Plant Cell, Tissue and Organ Culture (PCTOC) (2023) 154:297–309
https://doi.org/10.1007/s11240-023-02569-5

ORIGINAL ARTICLE

Growth and antioxidant defence in hypocotyl-derived calli of two cotton cultivars with contrasting salt tolerance

Melis Sacu[1] · Lale Yildiz Aktas[2] · Meltem Bayraktar[3] · Aynur Gurel[4]

Received: 6 April 2023 / Accepted: 5 July 2023 / Published online: 17 July 2023

Abstract

Soil salinity is one of the major abiotic stress factors that limits cotton (*Gossypium hirsutum* L.) production worldwide. Although cotton is categorised as a salt-tolerant plant, its tolerance level can vary depending on the cultivar. The present study aimed to evaluate the growth and biochemical responses of two cotton cultivars (Carmen and NM-503) to salt stress under tissue culture conditions by using an in vitro selection technique. Hypocotyl explants were cultured on callus formation medium (full-strength MS (Murashige and Skoog medium) macro- and micro-salts + B5 (Gamborg medium) vitamins + 0.1 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) + 0.5 mg/L kinetin) containing 0, 100, 200 or 400 mM NaCl for 4 weeks. Compared to Carmen cultivar, NM-503 cultivar showed an improved growth performance under non-stress control conditions; however, both cultivars showed similar growth and browning tendencies when exposed to salinity stress. Although both cultivars showed a significant decrease in biomass accumulation and relative growth rate following treatment with 200 and 400 mM NaCl, they were tolerant to NaCl stress up to 200 mM in terms of callus survival rate. Carmen cultivar showed the lowest photosynthetic pigment content after treatment with 400 mM NaCl; however, both cultivars showed no significant differences in the photosynthetic pigment content between NaCl concentrations. Furthermore, in contrast to Carmen cultivar, NM-503 cultivar did not accumulate proline in response to 200 and 400 mM NaCl treatment. Under salinity stress, NM-503 cultivar exhibited lower lipid peroxidation level than Carmen cultivar. Both cultivars showed no significant differences in superoxide dismutase activity in control and NaCl-treated groups, except for the 400 mM NaCl-treated group in Carmen cultivar. Apart from constitutive enzyme activity, both cultivars showed similar catalase activities at all concentrations of NaCl treatment. In both cultivars, peroxidase and ascorbate peroxidase activities increased in response to an increase in NaCl concentrations. While glutathione reductase activity gradually increased in NM-503 cultivar in response to NaCl treatment, it gradually decreased in Carmen cultivar. In conclusion, both cultivars showed similar growth response under salinity stress, and this growth response was significantly restricted by 200 and 400 mM NaCl treatment. Among all studied parameters, the most distinct response of the cultivars to salinity stress was reflected by the lipid peroxidation level, which indicates the differences in the mechanism of cellular antioxidant protection between the cultivars. Despite the absence of a salt exclusion mechanism used by intact plants, the high level of resilience shown by the calli of NM-503 cultivar against severe salt stress conditions may be attributed to its effective cellular antioxidant defence mechanism.

Key message

Salinity stress tolerance of two cotton cultivars (Carmen and NM-503) was evaluated by in vitro selection. Among the growth and biochemical parameters, the lipid peroxidation level was the most decisive parameter, and NM-503 cultivar was more salt tolerant than Carmen cultivar.

Keywords Antioxidant enzymes · Cotton · *Gossypium hirsutum* L. · *In vitro* selection · NaCl · Salt tolerance

Communicated by Christell van der Vyver.

Extended author information available on the last page of the article

Introduction

Abiotic stress factors cause adverse effects on crop plants by preventing them from reaching their full genetic potential and limiting their productivity, resulting in enormous economic losses worldwide (Mahajan and Tuteja 2005; Rai et al. 2011). Soil salinity is one of the primary abiotic stress factors that affects agricultural crop production worldwide. High salinity level in the soil inhibits plant growth and significantly limits the yield of crops (Liang et al. 2018). Salinity-induced osmotic stress and ion toxicity cause damages in the structure of plant cell membrane, restrict photosynthesis and induce the accumulation of reactive oxygen species (ROS) and toxic molecules (Munns and Tester 2008). Thus, together with the nutritional imbalance in plants, salinity leads to decline in plant productivity and even death (Guo et al. 2019).

Salt-tolerant plant species have evolved several defence mechanisms, including osmoregulation, ion homeostasis, hormonal regulation and antioxidant systems, to counteract salinity (Cha-um et al. 2013). Under salt stress, plants produce high levels of ROS. The increased formation of ROS (including OH^-, H_2O_2, $^1O_2$ and $O_2^{\cdot}$) induces cellular oxidative stress in plants, which damages membranes and macromolecules. These alterations lead to irreversible metabolic dysfunction and even cell death (Zhang et al. 2021; Mohsin et al. 2022). Plants have developed complex mechanisms to avoid the harmful effects of salt stress (Mohsin et al. 2022), such as elimination of excess ROS by enzymatic and non-enzymatic antioxidative systems. The enzymatic antioxidative systems include catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), while the non-enzymatic antioxidative systems include carotenoids, ascorbate, reduced glutathione (GSH) and flavonoids (Zhang et al. 2021). The activation of the antioxidant defence system in plants has been positively associated with salt tolerance, and the same pattern has been observed in in vitro cultures (Pérez-Clemente and Gómez-Cadenas 2012). Proline is a common compatible soluble organic compound synthesised to maintain osmotic balance, and it is accumulated in response to abiotic stress, including salt stress. Plant growth inhibition is also suggested as one of the criteria to identify salt-tolerant/salt-sensitive genotypes (Cha-um et al. 2013); this phenomenon may reveal the sum of consequences of metabolic deterioration in salt-exposed plant cells.

Cotton (*Gossypium hirsutum* L.) is one of the world's most economically important crops because of its fibre and seeds. Cotton yield is highly affected by abiotic stresses (Mohsin et al. 2022). Although cotton is considered a pioneer crop for use on lands with salinity and alkalinity because of its moderate salinity tolerance, high salinity still negatively affects cotton growth, development, biological functions and productivity (Zhang et al. 2021). Salinity stress results in poor fibre quality and low yield (reduction in boll number and weight) (Mohsin et al. 2022) and can reduce cotton yield by up to 50–67% (Zheng et al. 2022). Thus, efforts to develop or select varieties more tolerant to salinity stresses are critical to increase crop productivity. In addition to the traditional breeding technologies, genetic engineering and plant tissue culture techniques are used as a biotechnological approach for developing stress-tolerant plants (Rai et al. 2011). Regarding cotton, in the last two decades, genetic engineering has offered an important tool to transfer foreign genes into commercially important cotton varieties, and presently, 81% of the total cotton grown is genetically modified. Despite these advancements, there are still several limitations for engineering the salt-tolerant cotton variety because of the multigenic characteristics of the abiotic stress responses (Juturu et al. 2015; Pérez-Clemente and Gómez-Cadenas 2012).

The mechanisms of salt stress response and tolerance have been investigated at the whole-plant level; however, the structural complexity of the whole plant makes it difficult to differentiate between salinity tolerance mechanisms at the systemic and cellular levels (Queirós et al. 2007). In vitro experimental systems allow an accurate measurement of growth and cellular responses to salinity as all cells of callus tissue directly interact with the stressor (Shibli and Al-Juboory 2002; Mohanraj 2016). Both cell culture and callus culture allow researchers to focus on the physiological and biochemical mechanisms that aid plant stress tolerance (Kruglova et al. 2018).

The ability of plants to resist and develop in salinity conditions varies extensively between genotypes and species according to their sensitivity and growth stages (El-Mahdy et al. 2022). The effects of increasing NaCl concentration on callus growth and antioxidant enzyme activities have been studied in callus tissues of some salt-tolerant and salt-sensitive cotton genotypes, and variations in salt tolerance have been observed among these genotypes (Gossett et al. 1994a). Therefore, it is essential to investigate the salt tolerance potential for high-yielding genotypes. Nazilli M-503 (NM-503) and Carmen are commercial cotton cultivars widely grown in Turkey (Aydin et al. 2004; Basal et al. 2011). According to the greenhouse study conducted by Basal (2010), NM-503 genotype was categorised as salt tolerant, while Carmen genotype was considered moderately salt tolerant based on the biomass production of these genotypes under salt stress conditions (up to 250 mM NaCl). However, there is no information regarding the biochemical mechanisms involved in the stress responses in both cultivars. Therefore, the present study aimed to determine the growth and biochemical responses of these two cotton

cultivars to salinity stress by applying different concentrations of NaCl (100, 200 and 400 mM) to callus cultures to contribute to better understanding of the mechanisms underlying salt tolerance by using an in vitro selection technique.

Materials and methods

Plant material and sterilisation

Seeds of *G. hirsutum* L. cultivars Carmen and NM-503 were provided by the Nazilli Cotton Research Institute (Aydın, Turkey). First, the seeds were delinted by using 98% sulphuric acid (H_2SO_4) (Merck) for 3–4 min to thin the seed coat and ease germination. The seeds were then rinsed with sterile distilled water. The acid-delinted seeds were surface sterilised in 20% (v/v) sodium hypochlorite solution (NaOCl: available chlorine, 4–5%) containing 0.1% (v/v) Tween 20 (Merck) for 20 min and subsequently washed three times with sterile distilled water. They were finally kept in sterile distilled water for 24 h.

In vitro germination

The seeds kept in sterile water for 24 h after sterilisation were transferred individually into glass culture jars (210 cc) containing 25 mL of ½ Murashige and Skoog medium (MS; Murashige and Skoog 1962) supplemented with 1.5% (w/v) sucrose and solidified with 0.3% (w/v) Gelrite (Duchefa-Biochemie) (pH 5.8). In vitro germination was induced by incubation at 25 ± 2 °C under a 16-h photoperiod (3500 lx) provided by white light-emitting diodes.

Establishment of callus culture

Callus was induced from aseptically excised hypocotyl explants (1.0–1.5 cm in length) of 7-day-old in vitro germinated seedlings. The hypocotyl explants were transplanted in glass culture jars (210 cc) (one explant per jar) each containing 25 mL of callus formation medium (CFM) containing full-strength MS macro- and micro-salts and B5 vitamins (Gamborg et al. 1968) supplemented with 0.75 g/L $MgCl_2$, 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/L kinetin and 3% (w/v) glucose; the medium was solidified with 0.3% (w/v) Gelrite (pH 5.8) (Trolinder and Goodin 1987). The culture conditions were the same as those used for in vitro germination. After 3 weeks of culture, the induced calli were excised from the hypocotyl explants and then sub-cultured in CFM. To increase callus amount, cultures were sub-cultured four times at 3-week intervals. Salt treatments were initiated after four subcultures.

Salt treatments for in vitro selection

Callus masses with the same colour and texture were weighed to 1.0 g and cultured in the CFM containing 0, 100, 200 or 400 mM NaCl (Merck). The callus explants were transferred into glass culture jars (210 cc) containing 25 mL of medium. An NaCl-free medium was used as a control. The experiments were conducted in three replicates with seven callus masses in each replicate. Twenty-one callus masses were tested in total per treatment. The culture conditions were the same as those used for the in vitro germination process. After 4 weeks of culture initiation, different morphological, physiological, and biochemical characteristics were identified.

Growth parameters

Callus survival rate (%)

The callus masses which survived and continued to grow were counted, and the callus survival rate (CSR) was calculated as follows:

$$CSR(\%) = \left(\frac{Number\ of\ callus\ masses\ showing\ growth}{Total\ number\ of\ callus\ masses\ cultured}\right) \times 100 \quad (1)$$

Fresh weight and dry weight

Callus masses obtained from the control culture and all NaCl-treated cultures were collected after 4 weeks of culture initiation, and the final fresh weight (FW) was determined immediately. Callus masses (1 g) from each treatment group and the control group were placed in an oven and dried at 70 °C for 72 h to determine dry weight (DW).

Relative growth rate

The relative growth rate (RGR) of callus masses was calculated according to Patade et al. (2012) as follows:

$$RGR = \frac{Final\ fresh\ weight\ (g) - Initial\ fresh\ weight\ (g)}{Initial\ fresh\ weight\ (g)} \quad (2)$$

Callus browning rate

Callus masses obtained from each treatment group were separated according to their colours (green/cream and brown) and then weighed at the end of the culture period. The callus browning rate (CBR) was calculated as follows:

$$CBR(\%) = \left(\frac{Fresh\ weight\ of\ brown\ callus\ (g)}{Fresh\ weight\ of\ total\ callus\ (g)}\right) \times 100 \quad (3)$$

Biochemical parameters

Total chlorophyll and carotenoid contents

Total chlorophyll (TChl) and carotenoid contents were determined based on the method of Arnon (1949). Freshly weighed callus samples (100 mg) were homogenised in 15 mL of 80% aqueous acetone by using a porcelain mortar and pestle. Subsequently, the samples were filtered through a filter paper. The colour intensity of the samples was measured using a spectrophotometer (UV-1900i UV–VIS, Shimadzu, Japan) at 663, 646 and 470 nm. The chlorophyll content (mg/g FW) was calculated according to the method of Lichtenthaler and Wellburn (1983).

Proline content

Endogenous-free proline content was determined according to the modified acid ninhydrin method of Bates et al. (1973). Fresh callus tissues (1 g) were homogenised with 3% (w/v) sulfosalicylic acid, and a 2 mL aliquot was mixed with 2 mL of acetic acid and 2 mL of ninhydrin reagent. The reaction mixture was incubated in a boiling water bath for 1 h and then treated with toluene. The absorbance of the collected toluene phase was read at 518 nm by using toluene as a blank.

Lipid peroxidation

Lipid peroxidation was measured based on the content of total thiobarbituric acid-reactive substances (TBARS), as described by Cakmak and Horst (1991). Fresh callus tissues (0.3 g) were homogenised in 3 mL of 1% (w/v) trichloroacetic acid (TCA) (Sigma, Germany). The homogenate was centrifuged at 10,000× *g* for 20 min, and 1.5 mL of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) (Sigma, Germany) solution was then added to a 0.5 mL aliquot of the supernatant. The mixture was heated in a boiling water bath for 30 min and then cooled on ice. The absorbance of the supernatant was measured at 532 nm. The malondialdehyde (MDA) extinction coefficient (0.156 $\mu M^{-1}\ cm^{-1}$) was used for calculations.

Preparation of the enzyme extract for total protein content and antioxidant enzyme activities

Fresh callus tissues (1 g) were homogenised in an ice-cooled mortar with 2% (w/v) polyvinylpyrrolidone (PVP) (Sigma, Germany) and 100 mM potassium phosphate buffer (pH 7.8) (Carlo Erba) containing 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma, Germany), 10% (w/v) glycerol (Isolab) and 1 mM phenylmethanesulfonyl fluoride (PMSF) (Alfa Aesar). The homogenate was centrifuged at 12,000× *g* for 30 min at 4 °C; the supernatant was used for protein assay and enzyme determination. The total protein content in enzyme extracts was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Superoxide dismutase (EC 1.15.1.1)

SOD activity was assayed using the method of Beauchamp and Fridovich (1971). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 33 µM nitroblue tetrazolium (NBT) (Duchefa, Germany), 10 mM L-methionine (Duchefa), 0.66 mM EDTA, and 0.0033 mM riboflavin (Alfa Aesar). Absorbance was recorded at 560 nm, and the non-irradiated reaction mixture served as a control. One unit of enzyme was defined based on the inhibition of 50% of the reaction.

Catalase (EC 1.11.1.6)

The activity of CAT was assayed by monitoring the consumption of H_2O_2 at 240 nm according to the method of Bergmeyer (1970). The reaction mixture comprised 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 30% (w/v) H_2O_2 (Carlo Erba). The decrease in mixture absorbance was recorded for 3 min.

Peroxidase activity (EC 1.11.1.7)

POX activity was assayed by measuring the oxidation of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Herzog and Fahimi 1973). Three millilitres of the assay mixture contained DAB solution (Fluka-Sigma, Germany) (including 0.4 mM DAB, 50% (w/v) gelatin (BD), and 150 mM sodium phosphate-citrate buffer (pH 4.4), 50 µL of enzyme extract, and 3 mM H_2O_2. The increase in absorbance was recorded at 465 nm for 3 min.

Ascorbate peroxidase (EC 1.11.1.11)

APX activity was measured spectrophotometrically based on the oxidation of ascorbate (González et al. 1998). The assay mixture (3 mL) comprised 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (pH 7.8), 0.03% H_2O_2, 30 mM ascorbate (Merck), and 0.1 mL of enzyme extract. Ascorbate oxidation was determined by a decrease in absorbance at 290 nm.

Glutathione reductase (EC 1.6.4.2)

GR activity was assayed by monitoring the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) according to the method described by Carlberg and Mannervik (1985). The reaction mixture (3 mL) contained 60 mM potassium phosphate buffer (pH 7.4), 1 mM oxidised glutathione (GSSG) (Sigma, Germany), 0.1 mM NADPH (Sigma), and 0.2 mL of the enzyme extract. NADPH oxidation was measured by a decrease in absorbance at 340 nm.

Statistical analysis

The data were analysed by analysis of variance (ANOVA) using the general linear model (MINITAB 17.0 Statistical Software, Minitab Inc., State College, PA) at the significance level of $P \leq 0.05$. The values were expressed as mean ± standard error (SE) values. All data were subjected to analysis, and the significant differences among the mean values were compared by Tukey's test at $P \leq 0.05$.

Results

In this study, callus cultures of two cotton (*G. hirsutum*) cultivars Carmen and NM-503 were in vitro exposed to different concentrations of NaCl (0, 100, 200 and 400 mM) to evaluate their growth and biochemical responses to salt stress through in vitro selection.

Growth parameters

Both cultivars showed similar callus formation from the hypocotyl explants of in vitro germinated seedlings (86.66% in Carmen and 87.77% in NM-503) under control culture conditions (data not shown); however, the CBR was 53.33% in Carmen cultivar and much less (3.33%) in NM-503 cultivar (Table 1). This phenomenon could be the reason for the fundamental differences in FW and DW in the callus culture of the control groups of the two cultivars (Table 1). The browning rate of the callus for both cultivars increased following an increase in the concentration of salt in the culture medium (63.33% and 76.66% in Carmen and NM-503 cultivars, respectively).

The CSR (%) was reduced slightly in both cultivars treated with up to 200 mM NaCl in comparison to the control group; however, no significant differences were observed among the concentrations in comparison to the control group (Table 1). Severe stress caused by treatment with the highest concentration of salt (400 mM) led to a significant decrease in CSR (approximately 52.38% and 57.14% in Carmen and NM-503 cultivars, respectively).

The constitutive distinction between the cultivars was observed in the callus growth and biomass accumulation parameters. NM-503 cultivar reached the highest callus FW (5.70 g) and DW (0.91 g) (Table 1), thus indicating that the cultivar positively responded to the culture media. This phenomenon affected all measured parameters when we compared the two cultivars at the control level. However, the FW and DW of callus tissue of both cultivars were similar and in the same statistical group following 100 mM NaCl exposure. A definite reduction in callus FW was recognised when 200 mM NaCl was added to the culture media; the decrease ratio was ca. 36.33% and 71.40%, while the reduction ratio was ca. 56.74% and 81.23% in Carmen and NM-503 cultivars, respectively, in the 400 mM salt treatment group. The 200 and 400 mM NaCl-treated groups of both cultivars showed no significant difference among the cultivars and NaCl concentrations. DWs of the callus revealed severe stress in the 400 mM NaCl-treated group, resulting in 60% and 85.71% decrease in DW in Carmen and NM-503 cultivars, respectively. Together with the FW of the callus, the RGR of both cultivars were significantly reduced in response to 200 and 400 mM NaCl treatment; however, the RGR of both cultivars were not significantly different with increasing salt concentrations in the culture media.

Table 1 Effects of different NaCl concentrations on callus growth parameters in *Gossypium hirsutum* cultivars Carmen and NM-503

Cultivar	Concentration of NaCl (mM)	Callus survival rate (%) ± SE	Biomass accumulation		Relative growth rate ± SE	Callus browning rate (%) ± SE
			Fresh weight (g) ± SE	Dry weight (g) ± SE		
Carmen	Control	100.00 ± 0.00 a	2.45 ± 0.11 bc	0.30 ± 0.01 cd	1.46 ± 0.11 bc	53.33 ± 3.33 b
	100	95.24 ± 4.76 a	2.95 ± 0.25 b	0.41 ± 0.04 bc	1.95 ± 0.25 b	53.33 ± 3.33 b
	200	85.71 ± 8.25 a	1.56 ± 0.09 d	0.22 ± 0.01 de	0.56 ± 0.09 d	50.00 ± 0.00 b
	400	47.62 ± 4.76 b	1.06 ± 0.02 d	0.12 ± 0.01 e	0.06 ± 0.02 d	63.33 ± 3.33 ab
NM-503	Control	100.00 ± 0.00 a	5.70 ± 0.31 a	0.91 ± 0.05 a	4.70 ± 0.31 a	3.33 ± 3.33 c
	100	100.00 ± 0.00 a	3.27 ± 0.33 b	0.52 ± 0.05 b	2.27 ± 0.33 b	56.67 ± 3.33 b
	200	80.95 ± 9.52 a	1.63 ± 0.15 cd	0.26 ± 0.02 d	0.63 ± 0.15 cd	60.00 ± 0.00 b
	400	42.86 ± 0.00 b	1.07 ± 0.02 d	0.13 ± 0.01 e	0.07 ± 0.02 d	76.67 ± 3.33 a

Mean (± SE) values in columns with different letters are significantly different at $P \leq 0.05$ according to Tukey's test

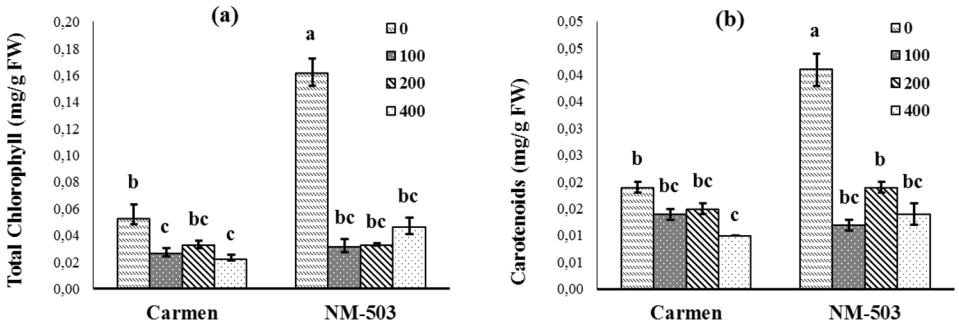

Fig. 1 Effects of different NaCl concentrations on total chlorophyll **(a)** and carotenoid **(b)** contents in the callus culture of *Gossypium hirsutum* cultivars Carmen and NM-503. Mean (± SE) values in columns with different letters are significantly different at $P \leq 0.05$ according to Tukey's test

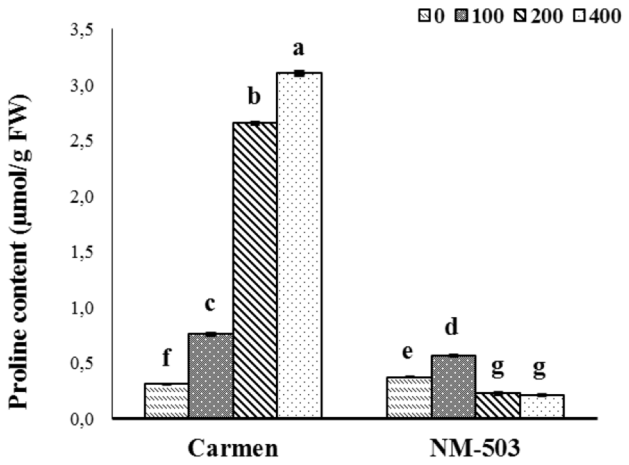

Fig. 2 Effects of different NaCl concentrations on proline accumulation in the callus culture of *Gossypium hirsutum* cultivars Carmen and NM-503. Mean (± SE) values in columns with different letters are significantly different at $P \leq 0.05$ according to Tukey's test

Biochemical parameters

TChl and carotenoid contents

A constitutive difference was observed between the cultivars in the TChl and carotenoid contents; NM-503 cultivar showed the highest contents of these photosynthetic pigments (Fig. 1a, b). This finding was consistent with the observation of the green callus formation in the control culture media. The presence of NaCl in the culture medium reduced the TChl and carotenoid contents at all levels in both cultivars. The lowest photosynthetic pigment contents were found in the 400 mM NaCl-treated group of Carmen cultivar, and the reduction rate was approximately 56.60% for the TChl and 47.37% for carotenoid content as compared to the respective controls. Despite these findings, the NaCl-treated groups of both cultivars did not reveal a significant difference regarding the TChl and carotenoid contents.

Proline content

The basic level of proline content was significantly different between the two cultivars. The callus of NM-503 cultivar had ca. 80.00% higher proline content than that of Carmen cultivar in the control culture medium (Fig. 2). Both cultivars showed a similar response to 100 mM NaCl treatment with an increase in the proline content of the callus (ca. 146% in Carmen and ca. 52% in NM-503).

Proline level as a response to NaCl treatment differed between the cultivars particularly at higher concentrations of NaCl (200 and 400 mM) in the culture medium. Carmen cultivar showed a consistent increase in the proline content with an increase in NaCl concentration. This increase was the highest in the callus grown on medium containing 200 and 400 mM NaCl, with an 8.65-fold and 10.11-fold increase in 200 and 400 mM NaCl treatment, respectively, compared to the control group. In contrast to Carmen cultivar, NM-503 cultivar showed a significant decrease in proline content (39.62% and 43.94%, respectively) in response to 200 and 400 mM NaCl treatment compared to the control group with no significant difference between the concentrations. These groups had the lowest level of proline between the cultivars and among the different NaCl treatments (Fig. 2).

Lipid peroxidation level

The lipid peroxidation level of the callus of the two cultivars were similar in the control culture medium. In Carmen cultivar, the content of MDA, which is a byproduct of membrane peroxidation, significantly increased with NaCl treatment regardless of the NaCl concentration in the culture medium. Compared to the control culture, an increase of 150%, 172% and 185.00% in the MDA content was detected in 100, 200 and 400 mM NaCl-treated callus tissues of Carmen, respectively. No significant differences observed among the other NaCl concentrations (Fig. 3). Although the MDA content was slightly increased in NM-503 cultivar treated with 400 mM NaCl as compared to that in the control group, the difference was not statistically significant. In contrast

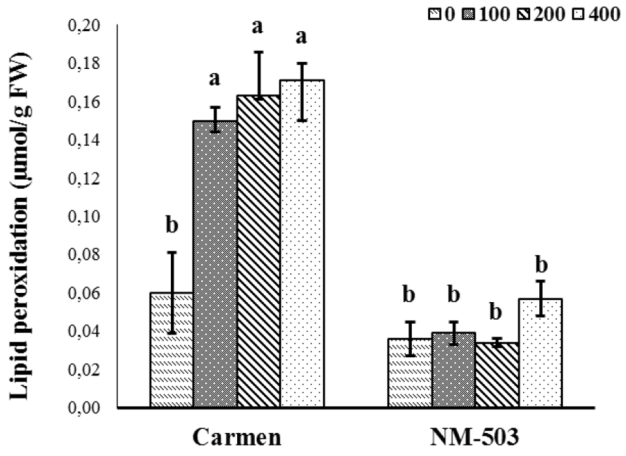

Fig. 3 Effects of different NaCl concentrations on lipid peroxidation in the callus culture of *Gossypium hirsutum* cultivars Carmen and NM-503. Mean (± SE) values in columns with different letters are significantly different at $P \leq 0.05$ according to Tukey's test

to Carmen cultivar, none of the NaCl concentrations led to membrane destruction in NM-503 cultivar (Fig. 3).

Antioxidant enzyme activities

Constitutive SOD activities of the cotton cultivars showed similarity in the control groups (Fig. 4a). The least SOD activity was measured in the callus tissues of Carmen cultivar exposed to 400 mM NaCl, which showed a significant decrease (91.29%) as compared to that of the respective control group. Other salt concentrations did not cause substantial differences in SOD activity between the cotton cultivars; SOD activity of the treated groups was similar to that of the control groups, except for the 100 mM NaCl-treated group (32.30% less activity than that of its respective control group [Fig. 4a]) in NM-503 cultivar.

Similar to SOD activity, the highest CAT activity was obtained for the NM-503 control culture, followed by the 200 mM NaCl-treated group of the same cultivar with 50.03% less activity than that of the control group. Nevertheless, all three salt treatment concentrations did not lead any alterations in CAT activity in callus tissues of Carmen cultivar. The CAT activities in the 100 and 400 mM NaCl-treated groups of NM-503 cultivar and all groups of Carmen cultivar were identical (Fig. 4b).

The basic level of POX activities among the groups differed from each other; there was approximately 2.4-fold higher activity in the control callus tissue of Carmen cultivar than in NM-503 cultivar (Fig. 4c). However, a progressive increase in NaCl concentrations in the culture medium led to an increase in POX activity in both cultivars. Although the highest POX activity (80.56% increase) was observed in the 400 mM NaCl-treated group of Carmen cultivar, when both cultivars were compared with their own control culture, the highest increase ratio in POX activity (2.90-, 3.27- and 3.15-fold) was observed in NM-503 cultivar with the increase in salt concentrations. Salt existence in the culture medium stimulated POX activities in the salt-treated groups of NM-503 cultivar regardless of NaCl concentrations (Fig. 4c).

APX activities of both cultivars were significantly similar in the control and NaCl-treated groups, except for the 400 mM NaCl-treated group of NM-503 cultivar. The highest APX activity (120.00% increase) was obtained for 400 mM NaCl-treated NM-503 callus tissue; however, the activity was still in the same statistical group for the 200 and 400 mM NaCl-treated groups of Carmen cultivar (Fig. 4d).

The effect of NaCl on GR activity varied between the cultivars. A progressive increase in NaCl concentrations in the culture medium decreased GR activity in Carmen cultivar, but increased GR activity in NM-503 cultivar (Fig. 4e). The constitutive GR activity of Carmen cultivar was significantly higher than that of NM-503 cultivar. GR activity was ca. 40.51%, 58.23% and 55.06% lower in callus tissues grown in 100, 200 and 400 mM NaCl, respectively, than those of the respective control culture. In contrast to the response of Carmen cultivar, in NM-503 cultivar, GR activity increased to 66.67%, 98.61% and 170.83% following treatment with 100, 200 and 400 mM NaCl, respectively, compared to the control culture.

Discussion

The in vitro selection method has been extensively used to understand the mechanisms of salt tolerance and sensitivity of plants to salinity (El-Mahdy et al. 2022). Because of their controllable conditions, in vitro callus culture systems can be used as an alternative model system to in vivo systems for studying the effects of stress factors on plant mechanisms and for determining plant responses to various stress factors at the cellular and tissue levels (Kruglova et al. 2018). The phenomenon reveals the cellular protection mechanisms by excluding other salt tolerance mechanisms such as roots, aged leaves or salt blades/hairs, which limit the distribution of salt to shoots and cytoplasm in the whole plant. In the present study, callus tissues of two cotton cultivars (Carmen and NM-503) were treated with different NaCl concentrations to evaluate the response of these cultivars to salinity stress.

Both cultivars had similar callus formation ratio from the hypocotyl explants; however, the responses of both cultivars to in vitro culture conditions varied during callus growth (4 weeks). A considerably less browning rate was observed in NM-503 cultivar under control culture conditions (Table 1).

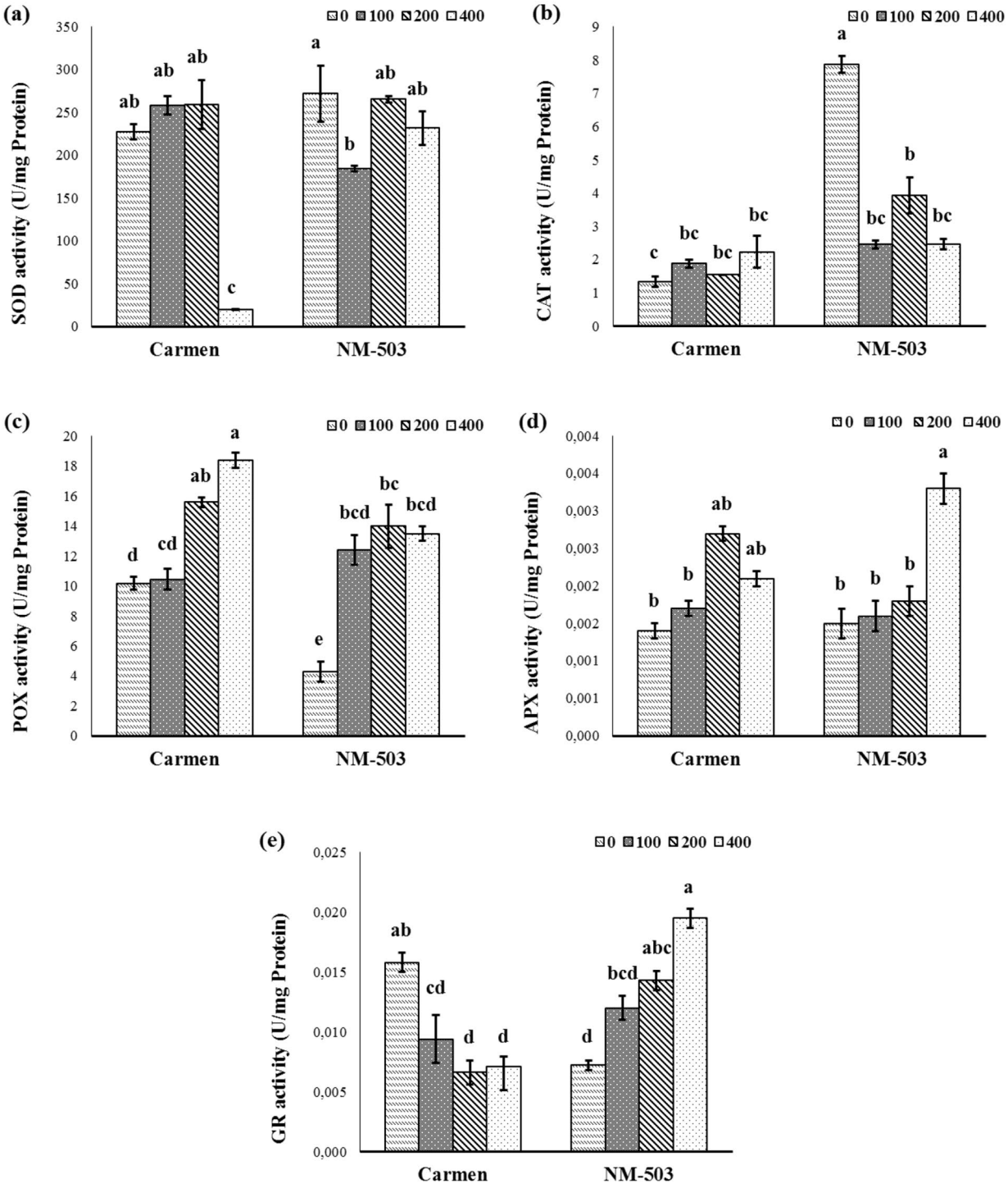

Fig. 4 Effects of different NaCl concentrations on SOD **(a)**, CAT **(b)**, POX **(c)**, APX **(d)** and GR **(e)** activities in the callus culture of *Gossypium hirsutum* cultivars Carmen and NM-503. Mean (±SE) values in columns with different letters are significantly different at $P \leq 0.05$ according to Tukey's test

Better growth performance trend of NM-503 cultivar then Carmen cultivar in the in vitro culture medium was evident only in control culture; NM-503 cultivar showed similar growth and browning tendency to Carmen cultivar when exposed to salinity stress. In a previous study, NaCl concentrations higher than 50 mM led to growth inhibition and tissue browning in *Solanum tuberosum* L. callus cultures (Queirós et al. 2007).

Cotton is a moderately salt-tolerant plant with a salinity threshold level of 7.7 dS m^{-1} (corresponding to 77 mM) (Ashraf 2002). In our results, the CSR was slightly reduced at 200 mM NaCl treatment in both cultivars in comparison with the control group. Baohong and Yun (1999) reported a slight impact of 5 g/L NaCl (corresponding to 125 mM) on the CSR of the cotton cultivar Coker 201; this finding is consistent with our results, thus indicating that both cultivars were moderately salt tolerant. The effect of severe stress appeared at the highest concentration of salt treatment, which significantly decreased the CSR whose level was still above 40% for both cultivars; this showed that even 400 mM NaCl concentration is not lethal for experienced cotton cultivars under in vitro conditions.

One of the processes to cope with stress is slowing down of the growth in plants (Hossain et al. 2007). In this regard, both cultivars maintained a high survival ratio in 200 mM NaCl treatment (approximately 85% and 80%) and in 400 mM NaCl treatment (approximately 47% and 42%) through substantial growth retardation under severe stress conditions.

The constitutive distinction between cultivars in callus growth and mass accumulation parameters demonstrated that NM-503 cultivar is more compatible to in vitro culture conditions than Carmen cultivar. Except for the control group, FW, DW and the RGR of callus tissue of both cultivars were similar and in the same statistical groups for all concentrations of NaCl. This shows that both cultivars had a similar growth response, which was significantly restricted by treatment with 200 and 400 mM NaCl (Table 1). The in vivo experiments results showed that 17 dS m^{-1} salinity (corresponding to 170 mM) causes 50% decrease in growth and seed yield of cotton (Ahmad et al. 2002), and it may vary among the cultivars (Akhtar et al. 2010). For in vitro conditions, salinity-tolerant cotton cultivars were defined by the stability of their characteristics at 8 g/L NaCl concentration (corresponding to 200 mM) (El Yacoubi et al. 2010).

Growth parameters are one of the most common parameters used to evaluate the salt stress tolerance of plants in in vitro selection studies (Chelli-Chaabouni et al. 2010). Osmotic stress caused by salinity mainly restricts the growth rate by inhibiting cell wall extension. Na^+ toxicity along with osmotic stress (Alhasnawi et al. 2016) and nutrient imbalance (Chelli-Chaabouni et al. 2010) in cells induce a decrease in biomass accumulation by inhibiting biosynthetic reactions. Therefore, the decrease in growth parameters with an increase in salt concentrations in shoots, cells, tissues or organs cultured in salt-stressed nutrient media is a common phenomenon encountered in in vitro selection (Ghane et al. 2014; Zhang et al. 2019; Singh and Kumar 2020). The reducing effect of NaCl on callus growth was reported by Gossett et al. (1994a) in callus tissue of salt-tolerant and salt-sensitive cotton cultivars. Growth reduction was also observed in 150 mM NaCl-treated greenhouse-grown whole-plant studies of the same cultivars and salt-tolerant and salt-sensitive cultivars (Gossett et al. 1994b). Apart from cotton, the embryogenic callus of indica rice (*Oryza sativa* L.) cv. MRQ74 subjected to 200 mM NaCl exhibited decreased callus FW, DW and RGR (Alhasnawi et al. 2016).

The fundamental components of the photosynthetic apparatus, namely the chlorophyll and carotenoid pigments, are used as salt tolerance markers in in vitro selection studies (Singh and Kumar 2020). TChl and carotenoid contents of NM-503 cultivar were higher than those of Carmen cultivar (Fig. 1a, b) in control conditions, thus indicating the compatibility of the former cultivar to in vitro culture in parallel to the results of high mass accumulation. Although the lowest photosynthetic pigment contents were observed in the 400 mM NaCl-treated group of Carmen cultivar, both cultivars did not show significant differences between NaCl concentrations. This result is consistent with the results of greenhouse-grown cotton genotypes (Munavar et al. 2021). The chlorophyll and carotenoid contents of leaves reduce under salt stress; however, it also significantly varies depending upon the tolerance level of cotton cultivars (Nascimento et al. 2019). It can be speculated that the NaCl exposure concentrations in the present study were inadequate to induce toxicity to inhibit the synthesis or accelerate the degradation of chlorophyll pigments in the callus cells of both cultivars.

Salinity induces Na^+ toxicity, while osmotic stress disrupts cellular homeostasis. This may eventually result in oxidative stress through the accumulation of toxic ROS. Therefore, the cellular protection mechanism against salinity stress relies on Na^+ sequestration and osmoprotection. In contrast to the whole-plant experiments, callus culture could provide better explants to understand the cellular protection mechanisms against salinity stress. The lipid peroxidation level of the cultivars indicated the presence of an efficient antioxidant mechanism and low consequences of salinity in NM-503 cultivar. This conclusion could be supported by the proline level in this cultivar; proline was not accumulated at 200 and 400 mM NaCl treatments. Together with the lipid peroxidation results, it could be suggested that proline accumulation varied based on the cellular stress level experienced by the cultivars. Gandonou et al. (2005) observed a similar situation in sugarcane calli exposed to different NaCl concentrations. They reported that proline accumulation was higher in the salt-sensitive variety than in the salt-tolerant cultivar; this situation was explained by the observation that proline acts a response factor in stressed sugarcane calli rather than as an indicator of tolerance.

Proline accumulation is reported as one of the most evident metabolic alterations under stress condition (Alhasnawi et al. 2016). Therefore, its cytoplasmic accumulation

in salt-stressed plants is generally used as a biochemical indicator to select salt-tolerant cultivars as it is involved in cellular osmotic adjustment between the cytoplasm and vacuole, cellular membrane stabilisation, and as a protective agent of enzymes and intracellular structures or as a free radical scavenger (Ashraf and Harris 2004; Gandonou et al. 2005; Cha-um et al. 2013; Singh and Kumar 2020). Nevertheless, there is uncertainty regarding the role of proline in plant salt tolerance and its specificity toward salt stress. Although high proline accumulation is associated with tolerance to stress in many plant species, instances of unaltered or reduced proline content have also been reported in the literature (Chelli-Chaabouni et al. 2010).

The generation of ROS is triggered by high NaCl concentrations. ROS can induce oxidative damage in crucial molecules, e.g., lipids, proteins and DNA. Membrane lipids have a crucial role to protect the structural and functional integrity of cells. Lipid peroxidation caused by ROS reduces membrane fluidity and selectivity and produces MDA, the level of which is regarded as a sign of stress-induced oxidative damage (Golkar and Taghizadeh 2018). Based on the salt-induced lipid peroxidation level, NM-503 cultivar showed better performance to limit the oxidative destruction in the membranes even under severe salt stress conditions than Carmen cultivar (Fig. 3). Cotton cultivars can be characterised according to their lipid peroxidation level, wherein the low lipid peroxidation level (namely low MDA) indicates a higher salt tolerance of the cultivar (Gossett et al. 1994b; Munavar et al. 2021). Among all studied parameters, the most distinct response of the cultivars against salinity is determined by the lipid peroxidation level, which represents the differences in cellular antioxidant protection between the cultivars. These results corroborate with those found by Basal (2010) who reported NM-503 cultivar as salt tolerant and Carmen as a moderately salt tolerant based on biomass production and/or reduction ratio under greenhouse conditions.

The ROS accumulated following high NaCl concentrations must be efficiently and continuously scavenged by the cellular antioxidant mechanism to prevent the potentially harmful effects of ROS (Alhasnawi et al. 2016). Plants have evolved a highly effective antioxidant defence mechanism comprising non-enzymatic and enzymatic components that generally maintain the balance of ROS within the cells (Queirós et al. 2007). Superoxide radicals produced in chloroplasts are reduced to H_2O_2 and O_2 by SOD. H_2O_2 and its derivatives are further scavenged by CAT, POX and APX (Zhang et al. 2019; Liang et al. 2018; Xiong et al. 2019). GR is a significant enzyme of the ascorbate-glutathione and glutathione peroxidase cycle (GPX) in antioxidant metabolism that catalyses the reduction reaction of oxidised glutathione to reduced glutathione (Mittler 2002). Because these antioxidant enzymes are crucial, the correlation between salt tolerance and antioxidant enzyme activities has been considered in studies on salt stress, and a positive correlation has been reported between salt tolerance and the activity of antioxidant enzymes (Singh and Kumar 2020).

Between the cultivars, no significant difference was observed in SOD activity for both control and NaCl-treated groups, except for the 400 mM NaCl-treated group in Carmen cultivar (Fig. 4a). The striking reduction in the SOD activity of callus tissue of Carmen cultivar may be because the 400 mM NaCl concentration inhibited the enzyme as a consequence of the cellular defence mechanism, i.e. ion homeostasis was maintained through vacuolar Na^+ sequestration ability, which was related to Carmen cultivar's lower salt tolerance than NM-503 cultivar. This hypothesis was also supported by the results of lipid peroxidation level and proline content. Apart from the constitutive enzyme activity, both cultivars expressed similar CAT activities at all concentrations of NaCl treatment. In agreement with our results, Gossett et al. (1994a) reported that the leaves of the more salt-tolerant cultivars had significantly greater constitutive levels of CAT than the more salt-sensitive cotton cultivars. However, the comparable level of CAT activity in response to salinity stress indicates that the enzyme activity may be an undistinctive parameter among the biochemical salt tolerance markers.

POX and APX activities increased in response to the increase in NaCl concentrations in both cultivars. The increase in the extent of POX activity was higher in NM-503 cultivar than in Carmen cultivar as compared to their respective control cultures. Combining the significant increase in APX following 400 mM NaCl treatment with the gradual increase in GR activity in response to salinity, it can be suggested that the role of the AsA–GSH cycle is obvious for scavenging ROS in the salt-tolerant NM-503 cultivar. Maintenance of the redox pool (AsA and GSH) of the cells was accompanied by the AsA–GSH cycle enzymes such as GR, MDHAR, APX and DHAR (Hasanuzzaman et al. 2019). In the higher salt tolerance ability of NM-503 cultivar, APX and GR played a crucial role to control the redox balance under severe salinity stress conditions, and these enzymes could be used as a biochemical marker to characterise salinity tolerance of cotton.

In conclusion, in the present study, the growth and biochemical responses of two *G. hirsutum* cultivars, Carmen and NM-503, were compared against salinity stress. In vitro germinated seedlings were used for callus culture, and they were exposed to mild and severe salinity stress for 4 weeks. Our study revealed that NM-503 cultivar is very compatible with in vitro growth conditions, and this cultivar could be used as a model cultivar to determine cellular and molecular aspects of abiotic stress tolerance for the breeding

programmes of cotton. Growth parameters, i.e. mass accumulation, CSR, RGR, and CBR, were significantly limited in the callus of Carmen and NM-503 cultivars under 200 and 400 mM salinity. This similarity of the two cultivars in response to severe salinity may indicate that the growth parameters alone may be insufficient as in vitro screening criteria for determining the salt tolerance level. A variation in response to salt stress between the two cultivars was revealed by the level of cellular lipid peroxidation and proline accumulation. NM-503 cultivar showed more salt tolerance than Carmen cultivar by maintaining a low level of lipid peroxidation under salt stress conditions. These results indicate that the lipid peroxidation level might be the most reliable selection criterion for salt tolerance of cultivars. In the absence of salt exclusion mechanisms used by intact plants, the high level of resilience shown by the calli of NM-503 cultivar against severe salt stress conditions may be attributed to its effective cellular antioxidant protection mechanism. Further research is required on the vacuolar Na^+ sequestration to elucidate the cellular ion homeostasis in the salt-tolerant cultivars of cotton.

Acknowledgements We are grateful to Ege University Planning and Monitoring Coordination of Organizational Development and Directorate of Library and Documentation for their support in editing and proofreading service of this study.

Authors' contributions All authors contributed to the study conception and design. Experiments were conducted by MS. Data collection and analysis were performed by MS, LYA and MB. The first draft of the manuscript was written by MB and critically edited by LYA and AG. All authors read and approved the final manuscript.

Funding This project was supported by the Ege University Scientific Research Projects Coordination Unit (project number: FYL-2018-20040).

Data Availability All data generated or analysed during this study are included in this published article as tables and figures.

Declarations

Ethics approval and consent to participate Not applicable.

Informed consent Not applicable.

Competing interests The authors declare that they have no competing interests.

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Authors and Affiliations

Melis Sacu[1] · Lale Yildiz Aktas[2] · Meltem Bayraktar[3] · Aynur Gurel[4]

✉ Meltem Bayraktar
meltembayraktar5@gmail.com; meltem.bayraktar@ahievran.edu.tr

[1] Department of Biotechnology, Graduate School of Natural and Applied Sciences, Ege University, Izmir 35040, Turkey

[2] Department of Biology, Faculty of Science, Ege University, Izmir 35040, Turkey

[3] Department of Genetic and Bioengineering, Faculty of Engineering and Architecture, Kırşehir Ahi Evran University, Kirsehir 40100, Turkey

[4] Department of Bioengineering, Faculty of Engineering, Ege University, Izmir 35040, Turkey