Effect of salt stress on some physio-biochemical traits and antioxidative enzymes of two *Brassica* species under callus culture

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Received: 1 April 2021 / Accepted: 2 July 2021 / Published online: 29 July 2021
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Abstract
The changes in lipid peroxidation, \( \text{H}_2\text{O}_2 \), proline, protein, involvement of different antioxidant systems (catalase, guaiacol peroxidase, ascorbate peroxidase) and callus-related traits were investigated under salt stress in the callus of two different ploidy levels of *Brassica* including *B. juncea* and *B. oleracea*. The calluses of *B. juncea* genotypes were less sensitive to NaCl stress in comparison with those of *B. oleracea* while increasing the concentrations of NaCl from 0 to 200 mM. Tetraploid genotype (*B. juncea* cr3356) showed a significant increase in the contents of protein and proline, and guaiacol peroxidase activity and catalase enzymes at higher salinity levels. In addition, a significant decrease occurred in the amount of \( \text{H}_2\text{O}_2 \) and malondialdehyde along with increasing the salinity intensity. Diploid cultivar (*B. oleracea* bra 2828) had the lowest enzymatic activities and the highest content of \( \text{H}_2\text{O}_2 \) and malondialdehyde along with an increase in the salinity level. Therefore, this genotype was identified as the most sensitive cultivar under the salinity stress. The salinity resistance difference between diploid and amphidiploid species could be attributed to the differences in the ploidy level of these species. This result underlines the fact that the tetraploid genome of *B. oleracea* could be considered as a suitable candidate for production under salinity conditions through maintaining higher activities of antioxidative enzymes.

Key Message
This study revealed the effects of salinity stress on *Brassica juncea* and *B.oleracea*, through callus culture. In sum, these species showed different response to the salinity stress.

Keywords *Brassica* · In vitro · Peroxidase · Salinity stress

Introduction
Salinity stress is considered as a major limiting factor for the crop productivity in arid and semi-arid regions of the world (Parihar et al. 2015). It is estimated that about 50% of the world’s land will be saline by the middle of the twenty first century (Mahajan and Tuteja 2005). Many physiochemical processes are influenced due to the toxicity effects of salinity stress at the whole and cellular levels of the plant (Arzani 2008; Van Zelm et al. 2020).

Excess formation of reactive oxygen species (ROS) is occurred in plants in response to salinity stress conditions (Arora et al. 2002). Different biochemical processes occur to scavenge the ROS under the salinity stress in plants (Ashraf and Foolad 2007). In particular, increasing \( \text{H}_2\text{O}_2 \) is a good marker of the extent of oxidative stress under the environmental stresses (Sofo et al. 2015). The production of \( \text{H}_2\text{O}_2 \) in plant cells, under the drought stress is accomplished with the roles of both signaling molecule and a regulator compound for increasing certain genes’ expression (Sofo et al. 2015). Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells under salinity stress (Parida and Das 2005). Different metabolic
adjustments are directed to such organic solutes’ accumulation under the plant adaptation process against the salinity stress (Ashraf and Foolad 2007). Proline is known as a compatible solute under the environmental stresses, particularly the salinity that protects plant cells by scavenging ROS (Mittler et al. 2004). Plants employ different antioxidant defense systems as well as antioxidant enzymes (e.g. catalase, ascorbate peroxidase, and guaiacol peroxidase) in order to mitigate the oxidative damage via ROS under salinity conditions (Gill and Tuteja 2010; Foyer and Noctor 2011).

The catalase (CAT) acts as an antioxidant enzyme by dismutating $\text{H}_2\text{O}_2$ into $\text{H}_2\text{O}$ and $\text{O}_2$ (Sudhakar et al. 2001; Sofo et al. 2015). Peroxisomes, cytosol, and mitochondria are considered as the main organs for its accumulation in plant cells (Sofo et al. 2015). The produced $\text{H}_2\text{O}_2$ is then scavenged by CAT and several classes of peroxidase enzymes (Gill and Tuteja 2010; Sofo et al. 2015).

At the first step of the ascorbate–glutathione cycle in the plant cells, ascorbate peroxidase (APX) uses ascorbate ($\text{C}_6\text{H}_7\text{O}_6\text{H}_2\text{O}_4$) as the electron donor for $\text{H}_2\text{O}_2$ detoxification (Noctor and Foyer 1998).

Similarly, guaiacol peroxidase (GPX) is involved in a range of processes related to ROS-induced stress (Uarrota et al. 2016). It is located in the cytosol, vacuole, and apoplast of plant cells. The GPX decomposes $\text{H}_2\text{O}_2$ by oxidation of co-substrates such as phenolic compounds and/or ascorbate (Uarrota et al. 2016). Consequently, the balance of APX, GPX, and CAT activities, representing the main enzymatic $\text{H}_2\text{O}_2$ scavenging mechanism in plants, is crucial for the suppression of toxic $\text{H}_2\text{O}_2$ levels in a cell (Sofo et al. 2015).

The study of plants’ physiological responses to salt stress shows that in vitro callus culture technique could facilitate the preparation of a uniform environment (undifferentiated cells of callus) (Rai et al. 2011). This biotechnological method could eliminate impediments that arise from variability in genetic and morphological levels associated with plants’ tissues at the whole level. As a supplementary tool, identification and selection of the salt-tolerant genotypes could be considered for traditional breeding in salt-stressed regions (Rai et al. 2011). Identification of involved biochemical mechanisms in salt tolerance at callus level has been reported in several industrial species such as *Nigella sativa* (Golkar and Nourbakhsh 2019), sunflower (*Alvarez et al. 2003*), sugarcane (*Gandonou et al. 2006*), *Plantago ovata* (Golkar et al. 2017), and safflower (Golkar and Taghizadeh 2018).

Different members of the Brassicaceae family (about 3675 species) are considered as very important components for nutritional purposes (Shankar et al. 2019). Crop plants from this family are among the oldest recognized cultivated plants that are categorized as commercially significant vegetables (Purty et al. 2008). Interestingly, the members of the Brassicaceae family have very diverse genetic compositions through different within and between hybridizations. The cultivated *Brassica* species include both diploid and polyploid species (Purty et al. 2008); while, their response and adaptation to salinity are meaningfully different (Ashraf and McNeill 2004). Two basic approaches are currently used to obtain stress-tolerant *Brassica*, including: (i) screening of existing genotypes, and (ii) conventional breeding (Purty et al. 2008). It is indispensable to identify the morphological, physiological, and biochemical responses of diverse *Brassica* species for sustainable production in a saline environment.

Currently, most *Brassica* crop species are classified as moderately salt tolerant. The amphidiploid species of *B. juncea*, *B. napus*, and *B. carinata* reportedly have a rather higher tolerance than the diploids of *B. oleracea*, *B. nigra*, and *B. rapa* (Purty et al. 2008; Pavlović et al. 2019). In addition, it has been reported that *Brassica* species are more sensitive to the salinity tolerance at the seed germination and early growth stages (Ashraf and McNeill 2004).

However, according to literature review, there is no notable knowledge regarding the tolerance mechanisms in the Brassicaceae species at the callus level. Furthermore, this study was conducted to elucidate mechanisms responsible for in vitro salt tolerance at the callus level in two different species of Brassicaceae family with ploidy levels including *B. juncea* (amphidiploid) and *B. oleracea* (diploid).

**Materials and methods**

Two genotypes of *B. juncea* (common name: mustard) with amphidiploid level (2n = 36, AABB) (namely cr113 and cr3356) and three genotypes of *B. oleracea* (common name cabbage) with diploid level (2n = 18, CC) (namely br258 and br2828 and br2993) were deposited from IPK Research Institute, Gatersleben, Germany. Using 1.5% sodium hypochlorite, the seeds were sterilized for 15 min. Then the sterilized seeds were washed with double sterile water three times. After disinfecting the seeds, they were incubated following the protocol of Murashige and Skoog (1962) MS (Duchepha, Netherlands). The concentration of 30 g/L sucrose was used as the supplement, and the seeds were solidified with 8 g/L agar (Merck, Com.) for initiating the germination. After 7–10 days, germinated seeds were applied for dissecting hypocotyl explants.

Afterward, the MS (Duchefa, Haarlem, The Netherlands) media was supported with 8 g/L agar, 30 g/L sucrose, 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2, 4-D), and 0.5 mg/L Kinetin for the callus induction. The cultures were then incubated with 16/8 dark/light cycle in a growth chamber at 24 °C. After four weeks, the induced calli were delivered to MS medium and varied NaCl concentrations were added (0, 75, 100, and 200 mM) to it. The period of
incubation in the salt stress was 30 days. The treatments were composed of three replicates (Petri dishes); while, each of them included seven callus parts. After 30 days of the culture, the callus biochemical and physiological parameters were measured.

**Callus-related traits**

An oven setting at 65 °C was used for drying the callus species of specified fresh weight for 48 h at 65 °C for calculating its dry weight. Then, their weights were re-calculated, and the differences between initial and final masses were specified. Relative water content (RWC) was measured as \([\text{fresh weight of the callus − dry weight of callus}/\text{dry weight of callus} × 100]\) (Golkar et al. 2017).

For calculation of the relative growth rate (RGR), the calli were initially weighed at the time of their transfer (\(W_i\)) to the salinity stress, and finally after 30 days of the salt treatment (\(W_f\)), the mean of RGR for calli was calculated as the following formulae: \([W_f − W_i/ W_i] × 100\%\) (Lokhande et al. 2010).

**Malondialdehyde content (MDA)**

Firstly, 0.2 g of the fresh callus was homogenized in 3 mL of trichloroacetic acid (TCA, 10% w/v) and then heated the filtrates’ aliquots for 30 min in 0.25% thiobarbituric acid. Afterward, the final cooling phase was conducted using an ice bath. The solution absorbance was measured at 532 nm, and then, the nonspecific absorbance correlation was calculated at 600 nm. The MDA was specified as 155 mM−1/cm, according to the extinction coefficient.

**Hydrogen peroxide content**

The method proposed by Loreto and Velikova (2001) was used for estimating the \(H_2O_2\) content. To this end, using varied concentrations of \(H_2O_2\), a standard calibration curve was developed. Shortly, 5 mL of 0.1% (w/v) TCA was added to 0.07 g of the fresh callus, in order to make it homogenized. Then, centrifuging the homogenate was carried out for 15 min at 12,000 × g followed by the addition of 0.5 mL of potassium phosphate buffer (10 mm) (pH 7.0), and 0.5 mL of potassium phosphate buffer (10 mm) (pH 7.0). The supernatant absorbance was calculated at 390 nm, and the content of \(H_2O_2\) was measured in comparison with a standardized calibration curve.

**Total protein content**

After extracting and quantifying the protein and enzyme tests, fresh callus (1 g) was homogenized in 2 mL of an ice-cold buffer (62.5 mM Tris–HCl, pH 6.7) that contained 0.3 M sucrose by a pestle and pre-chilled mortar. Centrifuging the supernatant was implemented for 30 min of 20,000 × g at 4 °C. We used supernatant for assaying enzyme activities and protein content. Given the method proposed by Bradford (1976), enzyme extracts’ solvable protein content was specified by bovine serum albumin (BSA). Using the Shimadzu spectrophotometer (UV–Visible 160, Shimadzu, Japan), the spectrophotometric analyses were performed.

**Guaiacol peroxidase activity**

The method presented by Lin and Kao (1999) was used for determining GPX activity in a reaction mix (1.0 mL) composing 9 mM guaiacol, 50 mM sodium phosphate buffer (pH 7.0), 10 μL protein extract, and 19 mM \(H_2O_2\). After adding the protein extract, the rise in absorbance was recorded for 1 min at 470 nm. The quantification of enzyme activity was performed by tetraguaiacol amount developed by the use of its molar extinction coefficient (26.6 mM−1/cm).

**Ascorbate peroxidase (APX) assay**

The total APX activity was measured according to the method described by Jebara et al. (2005) through the measurement of the decline in A290 as ascorbate (\(E = 2:8\) mM−1/cm) was oxidised, for three min. 1 mL reaction volume consisted of 50 mM potassium phosphate buffer pH 7; 10 μL enzyme extracts, 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. The reaction was started by adding the \(H_2O_2\) and decreasing in absorbance at 290 nm. The activity of APX was calculated based on μmol/mg protein.

**Catalase activity**

The activity of catalase (CAT) was assayed based on Aebi (1983) that calculates the \(H_2O_2\) decline in 240 nm at the maximum absorption. The mixture of the reaction included 15 mM \(H_2O_2\), 50 mM potassium phosphate buffer pH 7.0, and 20 mL protein extract. The decline of \(H_2O_2\) level was quantified and monitored by the related molar extinction coefficient (36 M−1/cm), and the outcomes were provided as μmol \(H_2O_2/min/mg\) protein.

**Proline content**

The approach presented by Bates et al. (1973) was used for measuring the leaf proline. In short, 500 mg fresh leaves were milled in an aqueous solution in 10 mL of 3% sulfosalicylic acid, followed by the extract filtration. Afterward, 2 mL of freezing acetic acid and 2 mL of ninhydrin reagent were combined with 2 mL of the excerpt. Using a water bath (100 °C), the reaction mixture was boiled for 1 h. The resulting mixture was cooled on ice before adding 4 mL of the toluene. The toluene phase was isolated, and its absorbance
was evaluated against the toluene blank at 520 nm by a spectrophotometer (UNICO Model UV-2100).

**Statistical analysis**

The study was performed as factorial research with the Completely Randomized Design (CRD) with 3 repetitions for each treatment. We selected 10 calli per replication randomly and examined their various traits. Data analysis was performed using ANOVA by PROC GLM of SAS Institute (2011). The comparison of means was studied based on the Fisher’s least significant difference (LSD) test at the probability level of 0.05. The data were expressed as the mean ± standard error (SE).

**Results and discussion**

There was significant difference between different levels of salinity and genotypes for all of the studied traits, except for RWC (Table S1). The genotype × salinity interaction was significant for all traits, except for RWC and CAT (Table S1).

**Effects of salinity stress on different studied traits**

**Callus growth traits**

The salinity stress resulted in producing browner and more necrotic calli in both *Brassica* studied species. Higher salinity levels caused more coloration of the calli colors in all genotypes (data not shown). According to Fig. 1A, the RGR mean of genotypes ranged from 6.79% in br2993 genotype (*B. oleracea*) to 16.94% in cr3356 genotype (*B. juncea*). Adding NaCl to *B. juncea* and *B. oleracea* led to a significant decline in RGR content, under in vitro salt stress; however, in all salinity levels, the means of *B. juncea* was greater than *B. oleracea* for RGR (Fig. 2A). According to the salinity × genotype interaction, the salinity stress resulted in a reduction of RGR in all studied genotypes; nevertheless, its effect was not equal in all genotypes (Table 1). The bra2993 and bra2828 genotypes revealed more decline in RGR at high concentrations of the salinity (Table 1). The cultivar of cr3356 (*B. juncea*) did not demonstrate a significant decrease in RGR up to 200 mM (NaCl) (Table 1), which indicated high tolerance of this genotype to the salinity stress at callus level.

This phenomenon could be resulted from a water potential gradient between the nutrient medium and cell (Lokhande et al. 2010). This negative potential would result decreasing the growth rate of the calli in dehydrated cells under salinity stress (Golkar et al. 2020). The findings verified that the callus growth reduction is more severe in diploid than amphidiploid genotypes (Fig. 2A; Table 1). In addition, no significant difference was found among the means of genotypes, salinity levels, and genotype × salinity interaction in terms of the relative water content (Table S1).

**Malondialdehyde (MDA) content**

The highest (0.0051 MDA μg/g DW) and the least (0.002 MDA μg/g DW) content of MDA were observed at the genotypes of br2993 and cr113, respectively, averaged over all salinity levels (Fig. 1B). The mean comparison of MDA changes under different salinity levels demonstrated an increase in its content parallel with increasing the salinity levels (Fig. 2B). The mean of MDA was significantly increased in two salinity levels (150 and 200 mM NaCl) more in diploid genotypes than the tetraploid ones (Fig. 2B).

On one hand, the significant differences observed among MDA values at different salinity levels might be attributed to non-supplementary effects of adequate osmolites’ accumulation as proline in these species at the salinity conditions, through detoxification of ROS and subsequent protection of membrane integrity (Ashraf and Foolad 2007). On the other hand, this type of response might be associated with H$_2$O$_2$ content increase, which could be a drastic enhancement under the salinity stress among the plant species undergoing higher lipid peroxidation (Khan and Panda 2008).

The increased MDA content in salinity-stressed calli has been reported in other species such as safflower (Golkar and Taghizadeh 2018) *N. sativa* (Golkar et al. 2020) and *Acanthophyllum* (Niknam et al. 2011). In diploid genotypes, the amounts of MDA showed an increase along with intensification of the salinity levels except for bra2828 and bra258 under 75 mM NaCl, which had a significant decrease compared to the control (Table 1). The treatments of 150 and 200 mM NaCl had the highest rates in all three genotypes, which revealed significant differences with the control treatment (Table 1). This finding exhibited a peroxidation increase and severe degradation of lipids in these genotypes under the stress conditions similar to the report of *B. oleracea* (Sahin et al. 2018). The highest content of MDA for cr3356 (0.003 μg/g DW) and cr113 (0.0024 μg/g DW) was observed in the 200 mM treatment (Table 1). The highest content of MDA (0.006 μg/g DW) was observed at 200 mM NaCl for diploid genotypes. This result highlights a selective advantage of species with amphidiploid levels to struggle with deleterious effects of the salinity stress in *Brassica* species. Similar results in the *Acanthophyllum* calli confirm that the callus of hexaploid species has a lower level of MDA than the callus of tetraploid species under the salinity stress (Niknam et al. 2011). This phenomenon demonstrates a higher salinity tolerance in higher ploidy levels than that of lower ones.
An increase in the content of hydrogen peroxide leads to lipid peroxidation, which ultimately results in cell membranes’ destruction (Hossain et al. 2015). The mean comparison among different salinity levels ranged from 0.392 μmol/g DW under 200 mM NaCl to 0.330 μmol/g DW under the control conditions. The H$_2$O$_2$ content presented...
no significant difference between both species under non-salinity condition; whereas, they exposed different trends along with increasing the salinity levels (Fig. 2C).

The highest (0.45 μmol/g DW) and the least (0.25 μmol/g DW) content of H$_2$O$_2$ were observed in genotypes *B. oleracea* br2993 and *B. juncea* cr113, respectively (Fig. 1C). According to the genotype × salinity interaction, H$_2$O$_2$ content ranged from 0.529 (μmol/g DW) in *B. oleracea* br2993 under 200 mM NaCl to 0.211 (μmol/g DW) in *B. juncea* cr113 under 150 Mm NaCl condition (Table 1). Significant differences were revealed among different genotypes under the control condition (Table 1). However, this difference was not significant between *B. oleracea* br2828 and *B. oleracea* br2993 cultivars (Table 1).

In three diploid genotypes, the amount of hydrogen peroxide was increased along with increasing the salinity levels (Table 1). However, the genotype *B. oleracea* br2993 had a significant reduction under 150 mM of NaCl compared to the control treatment (Table 1). Three diploid genotypes, at 200 mM NaCl displayed a significant difference with the control treatment, which indicates high sensitivity of these genotypes to the salinity at high NaCl concentrations (Table 1). Dissimilar to the diploid genotypes, amphiplipid ones (*B. juncea*) showed a decrease in H$_2$O$_2$ content with an increase in the salinity levels (Fig. 2C). This finding demonstrated a higher activity of CAT and APX in amphidiploid rather than the diploid genotypes, with H$_2$O$_2$ content reduction under the salinity stress.

### Protein content

Protein synthesis can be affected by different environmental stresses including the salt stress (Muchate et al. 2016). Several salt-induced proteins have been identified, belonging to
two distinct groups: salt stress proteins that are accumulated only due to salt stress, and stress-associated proteins, which are stored in response to various environmental stresses including salinity (Ashraf and Harris 2004). Proteins accumulated in grown plants under saline conditions may provide a storage form of nitrogen, which could be reutilized when stress is raised and in this situation, they may play a role in osmotic adjustment (Muchate et al. 2016). In overall salinity levels, the highest (1.05 μg/g DW) and the lowest (0.68 μg/g DW) contents of the total protein were observed in B. juncea cr3356 and B. oleracea br258 genotypes, respectively (Fig. 1D).

The mean of the protein content showed a significant increase from the control (0.79 μg/g DW) to 0.90 (μg/g DW) under 200 mM NaCl. In all salinity levels, the means of B. juncea species were more than B. oleracea species in terms of protein content (Fig. 2D).

The mean comparison of the salinity × genotype interaction (Table 1), indicates an increase in the total protein content and sodium chloride concentration in all genotypes of the culture medium. According to Table 1, the highest content of protein (1.12 μg/g DW) was observed at 200 mM NaCl in B. juncea cr3356 genotype, which indicates a significant difference with other treatments and cultivars (Table 1). Similar results were obtained as the protein content increase in B. juncea genotypes; nonetheless, this increase was detected more in B. juncea rather than B. oleracea genotypes. Comparable to this finding, the protein content increase was also reported in B. oleracea calli at 100 mM NaCl (Mukhtar and Hasnain 1994) as well as other species as Acanthyphyllus (Niknam et al. 2011) and Broussonetia papyrifera (Zhang et al. 2013).

### Antioxidants enzymes’ activity

To prevent oxidative damage, plants improve their antioxidant enzymes activity such as catalase, guaiacol peroxidase, and ascorbate peroxidases. In addition, to counteract the toxic effects of increasing the extent of ROS under the salinity stress, various defense mechanisms including antioxidant enzymes activity are triggered in the plant (Gupta and Huang 2014). Under these conditions, the enzymes inhibiting ROS production are augmented to reduce the toxic effects of oxidative stress. In this study, a significant variation was found regarding the activity of APX and GPX. Amphidiploid genotypes generally showed higher antioxidant activity (APX and GPX) as compared to diploid ones. This suggests that high antioxidant enzymes activity has a significant role in imparting salt tolerance in these amphidiploid genotypes.

### Table 1

The mean of various Brassica genotypes measured for different physio-biochemical traits under in vitro salinity stress

| Genotypes | NaCl | RGR % | MDA μg/g DW | H₂O₂ μmol/g DW | Protein μg/g DW |
|-----------|------|-------|-------------|----------------|-----------------|
| B. juncea cr113 | 0 | 16.32 ± 0.46 (100) | 0.0015 ± 0.0003 (100) | 0.267 ± 0.008 (100) | 0.93 ± 0.006 (100) |
| | 75 | 14.97 ± 0.17 (91.7) | 0.0023 ± 0 (151.1) | 0.296 ± 0.006 (110.7) | 1.04 ± 0.012 (111.8) |
| | 150 | 13.13 ± 0.54 (80.4) | 0.0024 ± 0.0003 (157.8) | 0.211 ± 0.009 (78.9) | 1.02 ± 0.006 (109.7) |
| | 200 | 11.46 ± 0.24 (70.2) | 0.0024 ± 0.0011 (157.8) | 0.244 ± 0.006 (91.5) | 1.04 ± 0.004 (111.8) |
| B. juncea cr3356 | 0 | 19.22 ± 0.64 (100) | 0.0015 ± 0.0001 (100) | 0.372 ± 0.001 (100) | 0.957 ± 0.009 (100) |
| | 75 | 17.8 ± 0.34 (92.6) | 0.0024 ± 0.0002 (162.2) | 0.308 ± 0.003 (82.8) | 1.05 ± 0.015 (109.7) |
| | 150 | 16.64 ± 0.34 (86.6) | 0.0021 ± 0.0011 (137.8) | 0.278 ± 0.01 (74.8) | 1.077 ± 0.018 (112.5) |
| | 200 | 14.09 ± 0.5 (73.3) | 0.003 ± 0.0001 (197.8) | 0.261 ± 0.003 (70.3) | 1.12 ± 0.006 (117) |
| B. oleracea br258 | 0 | 12.76 ± 0.92 (100) | 0.0039 ± 0.0001 (100) | 0.226 ± 0.002 (100) | 0.627 ± 0.015 (100) |
| | 75 | 10.99 ± 0.33 (86.1) | 0.0036 ± 0.0001 (91.5) | 0.34 ± 0.003 (150.6) | 0.66 ± 0.012 (105.3) |
| | 150 | 7.43 ± 0.37 (67.6) | 0.0055 ± 0.0002 (140.2) | 0.469 ± 0.003 (207.4) | 0.73 ± 0.006 (116.4) |
| | 200 | 4.32 ± 0.33 (58.2) | 0.005 ± 0 (127.4) | 0.425 ± 0.002 (187.9) | 0.69 ± 0.006 (110) |
| B. oleracea br2828 | 0 | 11.07 ± 0.11 (100) | 0.0048 ± 0.0002 (100) | 0.309 ± 0.006 (100) | 0.757 ± 0.029 (100) |
| | 75 | 8.68 ± 0.41 (78.4) | 0.0042 ± 0.0001 (88.2) | 0.488 ± 0.018 (157.8) | 0.84 ± 0.017 (111) |
| | 150 | 5.77 ± 0.08 (66.6) | 0.0053 ± 0.0003 (111.1) | 0.462 ± 0.011 (149.4) | 0.87 ± 0.012 (114.9) |
| | 200 | 3.46 ± 0.44 (59.9) | 0.006 ± 0.0001 (125) | 0.52 ± 0.014 (168.4) | 0.84 ± 0.035 (111) |
| B. oleracea br2993 | 0 | 10.91 ± 0.15 (100) | 0.0048 ± 0.0001 (100) | 0.479 ± 0.001 (100) | 0.7 ± 0.012 (100) |
| | 75 | 8.33 ± 0.28 (76.4) | 0.0053 ± 0 (110.4) | 0.488 ± 0.003 (101.9) | 0.75 ± 0.012 (107.1) |
| | 150 | 5.8 ± 0.33 (46.6) | 0.0054 ± 0.0002 (111.8) | 0.314 ± 0.014 (65.5) | 0.74 ± 0.012 (105.7) |
| | 200 | 2.82 ± 0.16 (25.9) | 0.006 ± 0.0004 (124.3) | 0.529 ± 0.001 (110.5) | 0.8 ± 0.012 (114.3) |

The values in parentheses indicate changes’ percentage rather than respected control.

RGR relative growth rate, MDA malondialdehyde.
GPX activity

The GPX is involved in numerous physiological processes and characterized as an electron donor (Ahire et al., 2013). In this study, the content of GPX for genotypes ranged from 0.32 μmol/mg protein in B. juncea (cr3356) to 0.007 μmol/mg protein in B. oleracea br258 genotype (Fig. 3A). The content of GPX showed an increasing trend from the control (0.084 μmol/mg protein) to 0.12 (μmol/mg protein) under 200 mM NaCl. In all salinity levels, particularly at 200 mM NaCl, the means of B. juncea species were more than the means of B. oleracea for GPX activity (Fig. 4A). This increase under in vitro salinity culture was similar to previous reports on Bacopa monnieri (Ahire et al. 2013).

Fig. 3 The activity of guaiacol peroxidase (A), ascorbate peroxidase (B), catalase (C) and proline content (D) of different genotypes of B. oleracea and B. juncea averaged over different salinity levels (0 to 200 mM NaCl). Error bars’ standard error (n = 12). Within each set of experiments, the means with different letters are significantly different at P<0.05.
and Spinacia oleracea (Muchate et al. 2019). The comparison of the salinity × genotype interaction for GPX activity, showed that the highest activity (0.39 μmol/mg protein) was observed at 200 mM NaCl in B. juncea cr3356 genotype (Table 2); while, the lowest activity (0.002 μmol/mg protein) was related to the genotype of bra2993 at 200 mM NaCl, which was not significantly different from the control treatment in the same genotype. According to Table 2 in diploid cultivars (B. oleracea br2993 and B. oleracea br258) no significant difference was observed in GPX activity between different salinity levels.

The GPX activity in B. oleracea br2993 was increased up to the level of 150 mM NaCl and then sharply decreased at 200 mM NaCl, which was not significantly different from the control treatment (Table 2). However, in two amphiploid genotypes, the GPX activity revealed a significant increase along with NaCl concentration increase (Table 2). The results of the previous reports uncovered that in amphiploid species of Brassica, the GPX activity was higher than its activity in diploid species under non-salinity stress (Meng et al., 2011).

**APX activity**

Peroxidases are a set of glutathione reductase ascorbate cycle enzymes that can convert oxygenated water to water through removing H₂O₂ (Das and Roychoudhury 2014). The APX is the most important reducing substrate that carries the dismutase of H₂O₂ to water. The mean comparison of genotypes showed a variation from 0.28 μmol/mg protein in B. juncea cr3356 to 0.07 μmol/mg protein in B. oleracea br258 (Fig. 3B). The APX content varied from 0.11 μmol/mg protein under 75 mM NaCl to 0.17 μmol/mg protein under 200 mM NaCl (Fig. 4B). The comparison of the salinity × genotype interaction with the activity of APX showed that the highest activity of APX (0.432 μmol/min/g of the
protein) belongs to the treatment of 150 mM NaCl on *B. juncea cr3356*, which was not significantly different from 200 mM NaCl treatment (Table 2). In addition, the lowest activity of APX was related to 150 and 200 mM NaCl on *B. oleracea br258*, which did not have any significant difference with the control and 75 mM NaCl treatments (Table 2). In *B. oleracea* br258, the activity of APX significantly decreased along with increasing the salinity levels; while, this trend was not significantly observed in other genotypes (Table 2).

As the duration of exposure to the salinity stress increased, the APX activity showed greater increase in the amphiploid genotypes, rather than the diploid genotypes (Fig. 4B). This finding was in line with the finding in different ploidy levels of turnip (Meng et al. 2011), the calli of eggplant (Yasar et al. 2006) and shoot culture in *S. oleracea* (Muchate et al. 2019) under the salinity stress.

Catalase activity

Plants are endowed with H$_2$O$_2$-metabolizing enzymes such as CAT and APX (Das and Roychoudhury 2014; Sofo et al. 2015). On average out of all salinity levels, the highest CAT activity (0.041 μmol/mg protein) was related to *B. juncea cr3356* genotype which had a significant difference with other cultivars (Fig. 3C). No significant difference was identified in relation to catalase activity of diploid genotypes. The CAT activity showed significant increase in both species until 150 mM NaCl; while, the trend of changes was different in both species under 200 mM NaCl (Fig. 4C). The tetraploid genotypes demonstrated significant increase; where, the diploids showed a decreasing trend (Fig. 4C). The considerable CAT activity increase, observed in the calli of different *Brassica* species under the salinity stress, is able to sustain electron flows that are the main producers and targets of the ROS action in *Brassica* species (Sofo et al. 2015). The main sites of the presence of catalase are peroxisomes, which convert H$_2$O$_2$ to H$_2$O and O$_2$ (Sofo et al. 2015). According to non-significant effect of the salinity × genotype interaction for the CAT activity, it can be concluded that trend responses of different genotypes were similar at different salinity levels.

Although the catalase activity increased with increasing the salinity levels, the catalase level decreased at 200 mM NaCl in the genotypes cr2993 and br2828 (Table 2). This finding was in contrast with other reports in the callus of melon under salinity stress (Kusvuran 2012) and shoot cultures of *S. oleracea* (Muchate et al. 2019). In general, as the chromosome number increases, the DNA content and enzyme activity per cell are also increased (Yıldız 2013).

### Table 2

The mean of various *Brassica* genotypes measured for antioxidants enzymes’ activity and proline under in vitro salinity stress.

| Genotypes | NaCl | GPX (μmol/mg protein) | APX (μmol/mg protein) | CAT (μmol/mg protein) | Proline (μg/g DW) |
|-----------|------|----------------------|----------------------|----------------------|------------------|
| *B. juncea cr113* | 0 | 0.059 ± 0.009 (100) | 0.151 ± 0.026 (100) | 0.018 ± 0.003 (100) | 241.74 ± 5.29 (100) |
|           | 75  | 0.068 ± 0.008 (115.8) | 0.115 ± 0.018 (76.1) | 0.021 ± 0.001 (114.8) | 212.72 ± 1.73 (88) |
|           | 150 | 0.105 ± 0.003 (178) | 0.151 ± 0.03 (99.8) | 0.023 ± 0.003 (127.8) | 507.18 ± 6.42 (209.8) |
|           | 200 | 0.176 ± 0 (298.9) | 0.351 ± 0.073 (232.7) | 0.044 ± 0.014 (244.4) | 639.77 ± 3.85 (264.7) |
| *B. juncea cr3356* | 0 | 0.285 ± 0.018 (100) | 0.072 ± 0.018 (100) | 0.025 ± 0.006 (100) | 198.31 ± 1.1 (100) |
|           | 75  | 0.292 ± 0.024 (102.5) | 0.229 ± 0.026 (318.4) | 0.033 ± 0.019 (133.3) | 586.32 ± 4.74 (295.7) |
|           | 150 | 0.312 ± 0.014 (109.5) | 0.432 ± 0.02 (600) | 0.05 ± 0.015 (201.5) | 599.81 ± 0.37 (302.5) |
|           | 200 | 0.39 ± 0.009 (136.8) | 0.388 ± 0.057 (538.9) | 0.053 ± 0.007 (213.3) | 629.04 ± 4.53 (317.2) |
| *B. oleracea br258* | 0 | 0.017 ± 0.003 (100) | 0.102 ± 0.014 (100) | 0.012 ± 0.001 (100) | 89.67 ± 0.91 (100) |
|           | 75  | 0.002 ± 0.001 (13.7) | 0.083 ± 0.02 (80.9) | 0.023 ± 0.006 (194.4) | 249.38 ± 2.65 (278.1) |
|           | 150 | 0.003 ± 0.001 (18.2) | 0.041 ± 0.019 (39.8) | 0.026 ± 0.005 (216.7) | 527.55 ± 9.93 (588.3) |
|           | 200 | 0.006 ± 0.001 (33.7) | 0.041 ± 0.015 (40.5) | 0.02 ± 0.003 (166.7) | 222.29 ± 1.33 (247.9) |
| *B. oleracea br2828* | 0 | 0.008 ± 0.002 (100) | 0.157 ± 0.022 (100) | 0.015 ± 0.001 (100) | 195.05 ± 2.89 (100) |
|           | 75  | 0.004 ± 0.001 (50) | 0.101 ± 0.007 (64.5) | 0.021 ± 0.001 (136.7) | 133.85 ± 3.24 (68.6) |
|           | 150 | 0.026 ± 0.003 (328.8) | 0.093 ± 0.012 (59.1) | 0.037 ± 0.014 (248.9) | 122.88 ± 1.59 (63) |
|           | 200 | 0.019 ± 0.001 (235.8) | 0.166 ± 0.013 (105.5) | 0.01 ± 0.001 (68.8) | 358.94 ± 7.11 (184.1) |
| *B. oleracea br2993* | 0 | 0.006 ± 0.001 (100) | 0.067 ± 0.011 (100) | 0.027 ± 0.001 (100) | 152.51 ± 10.61 (100) |
|           | 75  | 0.049 ± 0 (818.9) | 0.062 ± 0.001 (92.9) | 0.011 ± 0.001 (42.3) | 175.39 ± 4.92 (115) |
|           | 150 | 0.065 ± 0 (1081.7) | 0.081 ± 0.034 (121.3) | 0.02 ± 0.01 (72.8) | 47.71 ± 3.82 (31.3) |
|           | 200 | 0.002 ± 0 (31.1) | 0.117 ± 0.021 (174.1) | 0.009 ± 0.001 (32.1) | 183.42 ± 2.83 (120.3) |

The values in parentheses indicate changes’ percentage rather than respected control.

GPX guaiacol peroxidase, APX ascorbate peroxidase, CAT catalase.
Proline content

Proline, as an important buffer in the maintenance of osmotic homeostasis, exhibited significant dose-dependent concentration increases upon the salt treatment (Sofo et al. 2015). The mean comparison of genotypes in all salinity levels for proline varied from 139.8 μg/g DW in B. oleracea br2993 to 503.4 μg/g DW in B. juncea cr3356 (Fig. 3D). In Brassica calli, the average proline content out of all genotypes dramatically increased with increasing the salt from 175.5 μg/g DW in control to 406.7 μg/g DW in 200 mM NaCl (NaCl). Our findings related to proline accumulation in response to salt concentration increase, have been confirmed in various in vitro salt stress systems (Lokhande et al. 2010; Niknam et al. 2011; Golkar et al. 2017; Golkar and Taghizadeh 2018). The mean interaction of genotypes × salinity showed that the highest amount of proline (639.77 μg/g DW) was related to B. juncea cr113 genotype under 200 mM NaCl, which had no significant difference with B. juncea cr3356 genotype under 200 mM NaCl (Table 2). While, the lowest amount of proline (47.70 μg/g B. juncea cr3356 genotype under 200 mM NaCl) was related to Brassica genus, the higher ability for the salt tolerance observed in B. juncea, might be due to the greater polyplody level in this species compared to B. oleracea. Eventually, it should be underlined that the higher salt tolerance of amphidiploids as B. juncea, has been compromised from the genomes of A (B. campestris) and C (B. oleracea L.) (Ashraf et al. 2001). In agreement with our results, higher ploidy levels in wheat (Chandra and Dubey 2010) and Acanthophyllus (Niknam et al. 2011) were significantly more salt-tolerant than lower ploidy levels.

Conclusion

This study showed a considerable inter-specific and intra-specific variation of the salt tolerance within Brassica species that should be exploited by screening and subsequently identifying the opposing genotypes that could function as auspicious novel varieties or superior genetic backgrounds for greater improvements in production of tolerant varieties. In conclusion, the salinity tolerance of B. juncea is associated with a higher antioxidant activity of GPX and APX, with more accumulation of proline than B. oleracea. Additionally, in our research we noticed the higher antioxidant activities of machinery APX, GPX, and CAT in the salt-treated callus cultures, which indicate their remarkable stress tolerance role in both Brassica species (B. oleracea and B. juncea). According to our findings, it could be beneficial to choose amphidiploid species over diploid species, since they show slightly higher resistance to salinity. Consequently, they could be more tolerant in saline climates. However, it is essential to accomplish a more elaborate screening of antioxidant responses so that these conclusions can be supported.

Further studies are recommended regarding the inherent effects of somaclonal variation in order to increase the salt tolerance in Brassica species. Besides, this research paves the way for the studies focusing on the molecular events related to the salinity in the species of Brassica with different life span and ploidy.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02138-8.

Acknowledgements The authors would like to appreciate Agricultural Sciences and Natural Resources University of Khuzestan for making available the plant materials, experimental locations and offering technical assistance.
Author contributions ES conceived and designed the research. SJ implemented all the experiments’ section under the supervision of ES and AAM. A-AM prepared technical materials. PPM analyzed the data. ES wrote the manuscript. All authors have read and approved the final manuscript.

Declarations

Conflict of interest the authors declare that they have no competing interests.

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