S.A. Bazaid. 2014. Improving drought and salinity tolerance in barley by application of salicylic acid and potassium nitrate. *J. Saudi Soc. Agric. Sci.*, 13: 45-55. http://doi.org/10.1016/j.jssas.2013.01.001

Ghosh, S., S. Mitra and A. Paul. 2015. Physiological studies of sodium chloride on mungbean (*Vigna radiata* L. Wilczek) and its possible recovery with spermine and gibberellic acid. *Sci. World J.*, Article ID 858016. https://doi.org/10.1155/2015/858016

Hossain, M.A., M.G. Mostofa and M. Fujita. 2013. Heat-shock positively modulates oxidative protection of salt and drought-stressed mustard (*Brassica campestris* L.) seedlings. *J. Plant Sci. Mol. Breed*., 2, Article ID 2. DOI: 10.7243/2050-2389-2-2

Hussain, K., K. Nawaz, Abdul Majeed, U. Ilyas, Fenglin, K. Ali and M.F. Nisar. 2011. Role of exogenous salicylic acid and applications for salt tolerance in violet. *Sarhad J. Agric.*, 27(2): 171-175.

Isik, G. 2022. Ecophysiological responses of *Solanum lycopersicum* L. to different levels of salt stress. *Pak. J. Bot.,* 54(1): 1-5. DOI: http://dx.doi.org/10.30848/PJB2022-1(20):

Jini, D. and B. Joseph. 2017. Physiological mechanism of salicylic acid for alleviation of salt stress in rice. *Rice Sci*., 24(2): 97-108. http://doi.org/10.1016/j.rsci.2016.07.007

Kaya, C., M. Ashraf, M. Dikilitas and A.L. Tuna. 2013. Alleviation of salt stress-induced adverse effects on maize plants by exogenous application of indole acetic acid (IAA) and inorganic nutrients – A field trial. *Aust. J. Crop Sci*., 7(2): 249-254.

Kumar, S., A. Raj, J. Prakash, M. Kumar and N.K. Shukla. 2016. Evaluation of RAPD technique to induced DNA damage by heavy metal to detect the genotoxic effect on mung bean (*Vigna radiata* (L.) wilczek) seedlings. *Int. J. Appl. Res. Technol.*, 1(1): 1-12.

Lutts, S., V. Majerus and J.-M. Kinet. 1999. NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. *Physiol. Plant*., 105(3): 450-458. https://doi.org/10.1034/j.1399-3054.1999.105309.x

Machado, R.M.A. and R.P. Serralheiro. 2017. Soil salinity: effect on vegetable crop growth. Management practices to prevent and mitigate soil salinization. *Horticulturae*, 3: Article ID 30. https://doi.org/10.3390/horticulturae3020030.

Mansoor, S. and F.N. Naqvi. 2013. Effect of heat stress on lipid peroxidation and antioxidant enzymes in mung bean (*Vigna radiata* L.) seedlings. *Afr. J. Biotechnol*., 12(21): 3196-3203.

Qirat, M., M. Shahbaz and S. Perveen. 2018. Beneficial role of foliar-applied proline on carrot (*Daucus carota* L.) under saline conditions. *Pak. J. Bot*., 50(5): 1735-1744.

Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorgensen and R.W. Allard. 1984. Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamic. *Proc. Natl. Acad. Sci. USA*., 81(24): 8014-8018. DOI: 10.1073/pnas.81.24.8014

Sahu, S.K., M. Thangaraj and K. Kathiresan. 2012. DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. *Intl. Schol. Res. Net. Mol. Biol*., Article ID 205049. https://doi.org/10.5402/2012/205049.

Sehrawat, N., K.V. Bhat, A. Kaga, N. Tomooka, M. Yadav and P.K. Jaiwal. 2014. Development of new gene-specific markers associated with salt tolerance for mungbean (*Vigna radiata* (L.) Wilczek). *Span. J. Agric. Res.*, 12(3): 732-741. DOI: 10.5424/sjar/2014123-4843

Shahid, S.A., M. Zaman and L. Heng. 2018. Soil salinity: historical perspectives and a world overview of the problem. In: *Guideline for salinity assessment, mitigation and adaptation using nuclear and related techniques*. Springer, Cham, pp. 43-53. https://doi.org/10.1007/978-3-319-96190-3_2

Shereen, A., Asma, M.U. Shirazi, M.A. Khan, M. Ali and M. Arif. 2022. Physio-biochemical analysis of salinity tolerance in sodium contrasting rice (*Oryza sativa* L.) genotypes. *Pak. J. Bot.*, 54(3): 787-794. DOI: http://dx.doi.org/10.30848/PJB2022-3(15)

Steel, R.G.D. and J.H. Torrie. 1997. *Principles and procedures of statistics*: *A biometrical approach*: Edition: 3. McGraw Hill Book Co., Singapore. https://trove.nla.gov.au/version/26560275.

Velikova, V., I. Yordanov and A. Edreva. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants, Protective role of exogenous polyamines. *Plant Sci*., 151(1): 59-66. https://doi.org/10.1016/S0168-9452(99)00197-1

Wilson, K. and J. Walker. 2005. Principles and techniques of biochemistry and molecular biology. Edition: 6. Cambridge University Press, U.K. DOI: https://doi.org/10.1017/CBO9780511813412

Yanik, F., O. Aytürk, A. Cetinbaş-Genç and F. Vardar. 2018. Salicylic acid-induced germination, biochemical and developmental alterations in rye (*Secale cereale* L.). *ACTA Bot. Croat*., 77(1): 45-50. DOI: 10.2478/botcro-2018-0003

(Received for publication 22 March 2021)