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Na+ and Cl− induce differential physiological, biochemical responses and metabolite modulations in vitro in contrasting salt-tolerant soybean genotypes

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Abstract

Chloride and sodium constitute as the major ions in most saline soils, contributing to salt-induced damage in plants. Research on salt tolerance has mostly concentrated on the sodium toxicity; however, chloride toxicity also needs to be considered to understand the physiological, biochemical, and metabolite changes under individual and additive salts. In this study, we investigated the effect of individual Na+ and/or Cl− ions (equimolar 100 mM NaCl, Na+ and Cl− salts) using in vitro cultures of four soybean genotypes with contrasting salt tolerance. In general, all the treatments significantly induced antioxidant enzymes activities such as catalase, ascorbate peroxidase, glutathione reductase, guaiacol peroxidase, and superoxide dismutase and osmolytes including proline, glycine betaine, and total soluble sugar (TSS). Both individual (Na+, Cl−) and additive (NaCl) stresses induced more pronounced activation of antioxidant enzyme machinery and osmolytes accumulation in the tolerant genotypes (MAUS-47 and Bragg). The sensitive genotypes (Gujosoya-2 and SL-295) showed higher accumulation of Na+ and Cl−, while the tolerant genotypes were found to maintain a low Na+/K+ and high Ca2+ level in combination with enhanced antioxidant defense and osmotic adjustment. Gas chromatography–mass spectrometry (GC–MS)-based metabolomic profiling depicted the association of certain metabolites under individualistic and additive salt effects. The genotype-specific metabolic changes indicated probable involvement of azetidine, 2-furanmethanol, 1,4-dioxin, 3-fluorothiophene, decanoic acid and 2-propenoic acid methyl ester in salt-tolerance mechanism of soybean.

Keywords Soybean · Na-dominant · Cl-dominant · NaCl stress · GC–MS

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Introduction

Soil salinity is a major threat to crop productivity and overall crop yield. It causes osmotic, ionic, and oxidative stress, which leads to decline in growth and plant development (Hossain and Dietz 2016). Globally, salinity affects almost 0.8 billion hectares of land, which is almost 6% of total land area (Muchate et al. 2016a; Nikalje et al. 2017). Accumulation of toxic Na+ and Cl− ions leads to decrease in activity of stomata and electron transport system (ETS), which generate excess reactive oxygen species (ROS) (Negrao et al. 2017). The ROS causes oxidation of proteins, carbohydrates, lipids, chlorophyll and nucleic acids, and this results in cell death (Hossain and Dietz 2016). To combat salinity, plants have developed adaptive defense mechanism which includes synthesis of compatible solutes, compartmentalization of toxic ions, and induction of ROS-scavenging system (Abogadallah 2010).

Saline soil is rich in cations like calcium (Ca^{2+}), magnesium (Mg^{2+}), and sodium (Na^+), and anions like carbonates (including bicarbonates), chloride (Cl^-), and sulfate (SO_4^{2-}) (Parihar et al. 2015). Sodium chloride is the most toxic salt comprising of sodium and chloride ions, which constitute more than 50% of total soluble salts (Li et al. 2017). The Na^+ and Cl^- ions dominate in the saline soils (Ismail et al. 2014). It is well established that the toxic Na^+ triggers stress to most plant species, but, in some plants, Cl^- ion is more toxic than Na^+ (Li et al. 2017). Apart from toxicity, Cl^- ions have regulatory role in the generation of turgor, stability of enzymes, alteration in membrane potential, pH, balance of charge, volume control, osmoregulation, and stomatal conductance which leads to prevention of water loss, high water use and photosynthetic efficiency (Franco-Navarro et al. 2016; Li et al. 2017). Therefore, it is essential to consider toxicity of both the ions, and study the effect of Cl^- and Na^+ ions alone and combination with NaCl form. In *Medicago, Lotus*, Faba bean, Barley, *Chrysanthemum*, Cucumber, and rice, it has been shown that controlled chloride transport and exclusion from shoot are well correlated (Sibole et al. 2003; Teakle et al. 2007; Tavakkoli et al. 2010, 2011; Guan et al. 2012; Huang et al. 2015; Khare et al. 2015). Most of the studies at cellular level have been carried out in the presence of NaCl salt (Rahnama et al. 2003; Rahnama and Ebrahimzadeh 2004; Kusvuran et al. 2016; Bezirganoglu 2017), but very meager information is available using individual Na^+ or Cl^- salts. In vitro culture techniques offer a significant tool to evaluate genotypes for salt tolerance in less time and space at cellular level under uniform and controlled growth and treatment conditions (Nikam et al. 2014; Piwowarczyk et al. 2016; Bezirganoglu 2017; Boamponsem et al. 2018). Physiological and biochemical mechanisms of salinity tolerance could be different at both cellular and whole plant levels (Nikam et al. 2014; Zhao et al. 2018). In addition, salinity tolerance related traits expressed at cellular level could be used to select tolerant cell lines which provide an important tool to understand mechanisms of salt tolerance (Rania et al. 2015). The differential responses to salinity in plants are depicted in terms of altered growth, compatible solutes and antioxidants (Kumar et al. 2017; Muchate et al. 2016a).

Soybean (*Glycine max* (L.) Merr.), one of the important leading economic crops, contains almost 20% oil and 40% protein in seeds (Amirjani 2010). It is also an attractive crop for biodiesel production and protein source for humans and other animals worldwide. Soybean enriches soil through symbiotic nitrogen fixation, which improves the growth and development of next crop (Piwowarczyk et al. 2016). Despite its wide adaptability to different agro-climatic zones, the productivity of Soybean is affected drastically due to salinity. The crop is categorized as moderately salt sensitive (Munns and Tester 2008; Cao et al. 2017). However, regarding salt tolerance and salt sensitivity, there is large variation among the soybean genotypes necessitating screening of available germplasm (Shelke et al. 2017). In vitro screening for salt tolerance can provide the most methodical, rapid, and resourceful route to isolate stress tolerant genotypes. Information on salt tolerance under individual and additive salt in soybean crop is limited. The present investigation was aimed to study toxicity caused by individual Na^+ or Cl^- and NaCl salt in callus cultures of different soybean genotypes with contrasting salt tolerance. Different physiological and biochemical parameters were considered for salt-sensitive or salt-tolerant response. Metabolic analysis using GC–MS was done to reveal the involvement of major metabolites responsible for salt-tolerance mechanism of soybean at cellular level.

Materials and methods

Plant material, seed germination, and callus formation

Soybean seeds (MAUS-47 and Bragg- salt tolerant and Gujosoya-2 and SL-295- salt sensitive) were collected from National Institute of Soybean Research, Indore, MP, India. Seeds were surface sterilized using $HgCl_2$ (0.1%, w/v) followed by rinsing in distilled water and inoculated on Murashige and Skoog medium consisting of 0.1% sucrose and 0.8% agar as a solidifying agent in test tubes. After 3 days of dark incubation, test tube-containing seeds were transferred to light for next 7 days for seed germination at 25 ± 2 °C and 14 h light (36 µmol m^{-2} s^{-1}) in culture room. After seed germination, the cotyledons were excised and inoculated on callus induction medium (MS + 10.74 µM NAA). After 45 days of growth callus was subjected to Na-dominant, Cl-dominant, and NaCl salt treatments.

Differential salt treatment

About 0.5 g callus was subjected to 100 mM Na-dominant (Na^+) (EC 10.4 ds m^{-1}), Cl-dominant (Cl^-) (EC 11 ds m^{-1}), and NaCl (EC 11.4 ds m^{-1}) salt-enriched callus induction medium. The callus grown on only callus induction medium was used as control. The 100 mM sodium dominant salt solution was prepared using Na_2SO_4 (15 mM), Na_2HPO_4 (15 mM), and $NaNO_3$ (40 mM). The chloride dominant salt solution was prepared using $MgCl_2$ (15 mM), $CaCl_2$ (15 mM), and KCl (40 mM) (Kumar and Khare 2016). After 15 days of salt treatment, callus was harvested for analysis.

Determination of growth attributes

The changes in growth attributes (fresh weight, dry weight, and %TWC) were measured to study callus growth. After harvesting, the fresh weight of the callus was immediately recorded and subjected to oven drying (at 60 °C) until constant dry weight. The %TWC (% tissue water content) was measured as per Muchate et al. (2016b).

Extraction and estimation of antioxidant enzymes

Fresh callus (0.5 g) was homogenized using chilled sodium phosphate buffer (50 mM, pH 7.0), EDTA (0.1 mM) and PVP (1%). The homogenate was centrifuged (15,000 rpm, 20 min, 4 °C) and supernatant was used as enzyme source. The Lowry's method (1951) was used to estimate soluble protein content.

Estimation of superoxide dismutase (SOD)

The activity of SOD was recorded as per Beauchamp and Fridovich (1971). The assay mixture consisted of phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), methionine (14.3 mM), NBT (82.5 μM), riboflavin (2.2 μM), and enzyme source (25 μl). After addition of enzyme source, the assay mixture was exposed to light (2000 lux light intensity) along with light blank (assay mixture without enzyme source) for 30 min. The assay mixture consisted of enzyme source served as dark blank and incubated in dark. The reduction of NBT was observed spectrophotometrically at 560 nm (Shimadzu UV-1800) and enzyme activity was expressed as Units SOD activity mg^{-1} protein.

Estimation of catalase (CAT)

The activity was assayed as per Cakmak and Marschner (1992) method. The assay mixture contained phosphate buffer (50 mM, pH 7.0) and H_2O_2 (15 mM). After addition of enzyme source (50 μl), the activity was measured as decrease in O.D. at 240 nm and expressed as μkat mg^{-1} protein (extinction coefficient = 36 mM^{-1} cm^{-1}).

Estimation of ascorbate peroxidase (APX)

The method described by Nakano and Asada (1981) was used for APX assay. The assay mixture (3 ml) consisted of phosphate buffer (50 mM, pH 7.0), ascorbate (0.5 mM), EDTA (0.1 mM), and enzyme source (100 μl). The decline in O.D. was observed and enzyme activity was expressed as μkat mg^{-1} protein (extinction coefficient = 2.8 mM^{-1} cm^{-1}).

Estimation of guaiacol peroxidase (GPOX)

The Hemeda and Klein (1990) method was used for the measurement of GPOX activity. The assay mixture (3 ml) consisted of phosphate buffer (50 mM, pH 6.6), guaiacol (1%, (w/v)), H_2O_2 (0.3%), and enzyme source (25 μl). Increase in O.D. (at 470 nm) was observed and enzyme activity was expressed as units mg^{-1} protein (1 unit = 1 μmol of guaiacol oxidized min^{-1}).

Estimation of glutathione reductase (GR)

The method described by Smith et al. (1988) was used for the measurement of GR activity. The assay mixture (3 ml) consisted of phosphate buffer (50 mM, pH 7.5), DTNB (3 mM), EDTA (1 mM), H_2O_2 (0.1 mM), NADPH (2 mM), enzyme source (25 μl), and GSSG (20 mM). Increase in O.D. (at 421 nm) was observed and enzyme activity was expressed as units mg^{-1} protein (1 unit = 1 μmol of GSSG reduced min^{-1}).

Extraction and estimation of osmolytes

Estimation of glycine betaine (GB)

The glycine betaine content was measured as per the method of Grieve and Grattan (1983). The fresh callus (0.5 g) was homogenized in 20 ml distilled water and placed on rotary shaker for 16 h. The homogenate was centrifuged at 1000 rpm for 10 min. The supernatant was mixed with 2N sulphuric acid (1:1) and mixture was incubated on ice water bath for 1 h followed by the addition of freshly prepared I_2-KI reagent. This mixture was incubated at 4 °C for 16 h and then centrifuged (10,000 rpm, 15 min at 0 °C). The supernatant was discarded, and the residue was recovered carefully and dissolved in 1,2-dichloroethane. After 2 h of incubation at room temperature, the O.D. was measured at 365 nm.

Estimation of proline

The homogenate was prepared from fresh callus (0.5 g) in aqueous 5-sulphosalicylic acid (3%) and was centrifuged at 10,000 rpm (for 10 min at 4 °C). The supernatant was mixed with glacial acetic acid and acid ninhydrin reagent (2 ml of each). The reaction mixture was incubated in boiling water bath for 1 h and reaction was terminated on ice bath. Then, 4 ml of toluene was added in cold reaction mixture and vortexed robustly for 15 s. The reaction blend lacking extract was used as a blank and the O.D. was measured at 520 nm.

The content of proline was calculated using the standard curve and expressed as mg of proline g^{-1}FW (Bates et al. 1973).

Estimation of total soluble sugars (TSS)

The fresh callus (0.2 g) was homogenized in ice-chilled 80% ethanol. The homogenate was centrifuged (5000 rpm for 10 min at 4 °C) and supernatant and anthrone reagent (1:3) incubated in water bath (100 °C) for 10 min. The reaction was terminated on ice bath and O.D. was measured at 620 nm. TSS content was determined using the standard D-glucose curve and expressed as mg glucose g^{-1}FW (Watanabe et al. 2000).

Estimation of ion content

The dry calli (50 mg) was digested in concentrated nitric acid for 24 h. Then, acid was evaporated at 100 °C for 2 h. The digested samples were dissolved in 10 ml deionized distilled water and used for the estimation of cation (Na^+, K^+, Ca^{2+}, Mn^{2+}, Fe^{2+}, and Mg^{2+}) content. The ion content was measured using atomic absorption flame photometer (Agilant, India). The Cl^- content was estimated as per Chapman and Pratt (1961) by potentiometric method.

GC–MS analysis

The fresh callus (5 g) was crushed in liquid nitrogen. The resultant powder was soaked in 10 ml methanol (95%) and placed on rotary shaker for 12 h, followed by sonication for 25 min (CD-4820 Ultrasonic cleaner). To this, sodium sulfate (2 g) was mixed thoroughly and the mixture was filtered using pre-wetted Whatman filter paper no. 1 with 95% ethanol. The resultant aliquot was passed through 0.2 μm syringe micro-filters. The aliquots (2 μl) were analyzed using the GC–MS system (Agilent technologies 7890B GC and 5977A MSD system) equipped with HP5 column. The helium was used as a carrier gas with 1 ml min^{-1} flow rate. The oven temperature of GC was raised successively from 110 °C to 280 °C at 5 °C min^{-1} except at former temperature held for 2 min and later for 9 min. The electron ionization and detector was used to obtain mass spectrum of compounds with mass scan range 45–450 amu. Total 34 min running time 45–450 Da fragments were analyzed in each 0.5-s scan period. Identification of compounds was done based on the calculated fragments, molecular structure, and molecular mass. The test material compound name, molecular formula, RT, similarity index, and peak area of the components were assessed (Supplementary Table 1). The Turbomas 5.2 software was used to compare the spectrum of unknown compounds with spectrum of the components deposited in the updated version of NIST library (Keskes et al. 2017).

Statistical analysis

The experiments were setup in completely randomized design with 20 replicates to study differential impact of Cl^- and Na^+ ions on callus growth of 4 soybean genotypes. The one-way analysis of variance was evaluated using statistical software SPSS 16.0. The data represented as mean ± standard error of biological triplicate, and means were compared using DMRT (Duncan's 1955) at $P \leq 0.05$.

Results

Influence of Na^+, Cl^-, and NaCl on growth and water status

The effect of individual ionic (Na^+ and Cl^-) and additive NaCl stress on callus cultures of four contrasting soybean genotypes under 100 mM of salt concentration was studied. A significant reduction was observed in callus FW and DW under Na-dominant, Cl-dominant, and NaCl treatments as compared to control, in the four soybean genotypes (Table 1). In all the genotypes, significantly higher reduction of %TWC was observed in Na-dominant treatment rather than NaCl and Cl-dominant treatments as compared to their respective controls. In the genotypes MAUS-47 and Bragg, %TWC was found to be better maintained than Gujosoya-2

Table 1 Physiological parameters under Na^+, Cl^-, and NaCl stress of four soybean genotypes in callus culture system

Genotypes	Treatment	FW	DW	%TWC
MAUS-47	Control	1.6 ± 0.2^{aB}	0.1 ± 0^{aB}	94 ± 0.2^{aA}
	Na-dominant	0.64 ± 0^{bA}	0.06 ± 0^{bAB}	90 ± 0.7^{cA}
	Cl-dominant	0.75 ± 0^{bA}	0.06 ± 0^{bBC}	92 ± 0.2^{abA}
	NaCl	0.71 ± 0^{bA}	0.06 ± 0^{bA}	91 ± 0.2^{bcA}
Bragg	Control	1.54 ± 0.05^{aB}	0.087 ± 0^{aB}	94 ± 0.1^{aA}
	Na-dominant	0.52 ± 0.02^{bB}	0.057 ± 0^{bB}	89 ± 0.6^{cA}
	Cl-dominant	0.67 ± 0.03^{bB}	0.059 ± 0^{bC}	91 ± 0.1^{bA}
	NaCl	0.6 ± 0.03^{bA}	0.057 ± 0^{bA}	90 ± 0.7^{bAB}
Gujosoya-2	Control	1.99 ± 0.1^{aA}	0.11 ± 0^{aA}	94 ± 0.2^{aA}
	Na-dominant	0.47 ± 0^{bBC}	0.07 ± 0^{bA}	84 ± 0.5^{cB}
	Cl-dominant	0.56 ± 0^{bB}	0.06 ± 0^{cAB}	90 ± 0.7^{bB}
	NaCl	0.57 ± 0^{bB}	0.07 ± 0^{bcA}	88 ± 0.4^{bB}
SL-295	Control	1.45 ± 0.1^{aB}	0.1 ± 0^{aB}	93 ± 0.2^{aB}
	Na-dominant	0.4 ± 0^{bC}	0.08 ± 0^{bA}	81 ± 1.7^{cC}
	Cl-dominant	0.56 ± 0^{bB}	0.06 ± 0^{bA}	90 ± 0.6^{abC}
	NaCl	0.52 ± 0^{bB}	0.07 ± 0^{bA}	86 ± 0.4^{bcB}

Each value is the mean (± SE) of 20 replicates (Duncan's test, $P \leq 0.05$) and different letters for each factor in each column indicate significant difference. Small letter denotes significant difference between treatments, and capital letter denotes significant difference between genotypes in each treatment at 0.05% significance level

and SL-295 under Na-dominant, Cl-dominant, and NaCl salt treatments.

Influence of Na⁺, Cl⁻, and NaCl on antioxidant enzyme activity

The analysis of variance (ANOVA) showed differential response of soybean genotypes to Na-dominant, Cl-dominant, and NaCl salt treatments for the studied antioxidant enzymes activity. This indicated a significant interaction between the genotypes and applied stresses. The Na-dominant salt treatment significantly increased SOD activity in MAUS-47 and Bragg (4.1 and 3.6-fold, respectively), while it decreased in Gujosoya-2 and SL-295 (1.4- and 2-fold, respectively) genotypes compared to their control. The Cl-dominant and NaCl salt also significantly increased the enzyme activity in MAUS-47 (2.5- and 3.4-fold, respectively), Bragg (2.2- and 2.7-fold, respectively), Gujosoya-2 (1.4- and 1.9-fold, respectively), and SL-295 (1.2- and 1.7-fold, respectively) genotypes (Fig. 1a).

The CAT activity varied significantly among the control and treatments in the tested genotypes. The Na-dominant treatment significantly induced CAT activity in MAUS-47 (3.6-fold) and Bragg (5.3-fold), while, in Gujosoya-2 and SL-295, the enzyme activity was decreased by 1.5- and 1.3-fold, respectively. The Cl-dominant and NaCl treatment also significantly induced the CAT activity in all the soybean genotypes (1.5- and 2.3-fold in MAUS-47, 3.4- and 4-fold in Bragg, 1.5- and 1.8-fold in Gujosoya-2, and 1.6 and 2-fold in SL-295, respectively) (Fig. 1b).

A similar trend was observed for APX activity, which was also varied significantly in all the tested genotypes under Na-dominant, Cl-dominant, and NaCl salt treatments. The Na-dominant salt treatment significantly induced higher APX activity in MAUS-47 (2.5-fold) and Bragg (2.8-fold), while it decreased in Gujosoya-2 (1.2-fold) and SL-295 (1.2-fold) genotypes compared to its control. The Cl-dominant and NaCl salt also significantly increased this enzyme activity in MAUS-47 (1.9- and 2.2-fold, respectively), Bragg (1.7- and 2.1-fold, respectively), Gujosoya-2 (1.5- and 1.8-fold, respectively), and SL-295 (1.6- and 1.9-fold, respectively) genotypes (Fig. 1c).

The GPOX activity under Na-dominant salt was significantly increased in MAUS-47 (11.2-fold) and Bragg (8.2-fold) as compared to Gujosoya-2 (1.6), while, in SL-295, the enzyme activity was decreased by 1.1-fold as compared to their respective controls. In Cl-dominant salt treatment, all the genotypes showed increase in GPOX activity as compared to their respective controls. Within genotypes, MAUS-47 (4.9-fold) showed highest induction followed by Bragg (4.9-fold), Gujosoya-2, and SL-295 (2.4-fold). Significantly increased GPOX activity was recorded under NaCl treatment in all the genotypes; genotypes MAUS-47 (7.8-fold)

and Bragg (5.7-fold) showed the highest GPOX activity than Gujosoya-2 (4.1-fold) and SL-295 (3.8-fold) (Fig. 1d).

The GR activity was the highest in MAUS-47 (5.8-fold) and Bragg (9.5-fold), while, in Gujosoya-2, no significant change was observed. However, decrease in GR activity (1.8-fold) under Na-dominant salt treatment was observed in SL-295. In the Cl-dominant salt treatment, the GR activity was significantly increased in MAUS-47 (2.9-fold) and Bragg (4.8) as compared to Gujosoya-2 (1.7-fold) and SL-295 (twofold). Similar to Cl-dominant, under NaCl treatment, MAUS-47 (3.4-fold) and Bragg (5.5-fold) genotypes showed increased GR activity as compared to Gujosoya-2 (2.3-fold) and SL-295 (2.9-fold) (Fig. 1e). Among the genotypes, MAUS-47 and Bragg showed significant induction of antioxidant enzyme activity than Gujosoya-2 and SL-295 under individual or additive salt treatments (Fig. 1).

Influence of Na⁺, Cl⁻, and NaCl on osmolytes accumulation

Comparative study of the accumulation pattern of glycine betaine, proline, and total soluble sugars in soybean genotypes revealed significant differences between the Na-dominant, Cl-dominant, and NaCl treatments. The increase in glycine betaine content was about 9.4- and 12.1-fold in MAUS-47 and Bragg genotypes respectively in Na-dominant salt treatments (Fig. 2a). The Cl-dominant and NaCl salts also significantly increased glycine betaine content in MAUS-47 (4.2- and 8-fold, respectively), Bragg (4.3- and 8.8-fold, respectively), Gujosoya-2 (1.6- and 3.2-fold respectively), and SL-295 (2.6- and 3.4-fold, respectively) genotypes (Fig. 2a).

The proline content in callus cultures of control and treatments differed significantly among the four tested genotypes. The Na-dominant salt treatment significantly induced higher proline content in MAUS-47 (9.7-fold), Bragg (sixfold), and Gujosoya-2 (onefold), while it was decreased in SL-295 (onefold) genotype compared to their control. The Cl-dominant and NaCl salt treatment also significantly increased the proline content in all the soybean genotypes. Proline content was 4.3- and 5.8-fold in MAUS-47; 3.2- and 3.7-fold in Bragg, 1.9- and 3.1-fold in Gujosoya-2; and 1.3 and 1.9-fold in SL-295, respectively (Fig. 2b). In general, in all the treatments, Gujosoya-2 and SL-295 showed significantly lower accumulation of proline.

A similar trend was observed for TSS accumulation, which also increased significantly in all the tested genotypes under Na-dominant, Cl-dominant, and NaCl salt treatments (Fig. 2c). The Na-dominant salt treatment significantly increased TSS content in MAUS-47 (3.3-fold) and Bragg (3.1-fold), while it was decreased in Gujosoya-2 (1.3-fold) and SL-295 (1.4-fold) genotypes compared to its control. The Cl-dominant and NaCl salt also significantly
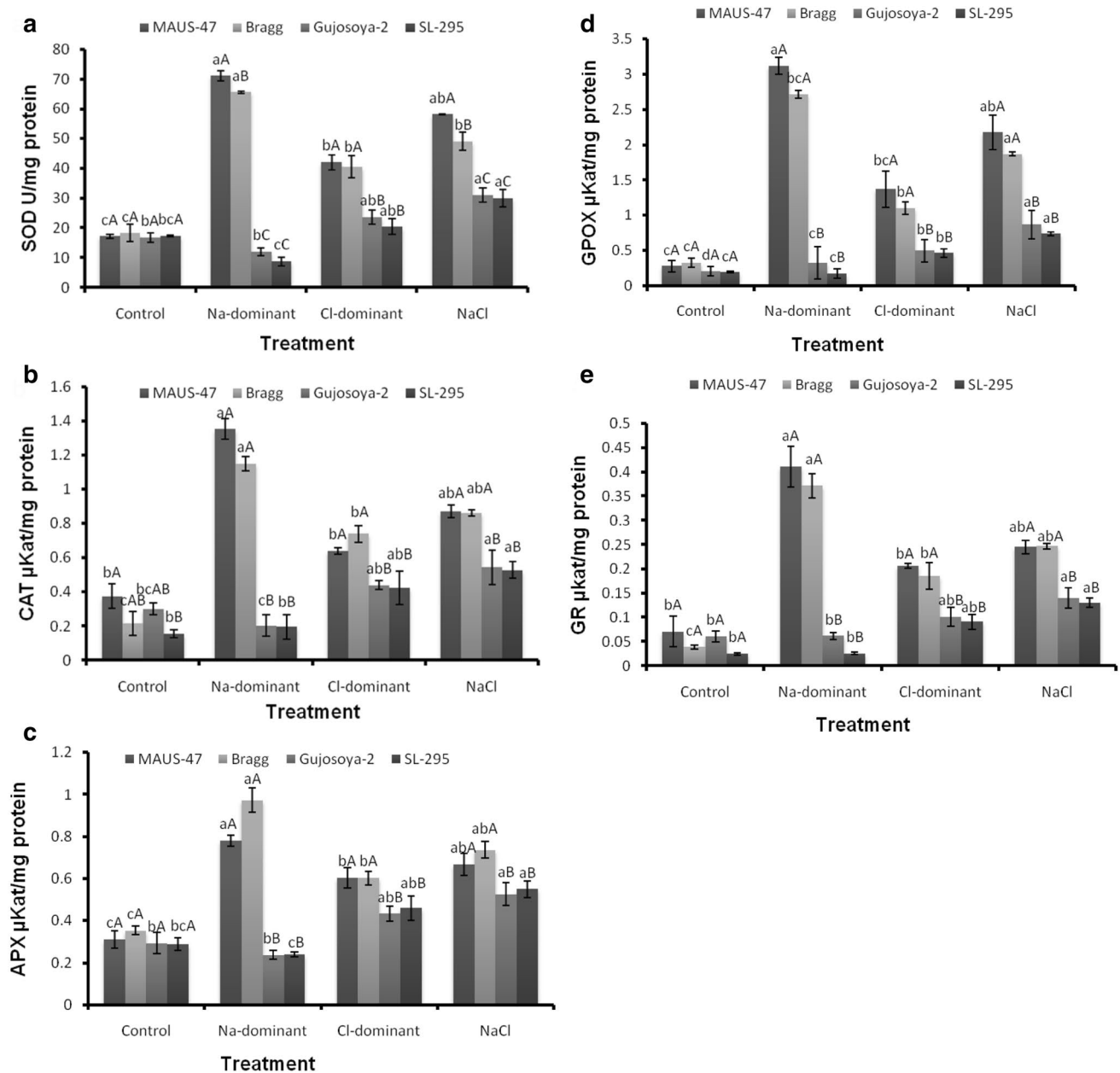
Fig. 1 Impact of individual (Na$^+$ and Cl$^-$) and additive (NaCl) salt on induction of **a** SOD, **b** CAT, **c** APX, **d** GPOX, and **e** GR activity of four soybean genotypes callus. Small letters denotes significant difference between treatment and capital letter denotes significant difference between genotypes in each treatment at 0.05% significance level. *SOD* superoxide dismutase, *CAT* catalase, *APX* ascorbic peroxidase, *GPOX* guaiacol peroxidase, *GR* glutathione reductase

increased TSS in MAUS-47 (1.3- and 2.2-fold, respectively), Bragg (1.3- and 2-fold, respectively), Gujosoya-2 (1.1- and 1.5-fold, respectively), and SL-295 (1.3- and 1.5-fold, respectively) genotypes (Fig. 2c). To summarize, the data showed that, in comparison with all the genotypes and treatments, Gujosoya-2 and SL-295 showed significantly lower glycine betaine, proline, and TSS accumulation than MAUS-47 and Bragg.

Influence of Na$^+$, Cl$^-$, and NaCl on ion content

The effects of Na-dominant, Cl-dominant, and NaCl salt treatments on ions homeostasis in callus cultures of four soybean genotypes are presented in Table 2. There was a significant Na$^+$ and Cl$^-$ ion accumulation (up to 10.4- and 32.6-fold, respectively) in the four tested genotypes. The Na-dominant salts treatment caused 7.2- and 5.6-fold higher Na$^+$
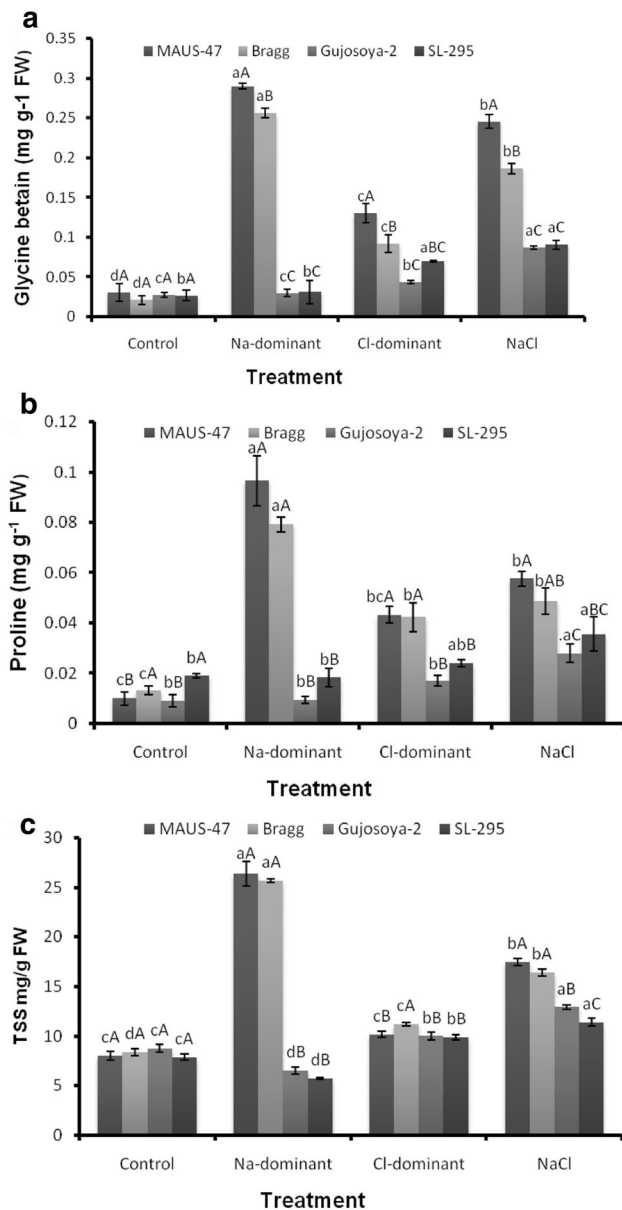
Fig. 2 Impact of individual (Na⁺, Cl⁻) and combined (NaCl) salt on accumulation of **a** glycine betaine, **b** proline, and **c** TSS of four Soybean genotypes callus. Small letter denotes a significant difference between treatments, and capital letter denotes significant difference between genotypes in each treatment at 0.05% significance level

ion accumulation in callus cells of MAUS-47 and Bragg, while it was 10.4- and 7.8-fold in Gujosoya-2 and SL-295 genotypes. A similar trend was observed with Cl-dominant salt treatment. The accumulation was 6.3- and 7.7-fold high Cl⁻ ion in MAUS-47 and Bragg, while 28.8- and 32.6-fold in Gujosoya-2 and SL-295 genotypes. On the other hand, the additive (NaCl) salt treatment induced 6.4- and 5.1-fold higher Na⁺ and 3.3 and 5.2-fold Cl⁻ in MAUS-47 and Bragg genotypes, while 10.8 and 7.6-fold high Na⁺ and 18.2 and 21.6-fold Cl⁻ was recorded in Gujosoya-2 and SL-295,

respectively. The MAUS-47 and Bragg genotypes displayed significantly lower Na⁺ and Cl⁻ accumulation than Gujosoya-2 and SL-295 genotypes. The MAUS-47 and Bragg genotypes showed efficient regulation of Na⁺ influx under Na-dominant and NaCl treatment which, in turn, could maintain lower Na⁺/K⁺ ratio, while uncontrolled influx in Gujosoya-2 (3.2 and 2.1 ratio, respectively) and SL-295 (2.1 and 1.8 ratio, respectively) could results in failure in maintaining the influx. In Na⁺ treatment, not only K⁺ but also Ca^{2+} content decreased significantly under Na-dominant and NaCl stress. The genotypes, Gujosoya-2, and SL-295 reported significantly lower accumulation of K⁺ and Ca^{2+} in all the salt treatments. Therefore, the results suggested that MAUS-47 and Bragg genotypes showed a better regulation of these ions than Gujosoya-2 and SL-295 genotypes. The analysis of Mg^{2+}, Mn^{2+}, and Fe^{2+} showed insignificant difference among the treatments except Cl-dominant treatment for Mg^{2+} in all tested genotypes (Table 2).

GC–MS-based metabolite profiling

To analyze the salt induced metabolic perturbations in the four contrasting soybean genotypes, we compared the GC–MS-based metabolic profile of MAUS-47, Bragg, Gujosoya-2, and SL-295 genotypes (Table 3). The identified peaks contained metabolites such as alcohols, fatty acids, carboxylic acids, sugars, and ketones. The 2-furancarboxaldehyde was observed in MAUS-47 under control, NaCl, Na⁺, and Cl⁻ stress, while 2-Furancarboxaldehyde, 5-methyl- were observed both in control and NaCl conditions. The 2-Furanmethanol was observed only in NaCl and Na⁺ salt. The phenylacetaldehyde was observed only under control condition, while (2H)-furan-3-one, 2-propenoic acid, methyl ester, 3-oxabicyclo[3.1.0]hexan-2-one, and pyrazole-4-carbaldehyde were detected in NaCl treatment. The dimethyl 2-(N-formyl-methylamino)butane-1,4-dioate, azetidine, dimethyl 2-hydroxy-4-[((tetrahydropyran-2-yl)oxy)methyl] benzene-1,3-dicarboxylate, and hexadecanoic acid were observed only in Na⁺ salt and (E)-5-Benzyloxypent-3-en-1-yne, silanediol, dimethyl- in Cl⁻ salt (Table 3). In Bragg, 2-Furanmethanol was observed in all the salt treatments, while N-Methylcyclopentane-1,1-dicarboximide and phenylacetaldehyde was found in the control and NaCl salt. 2-Propenoic acid, methyl ester was observed in NaCl, Na⁺, and Cl⁻. The 2-furancarboxaldehyde, 5-methyl- was recorded in NaCl and Cl⁻ and decanoic acid in NaCl and Na⁺ salt. The isoflavonoid azetidine was found in Na⁺ and Cl⁻ salt treatments in Bragg genotypes, while in Na⁺ salt in MAUS-47. The 1,3-dioxane-5-d, 2-(1,1-dimethylethyl)-4-methyl-, [2S-(2.alpha.,4.alpha.,5.alpha.)]-, 1,4-ethanonaphthalene-5,8-dione, 1,4,4a,8a-tetrahydro-1-methyl-4-(1-methylethyl)- and 3-pyridinamine were observed only under control condition of Bragg genotype, while 2-furan-carboxaldehyde,

Table 2 Ion accumulation in callus (mg g^{-1} DW) of four soybean genotypes under Na$^+$, Cl$^-$, and NaCl stress

Genotypes	Treatment	Na$^+$	K$^+$	Na$^+$/K$^+$	Ca^{2+}	Mg^{2+}	Mn^{2+}	Fe^{2+}	Cl$^-$
MAUS-47	Control	0.28±0.02[bAB]	2.55±0.06[bA]	0.1±0.01[bA]	0.93±0.08[aA]	0.125±0.04[bA]	0.051±0[aA]	0.31±0.01[aA]	12.9±2.1[cA]
	Na-dominant	1.98±0.28[aA]	1.98±0.17[cA]	1±0.1[aB]	0.42±0.02[bA]	0.127±0.02[bA]	0.075±0[aA]	0.45±0.1[aA]	10.8±2.1[cA]
	Cl-dominant	0.38±0.11[bA]	4.88±0.19[aA]	0.1±0.03[bA]	0.81±0.06[aAB]	0.258±0.01[aA]	0.058±0.0[aA]	0.49±0.09[aA]	81.5±7.5[aC]
	NaCl	1.76±0.33[aA]	2.18±0.07[bcA]	0.8±0.14[aB]	0.48±0.11[bA]	0.134±0.03[bA]	0.068±0.01[aA]	0.33±0.0[aA]	42±5.5[bD]
Bragg	Control	0.34±0.01[bA]	2.94±0.29[bA]	0.1±0.02[bA]	0.94±0.1[aA]	0.116±0.02[bA]	0.057±0.01[aA]	0.36±0.04[bA]	15±3.6[cA]
	Na-dominant	1.91±0.18[aA]	2.08±0.13[bA]	0.9±0.14[aB]	0.44±0.08[bA]	0.131±0.02[bA]	0.076±0.01[aA]	0.5±0.07[aA]	18.3±1.7[cA]
	Cl-dominant	0.3±0.02[bA]	5.35±0.47[aA]	0.1±0[bA]	0.85±0.04[aA]	0.274±0.02[aA]	0.057±0.01[aA]	0.43±0.02[abA]	114.8±6.2[aB]
	NaCl	1.84±0.01[aA]	2.1±0.11[bA]	0.8±0.12[aB]	0.5±0.1[bA]	0.134±0.03[bA]	0.068±0.01[aA]	0.33±0.0[bA]	77.8±6.2[bC]
Gujosoya-2	Control	0.21±0.01[bB]	2.81±0.63[aA]	0.1±0.02[bA]	0.74±0.09[aA]	0.077±0.03[bA]	0.052±0.0[aA]	0.37±0.01[aA]	6.7±2.08[cA]
	Na-dominant	2.17±0.14[aA]	0.69±0.31[cB]	3.2±0.55[aA]	0.25±0.01[cB]	0.105±0[bA]	0.067±0.0[aA]	0.36±0.05[aA]	15±3.6[cA]
	Cl-dominant	0.35±0.1[bA]	3.06±0.33[aB]	0.1±0.03[bA]	0.5±0.1[abC]	0.255±0.02[aA]	0.053±0.01[aA]	0.46±0.01[aA]	191.8±5.5[aA]
	NaCl	2.24±0.12[aA]	1.1±0.11[bB]	2.1±0.18[abA]	0.35±0.07[cA]	0.08±0.02[bA]	0.063±0.0[aA]	0.37±0.05[aA]	121±9.5[bB]
SL-295	Control	0.3±0.07[bAB]	2.74±0.18[aA]	0.1±0.02[bA]	0.85±0.03[aA]	0.091±0.01[bA]	0.045±0.0[aA]	0.37±0.02[aA]	6.7±4.2[cA]
	Na-dominant	2.34±0.03[aA]	1.09±0.19[bB]	2.3±0.51[aA]	0.26±0.02[cB]	0.085±0.02[bA]	0.067±0.01[aA]	0.32±0.01[aA]	17.1±2.1[cA]
	Cl-dominant	0.2±0.01[bA]	3.16±0.36[aB]	0.1±0.01[bA]	0.58±0.07[bBC]	0.242±0.04[aA]	0.046±0.0[aA]	0.46±0.02[aA]	216.7±11.5[aA]
	NaCl	2.27±0.28[aA]	1.24±0.24[bB]	1.9±0.24[aA]	0.36±0.04[cA]	0.098±0.01[bA]	0.068±0.01[aA]	0.38±0.03[aA]	143.9±4.2[bA]

Each value is the mean ($\pm$ SE) of three replicates (Duncan's test, $P \leq 0.05$) and different letters for each factor in each column indicate significant difference. Small letter denotes significant difference between treatments, and capital letter denotes significant difference between genotypes in each treatment at 0.05% significance level

cyclohexan-2,2,3,3,4-d5-ol, 4-methyl-, (1S-trans)-, N-Benzylindole, and 2-Octanone were found under NaCl treatment. The 1,4-dioxin, 2,4(1H,3H)-Pyrimidinedione, 5-methyl-, 3-Fluorothiophene, and syn-2-(2-Hydroxypropyl)-6-methyldihydropyran-3(6H)-ol were observed only under Na$^+$ salt. The (4S)-3,3-Dideuterio-4-methylcyclohexanone, (E)-6-Biphenyl-4-yl-4-oxohexanoic acid ethanolamide, (E)-6-Naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide, 7-Methoxy-2,3-dihydro-2-phenyl-4-quinolone, Ethanol, 2,2,2-trichloro-, propanoate, Methyl (2R)-2-[(tert-Butoxy)carbonylamino]-5,5-dimethyl-5-silahexanoate, and 2(5H)-furanone were observed under Cl$^-$ salt (Table 3).

In Gujosoya-2, phenylacetaldehyde was a common metabolite in control, NaCl, and Na$^+$ treatment. The (E)-6-Naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide, 2-Furanmethanol, 2-Propenoic acid, methyl ester, and 4-Amino-3,5-bis (dimethoxymethyl)-4H-1,2,4-triazole were observed in control and Na$^+$ treatments. N-Methyl-cyclopentane-1,1-dicarboximide was found under NaCl and Na$^+$ salt. Pentane, 3-bromo- only observed in Na$^+$ and Cl$^-$ salt. The 6-Methylhexahydrocycloprop[a]pentalen-3a,6-diol, Pent-4-enal and 2-Furancarboxaldehyde, 5-methyl- were observed only under controlled condition. The compound 1-(2,3,4,5,6-pentafluorophenyl)sulfanylhex-5-en-2-one was found only in Na$^+$ salt and (R)-16-Hydroxy-3-methylhexadecanoic acid, and butyl 2,4-dimethyl-2-nitro-4-pentenoate which were observed under Cl$^-$ treatment (Table 3). In SL-295, phenylacetaldehyde was observed in control, NaCl, and Na$^+$, while 2-Furanmethanol in NaCl, Na$^+$, and Cl$^-$ salt. The 2-Propenoic acid, methyl ester, and 3-Oxabicyclo[3.1.0]hexan-2-one were observed in control

and NaCl treatment. The (+/−)bis(2-aminobut-3-enyl) disulphide, (E)- 6-naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide, and 2-Furancarboxaldehyde 5-methyl- were synthesized in Na$^+$ and Cl$^-$ salt. The metabolites 1,1,4,4,7,7,10,10,13,13,16,16-Dodecachloro-hexasila-18-crow-6, 4-Amino-3,5-bis(dimethoxymethyl)-4H-1,2,4-triazole, N-Methylcyclopentane-1,1-dicarboximide, Pent-4-enal, 1,4-Ethanonaphthalene-5,8-dione, 1,4,4a,8a-tetrahydro-1-methyl-4-(1-methylethyl), and syn-2-(2-Hydroxypropyl)-6-methyldihydropyran-3(6H)-ol were observed only under control condition, while 4-endo-Amino-2-oxabicyclo[3.3.0]oct-7-en-3-one, N-Methylcyclopentane-1,1-dicarboximide, Pentane, 3-bromo were observed only under the NaCl treatment. The metabolites (E)-6-Biphenyl-4-yl-4-oxohexanoic acid ethanolamide, 2(5H)-furanone, 3,5-Dideutero-Aniline- and 3-Fluorothiophene observed only under Na$^+$ salt, while benzeneacetaldehyde, cyclooctylhydroperoxide, and methyl 3-hydroxy-2-(p-toluenesulfonyloxy)methyl-10-tetrahydropyranyloxydecanoate were found under Cl$^-$ salt (Table 3). The metabolic variation at certain level was observed in between genotypes and within treatment of each genotype.

Discussion

Studies on plant salt tolerance have contributed a great deal of information on plant responses and management of salt (NaCl) stress, and in most cases, effects of sodium toxicity have been the major focus. However, simultaneous comparison of the individual Na$^+$, Cl$^-$ ions, and NaCl salt effects are

Table 3 GC–MS-based metabolite profiling of MAUS-47, Bragg, Gujosoya-2, and SL-295 soybean genotypes callus under controlled and exposed to Na$^+$, Cl$^-$ and NaCl salt

Genotypes	Control	NaCl	Na-dominant (Na$^+$)	Cl-dominant (Cl$^-$)
MAUS-47	2-Furan-carboxaldehyde	2-Furan-carboxaldehyde	2-Furan-carboxaldehyde	2-Furan-carboxaldehyde
	2-Furancarboxaldehyde, 5-methyl-	2-Furancarboxaldehyde, 5-methyl-	Dimethyl 2-(N-formyl-methylamino) butane-1,4-dioate	(E)-5-Benzyloxypent-3-en-1-yne
	Phenylacetaldehyde	(2H)-Furan-3-one	Azetidine	Silanediol, dimethyl-
		2-Furanmethanol	2-Furanmethanol	
		2-Propenoic acid, methyl ester	Dimethyl 2-hydroxy-4-[((tetrahydropyran-2-yl)oxy)methyl]benzene-1,3-dicarboxylate	
		3-Oxabicyclo[3.1.0]hexan-2-one	Hexadecanoic acid	
		Pyrazole-4-carbaldehyde		
Bragg	1,3-Dioxane-5-d, 2-(1,1-dimethylethyl)-4-methyl-, [2S-(2.alpha.,4.alpha.,5.alpha.)]-	(+/−)Bis(2-aminobut-3-enyl)disulphide	1,4-Dioxin	(+/−)Bis(2-aminobut-3-enyl)disulphide
	1,4-Ethanonaphthalene-5,8-dione, 1,4,4a,8a-tetrahydro-1-methyl-4-(1-methylethyl)-	2-Furan-carboxaldehyde	2,4(1H,3H)-Pyrimidinedione, 5-methyl-	(4S)-3,3-Dideuterio-4-methylcyclohexanone
	2-Furanmethanol	2-Furanmethanol	2-Furanmethanol	2-Furanmethanol
	3-Pyridinamine	2-Propenoic acid, methyl ester	2-Propenoic acid, methyl ester	2-Propenoic acid, methyl ester
	N-Methylcyclopentane-1,1-dicarboximide	N-Methylcyclopentane-1,1-dicarboximide	3-Fluorothiophene	N-Methylcyclopentane-1,1-dicarboximide
	Phenylacetaldehyde	Phenyl acetaldehyde	Azetidine	Azetidine
		Decanoic acid	Decanoic acid	(E)-6-Biphenyl-4-yl-4-oxohexanoic acid ethanolamide
		Cyclohexan-2,2,3,3,4-d5-ol, 4-methyl-, (1S-trans)-	Syn-2-(2-Hydroxypropyl)-6-methyldihydropyran-3(6H)-ol	(E)-6-Naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide
		N-Benzylindole		7-Methoxy-2,3-dihydro-2-phenyl-4-quinolone
		2-Furancarboxaldehyde, 5-methyl-		2-Furancarboxaldehyde, 5-methyl-
		2-Octanone		Ethanol, 2,2,2-trichloro-, propanoate
				Methyl (2R)-2-[(tert-Butoxy)carbonylamino]-5,5-dimethyl-5-silahexanoate
				2(5H)-Furanone

Table 3 (continued)

Genotypes	Control	NaCl	Na-dominant (Na⁺)	Cl-dominant (Cl⁻)
Gujosoya-2	(E)-6-Naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide	N-Methylcyclopentane-1,1-dicarboximide	(E)-6-Naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide	(R)-16-Hydroxy-3-methylhexadecanoic acid
	Phenylacetaldehyde	Phenylacetaldehyde	Phenylacetaldehyde	Butyl 2,4-dimethyl-2-nitro-4-pentenoate
	2-Furanmethanol		2-Furanmethanol	Pentane, 3-bromo-
	2-Propenoic acid, methyl ester		2-Propenoic acid, methyl ester	
	4-Amino-3,5-bis(dimethoxymethyl)-4H-1,2,4-triazole		4-Amino-3,5-bis(dimethoxymethyl)-4H-1,2,4-triazole	
	6-Methylhexahydrocycloprop[a]pentalen-3a,6-diol		N-Methylcyclopentane-1,1-dicarboximide	
	Pent-4-enal		Pentane, 3-bromo-	
	2-Furancarboxaldehyde, 5-methyl-		1-(2,3,4,5,6-pentafluorophenyl)sulfanyl-hex-5-en-2-one	
SL-295	2-Propenoic acid, methyl ester	2-Propenoic acid, methyl ester	(+/−)Bis(2-aminobut-3-enyl) disulphide	(+/−)Bis(2-aminobut-3-enyl) disulphide
	3-Oxabicyclo[3.1.0]hexan-2-one	3-Oxabicyclo[3.1.0]hexan-2-one	(E)-6-Biphenyl-4-yl-4-oxohexanoic acid ethanolamide	(E)-6-Naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide
	Phenylacetaldehyde	Phenyl acetaldehyde	Phenyl acetaldehyde	Benzeneacetaldehyde
	1,1,4,4,7,7,10,10,13,13,16,16-Dodecachloro-hexasila-18-crow-6	4-endo-Amino-2-oxabicyclo[3.3.0]oct-7-en-3-one	2(5H)-Furanone	2-Furanmethanol
	4-Amino-3,5-bis(dimethoxymethyl)-4H-1,2,4-triazole	N-Methylcyclopentane-1,1-dicarboximide	2-Furancarboxaldehyde, 5-methyl-	2-Furancarboxaldehyde, 5-methyl-
	N-Methylcyclopentane-1,1-dicarboximide	Pentane, 3-bromo-	3,5-Dideutero-aniline	Cyclooctylhydroperoxide
	Pent-4-enal	2-Furanmethanol	2-Furanmethanol	Methyl 3-hydroxy-2-(p-toluenesulfonyloxy) methyl-10-tetrahydropyranyloxydecanoate
	1,4-Ethanonaphthalene-5,8-dione, 1,4,4a,8a-tetrahydro-1-methyl-4-(1-methylethyl)-		3-Fluorothiophene	
	syn-2-(2-Hydroxypropyl)-6-methyldihydropyran-3(6H)-ol		(E)-6-Naphthalen-2-yl-4-oxohex-5-enoic acid diethanolamide	

scarcely reported in field crops (Kumar and Khare 2016; Li et al. 2017). It is, therefore, important to study the individual effects of Cl^- and Na^+ ions on physiological and biochemical aspects of growth and development. In the present study, the relative impact of individual Cl^- and Na^+ and additive NaCl salt was analyzed using in vitro callus cultures of four contrasting soybean genotypes that were exposed to equimolar Na^+, Cl^-, and NaCl salt. Previous studies have shown that in vitro culture systems provide controlled environmental conditions to analyze the salt-stress effects at cellular level (Nikam et al. 2014; Rania et al. 2015). Both Cl^- and Na^+ exerted negative impact on dry matter (Table 1) and all the salt treatments caused reduction in FW and DW in all the genotypes as compared to their respective controls. NaCl stress-induced reduction in fresh and dry weight and TWC has been shown previously (Forooghian and Esfarayeni 2013; Balen et al. 2013). In the present study, both the ionic stresses were found to be genotype and treatment specific, and significantly affected FW, DW, and %TWC (Table 1). Among salt treatments, Na-dominant salt treatment showed significantly higher reduction in %TWC than NaCl and Cl-dominant. Gujosoya-2 and SL-295 showed more reduction in %TWC than MAUS-47 and Bragg genotypes in all the salt treatments. Such NaCl-induced reduction in %TWC in callus cultures was also reported in *Guizotia abyssinica* (Ghane et al. 2014) and *Saccharum officinarum* (Patade et al. 2008; Nikam et al. 2014).

Increasing salinity stress causes reactive oxygen species (ROS) burst in plant cells. To combat the ROS induced damage, plants deploy both non-enzymatic and enzymatic antioxidant systems. Several studies revealed that efficient and timely induction of these antioxidant systems plays a vital role in protection of plants from various abiotic stresses by scavenging and detoxifying ROS molecules (Hossain et al. 2007; Niknam et al. 2011; Kiani-Pouya 2015). The salt-tolerance ability of tolerant germplasm is often associated with effective antioxidant machinery as compared to their susceptible genotypes relatives (Yasar et al. 2013; Kiani-Pouya 2015). The present study revealed that MAUS-47 and Bragg genotypes showed significantly higher SOD, CAT, APX, GPOX, and GR activities than Gujosoya-2 and SL-295 (Fig. 1). Using in vitro callus cultures, several researchers analyzed salt-stress-induced differential antioxidant activities in callus of different plant species such as Sugarcane (Patade et al. 2012), Melon (Kusvuran et al. 2016), and Triticale (Bezirganoglu 2017). The Gujosoya-2 and SL-295 genotypes were unable to maintain higher enzymatic antioxidant activities to cope with Na^+ stress. The results suggested that Na^+ caused severe damage in Gujosoya-2 and SL-295 callus which could not be prevented by the induction of antioxidant activities. Similarly, under salt stress, in salt-sensitive sunflower callus, the increase in activities of antioxidant enzymes were observed (Davenport et al.

2003). The NaCl and Cl^- also induced significantly higher enzyme activities in MAUS-47 and Bragg genotypes than Gujosoya-2 and SL-295. The enhanced enzyme activities are well associated with salt-tolerance ability of genotypes (Gandonou et al. 2006; Sharma and Ramawat 2013). Based on our results, the stress-induced responses are suggested to be more pronounced under the Na^+ treatment followed by NaCl and Cl^-.

Increased accumulation of osmolytes is an essential adaptive response to salt stress (Ghane et al. 2014; Suprasanna et al. 2016). Osmotically dynamic compounds like sucrose, glycine betaine, proline, etc. are involved in salt-stress amelioration via maintenance of osmotic adjustment, stabilization of different proteins and their complexes, and scavenging the ROS under salt stress (Slama et al. 2015). In the present investigation, osmolyte accumulation (glycine betaine, proline, and TSS) was studied in the four genotypes for their ability to tolerate individual and combined salts. The results showed noticeably higher contents of glycine betaine, proline, and TSS in MAUS-47 and Bragg under Na^+ salt followed by NaCl and Cl^-. In Gujosoya-2 and SL-295 proline, glycine betaine and TSS contents were found low and equal to control and thus callus failed to combat against Na^+ and other stress treatments. The increased osmolyte content under salinity stress is a significant trait of the salt-tolerant genotypes (Rai et al. 2011; Patade et al. 2012; Nikam et al. 2014). Our results are indicative that the tolerant genotypes showed higher accumulation of osmolytes than sensitive genotypes, and the differential osmolyte accumulation was more under Na^+ salt than NaCl and Cl^- salt treatment in soybean.

Plant adaptation to salt stress, essentially, is dependent on efficient cellular ion homeostasis of net intracellular Na^+ and Cl^- uptake and its compartmentalization (Munns and Tester 2008). In the present study, increased Na^+ and Cl^- contents were observed under NaCl, Na^+, and Cl^- salt, but there was a significant variation among the soybean genotypes (Table 2). The increased Na^+ content under Na^+ and NaCl salt resulted in a significant drop in K^+ and Ca^{2+} levels (Table 2). The toxic impact of Na^+ in many plant species because of its role in nutrient imbalance has been demonstrated during salt stress (Ahmad et al. 2007; Kumar et al. 2008). Ability of Na^+ to compete with K^+ ions for absorption and binding sites of enzymes and subsequent inactivation of essential enzymes is the root cause of toxicity (Balen et al. 2013). Under saline condition, callus experienced dual cellular damage with low K^+ concentrations and high Na^+ toxicity (Errabii et al. 2007; Balen et al. 2013). In our study, both the sensitive genotypes, Gujosoya-2 and SL-295 exhibited an elevated Na^+/K^+ ratio compared with tolerant genotypes MAUS-47 and Bragg. This indicated inefficiency of the former to restrict the influx of toxic ions into cell during saline conditions. In contrast, tolerant genotypes (MAUS-47 and

Bragg) successfully employ ion exclusion, maintain better cellular K^+ level under NaCl and Na^+ stress, and achieve higher salt tolerance. Interaction between Na^+ and Ca^{2+} ions under salt stress disturbs ion transport and cell membrane properties, and leads to change in Ca^{2+} activity in cytoplasm. This resulted in altered physiological and biochemical attributes related to callus growth, nutrition, osmolyte, ion accumulation, and water transport (Summart et al. 2010; Ghane et al. 2014). In the present investigation, Ca^{2+} markedly decreased in Na^+, Cl^-, and NaCl stress, and it exhibited genotype-specific variation. Under these treatments, Gujosoya-2 and SL-295 showed lower accumulation of K^+ and Ca^{2+}, compared to treatment under Cl^- stress (because of KCl and $CaCl_2$ application) which showed significantly high accumulation of K^+ and Ca^{2+} which may render lower cellular toxicity. Similar results were revealed in sugarcane calli where the gathering of Na^+ and Cl^- content decreased K^+ and Ca^{2+} during salt stress (Patade et al. 2008). A higher concentration of Ca^{2+} in MAUS-47 and Bragg genotypes could help the replacement of displaced Ca^{2+}, which results in restoration of plasma membrane integrity and cell wall stability, facilitating higher Na^+/K^+ selectivity, increased Na^+ exclusion, and ultimately improvement in salt tolerance (Errabii et al. 2007; Summart et al. 2010; Nikam et al. 2014). The salt stress affects the allocation of nutrient elements and its uptake in callus tissues (Ahmad et al. 2009; Kumar et al. 2008).

Extensive research has been conducted on plant metabolomic responses under high salinity (Widodo et al. 2009; Zhang et al. 2016; Guo et al. 2017), but these studies were mostly limited to NaCl-induced stress. In the present investigation, involvement of certain metabolites under individual and additive salt effects of NaCl was studied. The capability of a plant to withstand against salt stress is primarily based on the induction of plant hormones, synthesis of osmolytes, induction of ROS scavengers, and modifications of cell membrane, and ensuing metabolite changes that could be useful in metabolic analysis to discriminate soybean genotypes. In the present study, among the metabolites, aldehydes and furans such as benzeneacetaldehyde, 2-furan-carboxaldehyde, 2-furancarboxaldehyde 5-methyl-, phenyl acetaldehyde, and 2-furanmethanol were found in salt-stressed calli. Some of these metabolites were previously observed in soybean due to lipid oxidation, degradation, and sugar dehydration (Pokorny et al. 2000; Lee and Ahn 2009). The present results showed that, under different salt treatments, soybean callus synthesized aldehydes and alcohol. The aldehydes and alcohol were previously shown to be induced in daffodil flowers under salinity stress (Koksal et al. 2015). Aldehydes are important intermediate of carbohydrates, vitamins, steroids, amino acids, and lipids anabolic and catabolic

pathways (Kirch et al. 2004). The various stresses induced the excess accumulation of aldehydes (Feder and Hofmann 1999) which negatively affect plant growth (Kotchoni et al. 2010). Plants regulate the cellular aldehyde level by limiting membrane peroxidation events by exploring enzymatic (SOD, CAT, and POD) antioxidant properties and directly through aldehyde dehydrogenases enzyme activity (Waheed et al. 2018). Some aldehydes and alcohol derivatives act like reactive molecules in plants have damaging effect on cell wall and cell membranes (Huang et al. 2011). The higher molecular weight methyl esters and acids were observed in differentially stressed calli samples which may be because of esterification of alcohol and free fatty acids (Lee and Ahn 2009). The fatty acids synthesized in *N. tangutorum* Bobr. suspension cells showed improved salinity stress tolerance (Ni et al. 2015). Ketones were also observed in differentially stressed calli samples possibly due to the degradation of lipids and/or amino acids (Su 1986). The MAUS-47 and Bragg under Na^+ and Cl^- salt was found to have azetidine isoflavonoid. Previously, it was reported that the proline analog azetidine-2-carboxylic acid accelerates the assembly of class I small heat shock proteins (sHSPs), which provides thermo-tolerance in soybean seedlings (Jinn et al. 2004), but it will be worthwhile to further explore any such role of this proline analog under individual and additive salt treatments. In this study, we have observed different ketones, aldehydes, and alcohols which were also detected. It was observed that lipoxygenases (LOXs) are an important class of non-heme iron enzymes yield flavor precursors hydroperoxides and further hydroperoxide lyase convert it in to flavor compounds such as ketones, aldehydes, and alcohols (Fauconnier and Marlier 1997). In our study, 1,4-dioxin and 2-octanone were detected in Bragg genotype under Na^+ and NaCl treatments, respectively. Vega et al. (2005) reported that activity of LOXs was reliant on the cosolvent concentration, which was augmented with 1,4-dioxane up to 5% (v/v), while its higher concentration decreased its activity and 2-octanone also showed its inhibitory effect on activity of LOXs. The data also showed the presence of 3-fluorothiophene compound under Na^+ salt stress in callus of Bragg genotypes. It was reported that it plays an inhibitory role in xanthine oxidase enzymes (patents/EP1783124A1). Xanthine oxidase (XOD) contributes to H_2O_2 production in plants during saline stress (Sharma et al. 2012). Sauter et al. (2002) reported improved stress tolerance under high oxidative stress through overexpression of ROS quenchers like chlorothiophene as a thiophene derivative. The 3-Fluorothiophene detected in tolerant genotypes may prompt further studies on its role in salinity tolerance. In view of the present results, we hypothesized that these metabolic changes could be used to unravel the salt tolerence mechanism in soybean and other crop plants.

Conclusion

In conclusion, our results on the effects of individual and additive sodium and chloride ions revealed that soybean genotypes differ in their physiological and biochemical responses. The tolerance mechanism in soybean genotypes MAUS-47 and Bragg was due to increased activity of antioxidant system, accumulation of osmolytes, and maintenance of Na^+/K^+ ratio and Ca^{2+} level. On the contrary, the response to salt stress was declined in sensitive genotypes SL-295 and Gujosoya-2. Among the salts, Na^+ was more detrimental followed by NaCl and Cl^-. Since the toxicity of Cl^- was enough to reduce plant growth and development, further investigation is needed to study the molecular mechanism. The genotype-specific occurrence of azetidine, 2-furanmethanol, 1,4-dioxin, 3-fluorothiophene, decanoic acid and 2-propenoic acid, and methyl ester metabolites indicate their probable role in salt tolerance. Detailed studies are warranted on the role of specific salt responsive metabolites in soybean which could be used in the improvement of salt-tolerant genotypes.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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