

Regulation of NPR1 Under Salinity and Osmotic Stress in Soybean (*Glycine max* L.) Leaves

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Aim of study: Non expressor of pathogenesis related gene (NPR1) is a key regulator of the SA dependent systemic acquired resistance (SAR) in plants. Although NPR1 is a well known important regulator of salicylic acid to biotic stress, researching on abiotic stress have not yet been well founded.

Materials and methods: With this aim, soybean (*Glycine max* L.) SA88 plants were grown with Hoagland solution for two weeks. Seedlings were treated with 200 mM NaCl, 10 % PEG 6000 and 200 mM NaCl + 10 % PEG 6000 and harvested at 2h, 4h, 6h (short term) and 7 day and 14 day (long term) of treatment.

Main results: The results showed that plants treated with NaCl showed a better defense response in physiological parameters than PEG. Additionally, PEG stress lead to more oxidative damage at long term, while combined stress at short term in soybean leaves. Otherwise, the highest accumulation of ABA, SA and proline level was with PEG treatment at both short term and long term of treatment. However, *GmNPR1* gene expressions were upregulated with PEG stress significantly at 7 day and combined stress at 14 day.

Discussion: Considering the short term effects on *GmNPR1* transcript levels, combined stress were more pronounced compared to NaCl and PEG stress alone.

Research highlights: Consequently, this work firstly determined that osmotic stress may role as a potential signal but not salt stress for the regulation of *NPR1* in soybean leaves.

Key words: *NPR1*, osmotic stress, salt stress, soybean

Salinity and osmotic stress are regarded as two important abiotic stress factors reduces plant growth and development. Under salt stress, plants accumulate excess Na^+ and Cl^- , which leads to limited water and nutrient uptake, altered metabolic and photosynthetic activity, and increased lipid peroxidation (Deinlein *et al.*, 2014; Liang *et al.*, 2018). Osmotic stress induces detrimental effects on crop productivity by arresting numerous plant metabolic processes such as loss of turgor, carbon assimilation rate, leaf gas exchange and increased oxidative damage. Although they have similar effects on plant metabolism, it has been suggested that the plants suffers from osmotic stress firstly then later affected by salinity (Munns *et al.* 2002). They have to cope with two these detrimental factors at the same time and consume more energy to survive.

Both stress increases the production of various forms of reactive oxygen species (ROS) such as superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radical in plants. The excessive ROS generation is found in the stressed plants and induces decomposition of membranes, inactivation of enzymes and alteration in gene expression leading to cell death (Singh *et al.* 2019). The ROS are scavenged by enzymatic and non enzymatic antioxidants to protect plants from stress. Also it has been known that ROS are regulated by the hormonal (SA, ABA, JA) signaling pathway (Herrera-Vasquez *et al.* 2015).

The phytohormone salicylic acid (SA) is a signaling molecule in regulating plant growth and development. Also it plays role in defence responses against biotic and abiotic stress factors such as pathogen infection, salinity, drought, heavy metals and chilling. In recent years, it was determined that exogenous addition of SA can improve photosynthetic capacity, enhance antioxidant enzymes, increase accumulation of soluble contents and maintain ion balance (K^+/Na^+) in many plants species (Hayat *et al.*, 2010). Beside this, SA stimulates the root development and stomata closure as well as induces the expression of defense genes and secondary metabolites (Miura and Tada, 2014).

Non expressor of pathogenesis-related genes 1 (NPR1) was detected firstly in the study of Arabidopsis

mutants against pathogens (Cao *et al.*, 1997). NPR1 is a key regulator of systemic acquired resistance (SAR) and regulates the salicylic acid (SA) dependent genes. NPR1 is an important regulator of responses downstream of SA (Mou *et al.*, 2003; Zhang *et al.*, 2003). During SA mediated plant defense responses, oligomeric NPR1 in cytoplasm is reduced to monomeric NPR1 and translocated to nucleus by thioredoxins (TRX) (Herrera Vaquez *et al.*, 2015). SA is essential for NPR1 redox modification but this is still not well explained. Given that, SA involves in the regulation of salt tolerance mechanisms by the activation of plant defense responses, NPR1 might play role in mediating defence responses, induction antioxidant defence system, redox signaling and maintaining hormone level.

Although the role of NPR1 has been determined under pathogen stress, some studies suggested that NPR1 is also effective on abiotic stress such as salinity, drought and heavy metal in plants. Only a few researchers underlined the importance of NPR1 but there are contraversial results in literature (Cao *et al.*, 1997; Zhang *et al.*, 2013, Jayakannan *et al.*, 2015; Liu *et al.*, 2017; Lee *et al.*, 2019). To understand the functions of "NPR1" will be guide for analyse in plant stress tolerance. With this aim, in this work, the comparative analysis of NPR1 under two different stress in soybean was studied which has never been addressed until now.

MATERIALS AND METHODS

Experimental design and plant material

Soybean (*Glycine max* L. Merr.) SA88 seeds were obtained from a commercial provider (Agrova, Adana, TR). The seeds were sown in plastic trays (10 cm×14 cm) filled with soil under dark conditions. After germination, seedlings were placed into a growth chamber at 25 °C with 16 h/8 h day/night photoperiod and light intensity of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with Hoagland solution for 2 weeks. Then, seedlings were treated with 200 mM NaCl, 10 % PEG 6000, 200 mM NaCl + 10 % PEG 6000 in Hoagland solution. After stress treatment, leaves were harvested at hours (2h, 4h, 6h), days (7 th and 14 th) and stored at -80 °C until further analysis.

Physiological parameters

Relative growth rate

The relative growth rate (RGR) of shoot was calculated from the dry mass data taken at initial and final harvests, using the formula given by (Hunt *et al.*, 2002). For dry weight (DW) calculations, shoots and roots were dried in the oven at 70 °C for 48 hours and then weight.

$RGR = (\ln(DW2) - \ln(DW1)) / (t2 - t1)$ where DW1 = dry mass (g) at time 1; DW2 = dry mass (g) at time 2; and t1 and t2 = initial harvest time 1 and final harvest time 2 in days.

Relative water content

The RWC was calculated using the following formula The relative water content (RWC) was calculated by (Smart and Bingham, 1974). Six leaf discs were obtained from plants in every group on hours, 2h, 4h and 6h and days 7 and 14 of salinity and PEG treatment. After FW of these discs were determined, they were floated on deionized water for 4h under low irradiance. Two turgid tissues were then quickly blotted dried prior to determining turgid weight. DW was then determined after oven drying at 70°C for 72h, the time point at which a constant weight was reached.

$$RWC (\%) = [(FW - DW) / (TW - DW)] \times 100$$

Relative electrolyte leakage

The relative electrolyte leakage (REL) was determined according to Singh *et al.* (2008). Leaf tissue was vibrated for 30 min in deionised water followed by measurement of conductivity of bathing medium (C₁). Boiled the samples for 15 min and again measured the conductivity (C₂) was measured. Percent relative electrolyte leakage (REL) was determined using the following formula:

$$REL = \left(\frac{C_1}{C_2} \right) \%$$

Biochemical analysis*Proline content*

The proline content of the leaves was determined according to Claussen (2005). The absorbance of the reaction mixture was determined at 546 nm. The proline

concentration was determined from a standard curve and calculated on fresh weight basis ($\mu\text{g proline g}^{-1} \text{FW}$).

Hydrogen peroxide content

The H₂O₂ content was determined according to Velikova *et al.* (2000). Fresh leaves (0.1 g) were homogenized in 5ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 12.000 rpm for 15 minutes. The supernatant (0.5 ml) was then mixed with 0.5 ml of buffer (10 mM potassium phosphate, pH 7) and 1ml of 1M KI. The absorbance reading was taken at 390 nm.

Malondialdehyde content

The level of lipid peroxidation in leaf samples was determined in terms of the malondialdehyde (MDA) content according to the method specified by Madhava Rao and Sresty (2000). The MDA content, an end product of lipid peroxidation, was determined by using the thiobarbituric acid reaction. The MDA concentration was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. An extinction coefficient of 155 mM⁻¹ cm⁻¹ was used to determine the MDA concentration.

Determination of hormone levels

SA and ABA level will be determined by using AOAC Official Method 2007. 01 Quechers extraction method with LC-MS/MS. 15 g sample will be weight into 50 ml Quechers extraction centrifuge tubes containing 6 g Mg sulfate, 1.5 g Na acetate. Then, 15 ml acetonitrile containing 1 % acetic acid will be put into tube. Then, this will be shaken vigorously for 1 minute. It will be centrifuged for 5 minute with 4000 cycle. 8 ml will be taken from upper phase. Then, it will be put into 15 ml teflon centrifuge tube containing 0.2 g primer second amine, 0.6 g magnesium sulfate. Then it will be shaken vigorously for 1 minute. It will be centrifuged for 5 minute with 4000 cycle. The upper phase will be taken into vials. The kit containing 0.04 g active carbon will be used for second phase. Calibration graphic will be prepared with 1-5-10-25-50-100-200 $\mu\text{g/kg}$ concentration (matrix-match). The residue concentration in sample will be calculated calibration graphic. The

results will be presented by multiplied with dilution factor.

Gene expression analysis

Non-expressor of pathogen related gene expression

Relative quantification of gene expression and statistical analysis of all qRT-PCR data (pair wise fixed reallocation randomisation test) were performed using the REST software according to Pfaffl *et al.* (2002).

RNA Isolation, cDNA Synthesis, and Real-time RT-PCR Assay

RNA extraction was performed using Tripure reagent (Roche) according to the manufacturer's instructions. The integrity of total RNA was checked spectrophotometrically using a NanoDrop Spectrophotometer ND-2000 (Labtech International), followed by gel electrophoresis. cDNA synthesis was performed from 4 g total RNA using a Transcriptor 1st strand cDNA synthesis kit (Roche) according to the manufacturer's instructions and cDNAs of independent biological replicates ($n = 3$) from same treatments were pooled into single samples. Subsequently, transcript levels were analyzed in a LightCycler 480II real-time PCR cycler (Roche) using a FastStart Essential DNA Probes Master kit (Roche) according to the manufacturer's instructions. Reaction conditions were 95 °C for 600s, followed by 45 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 °C for 1 s. Relative quantification of gene expression and statistical analysis of all qRT-PCR data (pairwise fixed reallocation randomization test) were performed using the REST software according to Pfaffl *et al.* (2002). GmNPR1-specific products were obtained using the following primers: forward primer, 5-TCAGATGATGTTGAGCTTGTTAAAC-3; reverse primer. ACCAAGTACCTCAGAAACAACCTT. Actin beta gene was used as reference gene. GmAct F; GAGCTATGAATTGCCTGATGG, GmAct R; CGTTTCATGAATTCCAGTAGC, Probe upl 61 (roche). Primer design was designed by using Acs number: XM, Acs number NM, NCBI and ensemble gene banks by us.

Statistical analysis

The experiment was conducted in a completely randomized design and measurements were performed

with 6 replicates ($n = 6$). Statistical variance analysis of the data was performed using ANOVA and differences among treatments were compared using Tukey's post-hoc analysis with least significant differences at the 5% level.

RESULTS

Relative water content is an indicator of water stress in plants. In this study, the results showed that short and long term of stress reduced RWC values in soybean leaves (Table 1, 2). Especially, alone PEG treatment induced damage in water status was more pronounced according to NaCl stress. This content was reduced by 17.76 % at 7 day, 20.89 % at 14 day comparing with controls. Otherwise, the reduction was 52.96 % at 2h and 36.53 % at 4h and 49.15 % at 6h in short term application with PEG stress. By the way, combined treatment lead the highest reduction in both short and long term of stress as compared to control groups.

Relative growth rate is generally reduced under stress conditions in plants. In our results, PEG treatment lead a more reduction in RGR with PEG treatment (60.71 % at 7 day, 60.66 % at 14 day) compared to salinity in soybean plants (Table 1). Considering the short term effects of stress, the results showed us that salinity or PEG stress had a similar effects on plants at 2 h, 4 h and 6 h while combined stress reduced RGR content by 48.57 % , 43.75 % and 69.69 % respectively (Table 2).

Relative electrolyte leakage is induced with salinity in plants. Long term of stress increased REL levels by salinity 66.20 % and 2 fold at 7 and 14 day (Table 1). Otherwise, combined stress increased this level under all treatment while it was 2 fold under PEG and salinity. Short term of salinity also induced REL levels but the highest increase was at 4h under all treatments. Proline is a compatible osmolyte in plants to tolerate salinity in plants. In results, PEG induced stress increased the proline content by 15.34 % and 37.52 % at 7 and 14 day. Also, PEG was more effective than NaCl stress at 4h and 6 h (46.47 % and 46.62 %) (Table 2).

Malondialdehyde content is an indicator for stress damage. Stress treatments induced MDA content under

all treatment but it was increased more by 26.06 % and 19.80 % at 7 and 14 day under long term effects of PEG stress as compared to salinity. Nevertheless, PEG and salinity lead to highest increase in MDA content by 28.28 % at 2h, 40.82 % at 4h and 2.16 fold at 6h according to control treatments (Fig.1). Hydrogen peroxide is produced via superoxide dismutase enzyme from superoxide anion radical in cells. PEG stress caused a higher increase in hydrogen peroxide level compared to salinity in both short and long term of stress. Beside this, PEG and salinity induced by 87.55 %, 3.98 and 5.60 fold at 2h, 4h and 6h as compared to control groups (Fig 2).

Abscisic acid levels was checked in this study to determine the effect of stress on soybean leaves. In results, all treatment induced ABA levels but PEG stress induced significantly by 45.99 % and 41.22 % at 7 and 14 day. Otherwise, considering the short term of stress, PEG treatment also induced ABA level (54.53 %, 71.42

%, 76.41 %) at 2h, 4h and 6h compared to salt stress but combined stress showed similar results to control treatments (Fig.3). Salicylic acid levels were determined with treated soybean leaves. In parallel with the results of ABA, PEG stress induced more SA levels according to salt stress. At 7 and 14 day of stress, 33.19 % and 3.77 fold increase was reported significantly (Fig.4). Similarly, short term of application of PEG stress induced SA levels in this plant but not as well as long term effect.

Non expresor of pathogen related gene 1 (*NPR1*) was upregulated with combined stress according to salt and PEG treatment alone at 4h. There was also upregulation in salinity or PEG stress but they were not significant. However, salinity induced *NPR1* gene expressions at 7 day of treatment, while it was also upregulated with combined stress at 14 day (Fig. 5).

Table 1. Long term (7 d and 14 d) effects of salt and osmotic stress on relative growth rate (RGR), relative water content (RWC), relative electrolyte leakage (REL), proline (PRO) of soybean (*Glycine max* L.) seedlings. Control (C), Osmotic Stress (PEG), Salt stress (NaCl), PEG+NaCl (Osmotic and Salt stress). Columns with different letters represent significantly different ($P < 0.05$) values

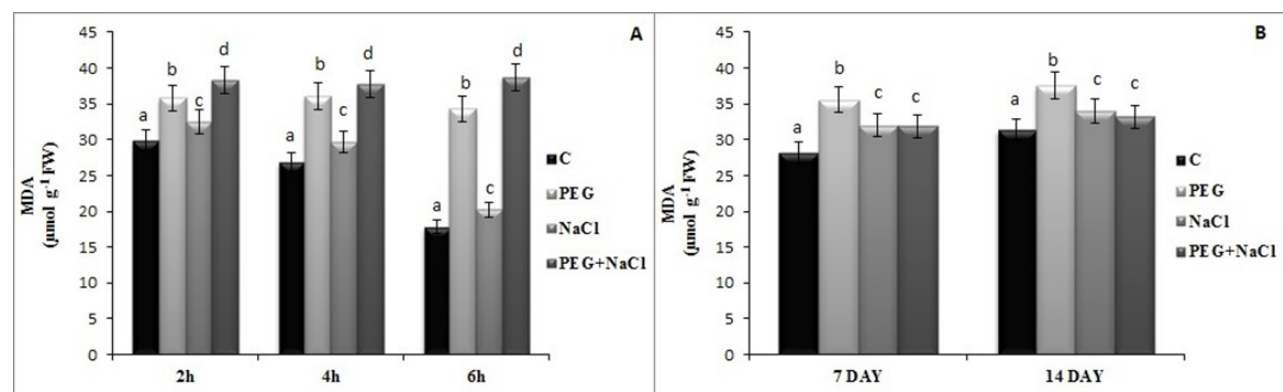
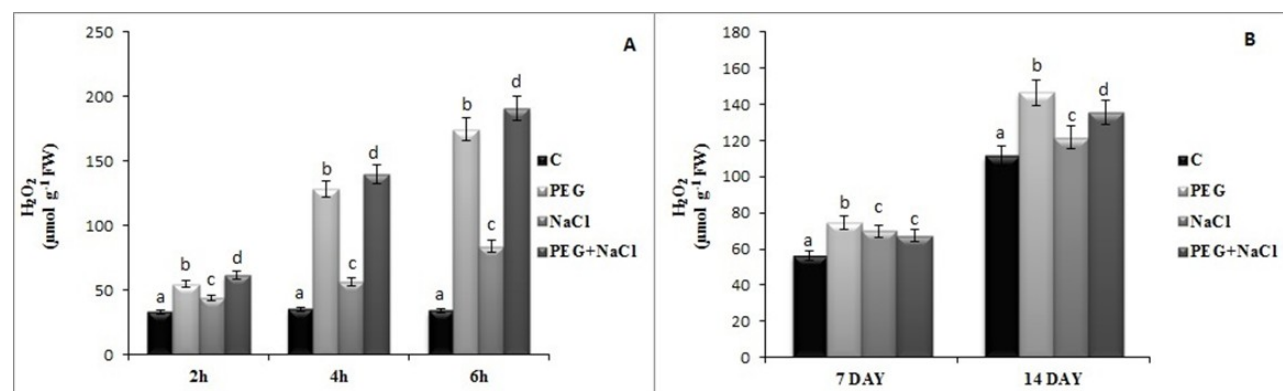
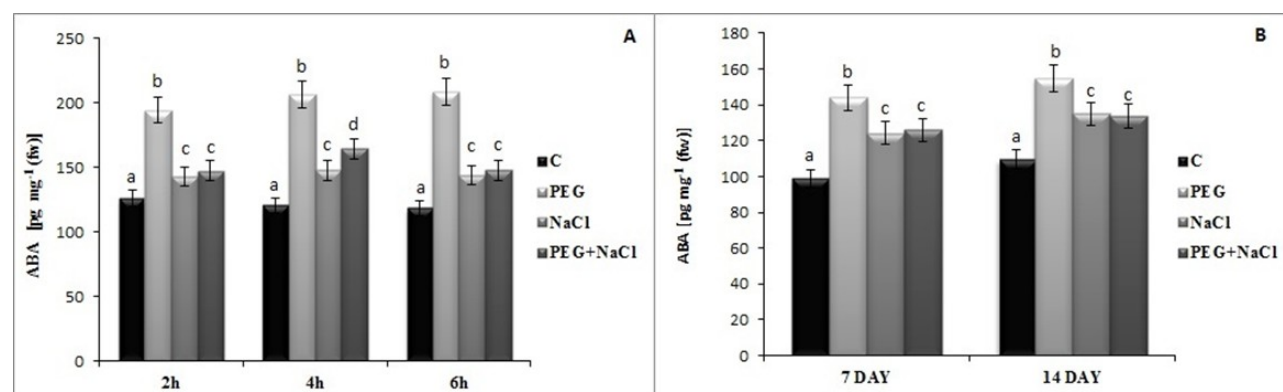
	RGR (%)		RWC (%)		REL (%)		PRO	
	7 DAY	14 DAY	7 DAY	14 DAY	7 DAY	14 DAY	7 DAY	14 DAY
C	0.28±0.05 ^a	2.39±0.1 ^a	86.69±2.4 ^a	77.78±1.6 ^a	22.4±2.2 ^a	32.08±1.9 ^a	535.30±3.4 ^a	573.21±7.4 ^a
PEG	0.11±0.03 ^b	0.94±0.07 ^b	71.29±1.9 ^b	61.51±1.8 ^b	26.6±2.6 ^b	36.08±0.3 ^b	632.67±0.5 ^b	788.75±8.05 ^b
NaCl	0.16±0.008 ^c	1.33±0.04 ^c	74.84±2.05 ^b	73.52±1.7 ^c	37.2±0.8 ^c	64.8±0.2 ^c	549.05±3.2 ^a	581.98±13.7 ^a
PEG+NaCl	0.14±0.004 ^c	1.2±0.04 ^c	65.54±2.5 ^c	54.08±3.1 ^d	46.48±2.8 ^d	55.8±1.6 ^d	567.43±3.4 ^c	468.9±7.5 ^c

Table 2. Short term (2h, 4h, 6h) effects of salt and osmotic stress on relative growth rate (RGR), relative water content (RWC), relative electrolyte leakage (REL), proline (PRO) of soybean (*Glycine max* L.) seedlings. Control (C), Osmotic Stress (PEG), Salt stress (NaCl), PEG+NaCl (Osmotic and Salt stress). Columns with different letters represent significantly different ($P < 0.05$) values.

	RGR (%)			RWC (%)			REL (%)			PRO		
	2h	4h	6h	2h	4h	6h	2h	4h	6h	2h	4h	6h
C	0.175±0.01 ^a	0.08±0.11 ^a	0.33±0.5 ^a	65.14±6.1 ^a	69.42±3.7 ^a	79.05±3.7 ^a	29.07±7.4 ^a	29.85±2.9 ^a	36.31±6.4 ^a	409.33±3.2 ^a	312.13±1.2 ^a	356.2±1 ^a
PEG	0.118±0.06 ^b	0.06±0.03 ^a	0.23±0.06 ^b	30.64±8.4 ^b	44.06±9.5 ^b	40.19±5.2 ^b	49.39±8.8 ^b	64.52±7.4 ^b	40.62±10.9 ^b	413.42±2.2 ^a	388.16±1.9 ^b	350.8±2.6 ^a
NaCl	0.115±0.03 ^b	0.06±0.04 ^a	0.25±0.1 ^b	45.92±5.4 ^c	46.39±3.9 ^b	58.08±4.2 ^c	37.83±3.7 ^c	118.51±7.8 ^c	61.57±4.5 ^c	435.05±12.2 ^b	457.38±1.2 ^c	522.2±3.1 ^b
PEG+NaCl	0.09±0.05 ^c	0.04±0.07 ^c	0.10±0.1 ^c	22.40±4.8 ^d	35.02±5.7 ^c	26.94±6.4 ^d	36.56±8.5 ^d	59.63±6.9 ^d	49.43±0.1 ^d	401.49±21 ^a	378.22±0.7 ^d	367.9±1.8 ^c

Table 3. Genes used in this study. Gene identification number (gene ID number), forward (OR), reverse and primer sequences, probe number and expected amplicon length (Bp).

Gene	Gene ID No	Foward Primer	Reverse Primer	Probe	
				Number	Bp
NPR1	NM_001251745.1	TCAGATGATGTTGAGCTTGTTAAAC	ACCAAGTACCTCAGAAACAACCTT	139	122
GmAct	XM_003547534.3	GAGCTATGAATTGCCTGATGG	CGTTTCATGAATTCAGTAGC	61	118

**Figure 1.** Short (A) and long term (B) effects of salt and osmotic stress on malondialdehyde (MDA) level of soybean (*Glycine max* L.) seedlings. Control (C), Osmotic Stress (PEG), Salt stress (NaCl), PEG+NaCl (Osmotic and Salt stress). Columns with different letters represent significantly different ($P < 0.05$) values.**Figure 2.** Short (A) and long term (B) effects of salt and osmotic stress on hydrogen peroxide (H₂O₂) level of soybean (*Glycine max* L.) seedlings. Control (C), Osmotic Stress (PEG), Salt stress (NaCl), PEG+NaCl (Osmotic and Salt stress). Columns with different letters represent significantly different ($P < 0.05$) values.**Figure 3** Short (A) and long term (B) effects of salt and osmotic stress on abscisic acid (ABA) content of soybean (*Glycine max* L.) seedlings. Control (C), Osmotic Stress (PEG), Salt stress (NaCl), PEG+NaCl (Osmotic and Salt stress). Columns with different letters represent significantly different ($P < 0.05$) values.

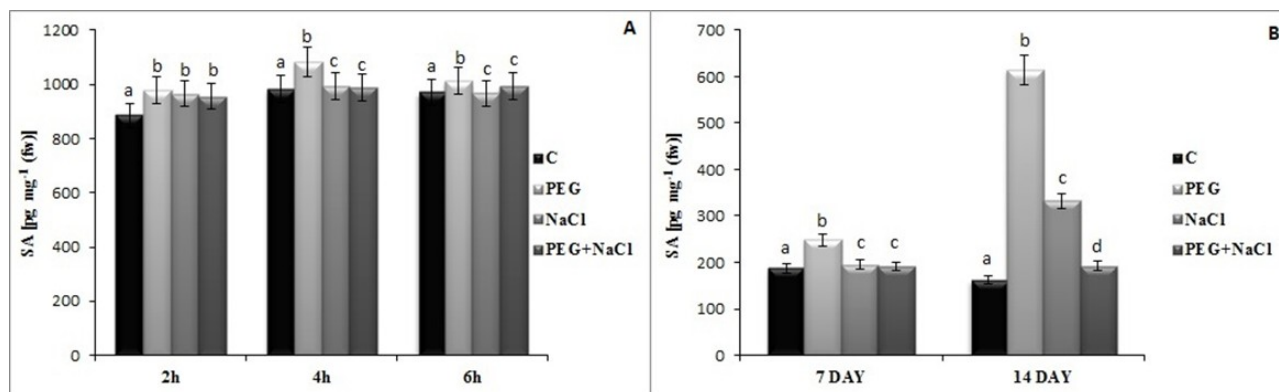


Figure 4 Short (A) and long term (B) effects of salt and osmotic stress on salicylic acid (SA) content of soybean (*Glycine max* L.) seedlings. Control (C), Osmotic Stress (PEG), Salt stress (NaCl), PEG+NaCl (Osmotic and Salt stress). Columns with different letters represent significantly different ($P < 0.05$) values.

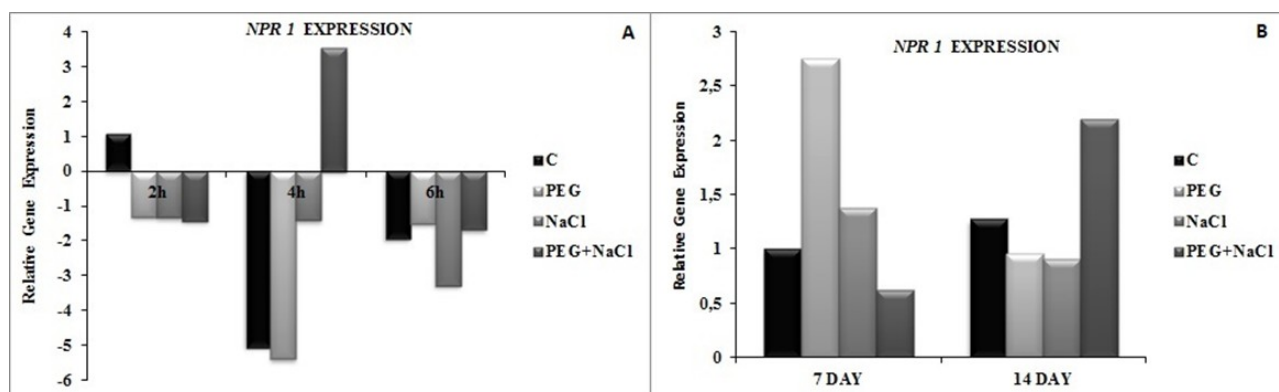


Figure 5 Short and long term effects of salt and osmotic stress on non expressor of pathogen related (NPR1) gene expressions of soybean (*Glycine max* L.) seedlings. Control (C), Osmotic Stress (PEG), Salt stress (NaCl), PEG+NaCl (Salt and Osmotic stress). Columns with different letters represent significantly different ($P < 0.05$) values

DISCUSSION

PEG is non ionic and non toxic osmoticum to study the water status of plants while NaCl is an ionic and is well known to produce specific ion toxicities (Handa *et al.*, 1982). Although salinity and osmotic stress have common effects on plant metabolism, there are many various results in literature (Perz-Alfocea, 1993; Slama *et al.*, 2007; Lokhande *et al.*, 2010). In the present work, plants treated with NaCl showed a better defense response in physiological parameters than PEG. This may be due to maintain of higher succulence under salt than PEG induced stress and higher viscosity traits of PEG as suggested by Chazen *et al.* (1995). In results, RWC content was decreased under all treatment but the highest inhibition was by combined stress comparing with NaCl or PEG treatment alone. Similarly, Bai *et al.* (2019) determined that water content and cell turgor of

soybean under drought and salinity were higher than those of drought alone. Given the duration of stress, short term treatment was more pronounced with a reduction of over 50 % with PEG stress in RWC values. This could be explained by rapid effect of osmotic stress within hours. Similarly, PEG treatment caused a more pronounced decrease in relative growth rate, when compared with NaCl or combined treatment, consistent with the findings of Silva *et al.* (2010). However, the most inhibition in growth was under combined stress of 6h in short term application different from long term. This finding could be explained with the maintain leaf water status, integrity of photochemical activity and loss of growth depending on time of the exposure of stress. These results are also agreement with the proline content. Liang *et al.* (2013) reported that proline is an osmolyte which acts as chelator, reactive oxygen scavenger and signal molecule under stress. There was

a slight increase in proline accumulation under stress in soybean leaves according to controls. In parallel to this results, previous studies determined that stress induces proline level in soybean to increase stress adaptation mechanisms (Sariso *et al.*, 2018). In the present study, proline content was higher in PEG induced stress as compared to NaCl at both short and long term of stress. This results are also agreement with the reports of Ahmad *et al.* (2007). Totally, it could be suggested that proline accumulation is the first response of plants exposed to osmotic stress and may be related to a higher mobilization of this metabolite synthesis as suggested by Grzesiak *et al.* (2013).

Relative electrolyte leakage is an important indicator for membrane damage or deterioration. Electrolyte leakage was enhanced with increasing salinity levels as compared to the control plants in many reports (Tang *et al.*, 2019). In our results, plants treated with NaCl had a higher REL values than treated with PEG at 7 and 14 day, while combined stress increased REL level more than the other treatments at all hours but the most at 4h. This results are also in agreement with the reports of Filek *et al.* (2012) and Patade *et al.* (2012).

Under abiotic and biotic stress conditions, plants produce reactive oxygen species include superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen (1O_2). These molecules are harmful for the proteins, lipids and DNA. One of the ROS radical, "hydrogen peroxide" is produced via superoxide dismutase enzyme from superoxide radical in plants. Beside this, H_2O_2 plays acts as a messenger molecule involved in acclimatory signalling, and it orchestrates programmed cell death at high concentrations (De Azevedo Neto *et al.*, 2005). In results, hydrogen peroxide is induced more by PEG stress as compared to NaCl at 7 and 14 day, while combined stress showed the highest increase at short term of treatment.

Malondialdehyde is a lipid peroxidation product of membranes in plants which is known as and indicator. Increase of accumulation of ROS content in plants lead to increase oxidative damage. It's well known that tolerant plants have low MDA content under stress

conditions. Similar to hydrogen peroxide, PEG treated plants showed higher MDA content according to NaCl at 7d and 14 day. However, short term application combined lead to much damage with higher MDA content as compared to other groups. This findings are also consistent with the RWC, RGR and REL values. Totally, this result made us think that soybean leaves were more sensitive to PEG induced stress condition than NaCl but this can be changed with the duration of stress.

Salicylic acid is known as a signalling molecule that modifies plant responses to stress factors. In this work, SA level was increased by PEG treatment in the long and short term of stress but it was more pronounced with daily practise (7 and 14 d). Several studies have indicated that exogenous application of SA improved stress tolerance and increase in SA accumulation is an important response to stress in plants. It could be suggested that the significant increase in SA with PEG stress was an evidence that soybean leaves were more effected from PEG. This results are also agreement with the report of Lee *et al.* (2019), who reported that the induce SA level in *Brassica napus* under drought stress. To the best of our knowledge, this is the first report of the comparative analysis of SA change under PEG and salinity in soybean.

ABA plays essential roles in seed maturation and germination, maintaining plant water status, stomatal closure, and stress-responsive gene expression under osmotic stress conditions (Yoshida *et al.*, 2019). In soybean leaves, NaCl induced ABA level but the most induction was under PEG induced stress at 7 day. In parallel to our results, 10 % PEG treated *Brassica* plant for 96 h, showed an induced ABA level (Sahay *et al.*, 2019). This findings are also consistent with the physiological parameters and MDA content. Given the results of SA, it could be suggested that two important phytohormone was responsible to struggle with water deficit and damage in soybean leaves.

The main trends observed were the significant induction of *GmNPR1* in PEG treated samples at 7 day, while combined stress treated leaves also showed significantly induced *GmNPR1* transcript levels at 14

day. This result was consistent with the endogenous SA accumulation during PEG treatment. In parallel to this finding, SA and ABA treatment leads to changes in *NPR1* gene expression (Ding *et al.*, 2016). Nevertheless, *GmNPR1* gene expressions were down regulated by short term of stress application but only the significant increase was under combined stress according to PEG or NaCl alone at 4h. As previously mentioned, salicylic acid is essential for NPR1 redox modification but it is not well documented. Oxidative stress also induces NPR1 monomerization but it has not been explained yet. Our results shows that PEG induced osmotic stress was more effective to induce *GmNPR1* upregulation. Similarly, Jayakannan *et al.* (2015) reported that NPR1 dependent SA signaling is important to the salt stress tolerance in Arabidopsis. In parallel to our results, Liu *et al.* (2017) determined that *GmNPR1* was upregulated under AI stress with increase SA level in soybean roots. Beside this, Sarısoy *et al.* (2018) showed an upregulation of *GmNPR1* transcript levels in soybean leaves under salinity. Interestingly, Zhang *et al.* (2013) determined that overexpressions of NPR1 increased salt tolerance but no effect on drought tolerance in Arabidopsis. However, Arabidopsis *npr1* mutant showed enhanced growth during salt stress (Hao *et al.*, 2012). Collectively, it could be said that the role of NPR1 is variable and is altered depending of stress type and time.

CONCLUSION

In conclusion, considering the short term effects on physiological parameters, REL values were remarkable with combined stress at 4h according to NaCl and PEG stress alone. From this result, it could be said that membrane damage of soybean leaves were higher at this situation. Beside this, the highest damage was at 6h according to MDA content. This paradox could be explained by the ion movement such as K⁺ with PEG stress. It is difficult to allege that there is a connection between REL values and *GmNPR1* gene expressions under combined stress but it is clear that PEG stress may role as a potential signal but not salt stress for the regulation of NPR1 in soybean leaves. The present work firstly intends to present the change of NPR1 gene

expressions with comparative analysis of PEG induced osmotic stress and salinity or combined effects in soybean leaves. In future, NPR1 could be used as a defense material, based on this results to improve resistant against to environmental stress factors in plants.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR'S CONTRIBUTIONS

BSD is the sole author of this article and responsible for the submission of final version of the manuscript

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