Effect of Salt Stress on Some Physio-Biochemical Traits and Antioxidative Enzymes of Two Brassica Species Under Callus Culture

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Abstract

The changes in lipid peroxidation, H_2O_2, proline, protein and the involvement of the different antioxidant system (catalase, gayacoule peroxidase, ascorbate peroxidase) and callus-related traits were investigated in relation to 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 than that of *B. oleraceae* by increasing concentrations of NaCl from 0 to 200 mM.Tetraploid genotype (*B. juncea* cr3356) showed significant increase in the contents of protein and proline, and also activity of giacle peroxidase and catalase enzymes at higher salinity levels. Also, a significant decrease in the amount of H_2O_2 and malondialdehyde occurred with increasing the salinity intensity. Diploid cultivar (*B. oleracea* bra 2828) had the lowest enzymatic activities and the highest content of H_2O_2 and malondialdehyde with an increase in the salinity level. Therefore, this genotype was identified as the most sensitive cultivar to the salinity stress. The difference in the salinity resistance between diploid and amphidiploid species may be due to differences in the ploidy level of these species. This result suggests that tetraploid genome of *B. oleraceae* could be considered as a suitable candidate for production under salinity conditions by maintaining higher activities of antioxidant enzymes.

Introduction

Salinity stress is considered as a major limiting factor for 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 21st century (Mahajan and Tuteja 2005). Many physiochemichal processes are affected due to toxicity effects of salinity stress at whole and cellular levels of plant (Arzani 2008; Van Zelm et al. 2020).

The excess formation of Reactive Oxygen Species (ROS) 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, the increase of cell H_2O_2, is a good marker of the extent of oxidative stress under environmental stresses (Sofo et al. 2015). Under drought stress, the production of H_2O_2 in plant cells, is done with both roles as a signaling molecule and a regulator compound for the increase in expression of certain genes (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 accumulation of such organic solutes under the process of plant adaptation to salinity stress (Ashraf and Foolad 2007). Proline is known as a compatible solute to environmental stresses, especially salinity which protects plant cells by scavenging ROS (Mittler et al. 2004). Plants employed different antioxidative defense system as well as antioxidative enzymes, (e.g. catalase, ascorbate peroxidas and guaiacol peroxidase) to mitigate the oxidative damage by ROS under salinity conditions (Gill and Tuteja 2010; Foyer and Noctor 2011).

Catalase (CAT) acts as an antioxidant enzyme by dismutating H_2O_2 in to H_2O and O_2 (Sudhakar et al. 2001; Sofo et al. 2015). Peroxisomes, cytosol, and mitochondria are the main organs for its accumulation in plant cells (Sofo et al. 2015). The produced H_2O_2 is then scavenged by CAT and several classes of peroxidases enzymes (Gill and Tuteja 2010; Sofo et al. 2015).

At the first step of ascorbate–glutathione cycle in plant cells, ascorbate peroxidase (APX) uses ascorbate ($C_6H_7O_6$) as the electron donor in the first step of the ascorbate–glutathione cycle for H_2O_2 detoxification

(Noctor and Foyer 1998).

Similarly, Gayacoul peroxidase (GPX), is involved in a range of processes related to ROS-induced stress (Uarrota et al. 2016). It is located in cytosol, vacuole, and apoplast of plant cells. The GPX, decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or ascorbate (Uarrota et al. 2016). As a consequence, the balance of APX, GPX, and CAT activities, representing the main enzymatic H_2O_2 scavenging mechanism in plants, is crucial for the suppression of toxic H_2O_2 levels in a cell (Sofo et al. 2015).

The study of the physiological responses of plants to salt stress shows that the technique of *in vitro* callus culture could facilitate the preparation of an uniform environment (undifferentiated cells of callus) (Rai et al. 2011). This biotechnological method could eliminate impediments which arise from variability in genetic and morphological levels associated with tissues of plants at the whole level. As a supplementary tool, identification and selection of salt tolerance genotypes could be considered for traditional breeding in salt-stressed regions (Rai et al. 2011). Identification of the 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 Brassicaceae family (in about 3675 species) are very important components for nutritional aims (Shankar et al. 2019). Crop plants from this family are among the oldest known cultivated plants which are categorized as commercially important vegetables (Purty et al. 2008). Interestingly, the members of 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). Thus, their response and adaptation to salinity differs greatly (Ashraf and McNeilly 2004). Three basic approaches that are currently being used to obtain stress-tolerant Brassica, including: (i) screening of existing genotypes, and (ii) conventional breeding (Purty et al. 2008). It is necessary to know the morphological, physiological and biochemical responses of different 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 somewhat higher tolerance than the diploids of *B. oleracea*, *B. nigra, and B. rapa* (Purty et al. 2008; Pavlović et al. 2019). Also, it has been reported that Brassica species are more sensitive to salinity tolerance at seed germination and early the growth stages (Ashraf and McNeilly 2004).

However, according to literature review, there is no knowledge regarding tolerance mechanisms in the Brassicaceae species at callus level. Also, this study was done to assist efforts to elucidate mechanisms responsible for in vitro salt tolerance at callus level in two different species of Brassicaceae family with ploidy levels including *B. junceae* (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 diploidy 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 sterilized for 15 min. Then the sterilized seeds were washed with double sterile water them 3 times. After disinfecting the seeds, they were incubated on Murashige & Skoog (1962) MS (Duchepha, Netherlands). The concentration of 30 g/L sucrose was used as supplementation, and the seeds were solidified with 8 g/L agar (Merck, Com.) for initiating germination. After 7 - 10 days, we used germinated seeds 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 callus induction. We incubated the cultures with 16/8 dark/light cycle in growth chamber at 24°C. After 4 weeks, we delivered the induced calli to MS medium and added varying NaCl concentrations (0, 75, 100, & 200 mM) to it. The period of incubation in salt stress was 30 days. The treatments were composed of 3 replicates (Petri dishes), and each of them included 7 callus parts. After 40 days of culturing, the callus' biochemical and physiological parameters were measured.

Callus-related traits

An oven setting at 65°C was used for drying 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 final and initial masses were specified. Relative Water Content (RWC) was measured as [(fresh weight of callus - dry weight of callus)/ dry weight of callus × 100] (Golkar et al. 2017).

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

Malondialdehyde content (MDA)

Firstly, we homogenized 0.2 g of the fresh callus 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. We measured the solution absorbance at 532 nm, and then, the nonspecific absorbance correlation was measured at 600 nm. According to the extinction coefficient as 155 mM^{-1} cm^{-1}, MDA was specified.

Hydrogen peroxide content

The method proposed by Loreto and Velikova (2001) was used for estimating the H_2O_2 content. To this end, using varying concentrations of H_2O_2, a standard calibration curve was developed. Shortly, we added 5 ml of 0.1% (w/v) TCA to 0.07 (g) of fresh callus, for making it homogenized. Then, centrifuging the homogenate was done for 15 minutes at 12,000 × g followed by addition of 0.5 ml of supernatant to 1 ml of KI (1 M)and 0.5 ml of buffer of potassium phosphate (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 Protein and enzyme tests, we homogenized fresh callus (1 g) in 2 ml of a buffer of ice cold (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 done for 30 min at 20,000×g at 4°C. We used supernatant for assaying enzyme activities and content of protein. Given the method proposed by Bradford (1976), enzyme extracts' solvable protein content was specified by Bovine Serum Albumin (BSA). Using Shimadzu spectrophotometer (UV-Visible160, Shimadzu, Japan), spectrophotometric analyses were performed.

Guaiacol peroxidase activity

The method presented by Lin and Kao (1999) was used for determining GPX content 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, we recorded the rise in absorbance for 1 minute at 470 nm. Quantification of enzyme activities was done by tetraguaiacol amount developed by the use of its molar extinction coefficient (26.6 mM^{-1} cm^{-1}).

Ascorbate peroxidase (APX) assay

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^{-1}) was oxidised, for 3 min. In one mL reaction volume containing 50 mM potassium phosphate buffer pH 7; 10 ml enzyme extracts 0.1 mM hydrogen peroxide and 0.5 mM ascorbate. The reaction was started with adding the H_2O_2 and the decrease in absorbance at 290 nm. The activity of APX was calculated based on µmol mg^{-1} protein.

Catalase activity

The activity of Catalase (CAT) was assayed based on Aebi (1983) that calculates the H_2O_2 decline at 240 nm at the max 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^{-1}), and the outcomes were provided as µmol H_2O_2 min^{-1} mg^{-1} protein.

Proline content

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

Statistical analysis

The study was performed as a 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) SAS (2011). The comparison of means was studied based on Fisher's Least Significant Difference (LSD) test at the level of probability of 0.05. Data were expressed as 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 1). The genotype × salinity interaction was significant for all traits, except for RWC and CAT (Table 1).

Effects of salinity stress on different studied traits

Callus growth traits

The salinity stress resulted to production of more brown and necrotic calli in the two studied species of Brassica (Fig. 1). Higher salinity levels resulted to more coloration in the calli colors in all genotypes (Fig. 1). According to Fig. 2A, the RGR mean for genotypes ranged from 6.79 % in br2993 genotype (*B.oleracea*) to 16.94 % in cr3356 genotype (B*. junceae*) . The RGR ranged from 14.06 % in control to 7.32 % under 200 mM NaCl (Fig. 3A). Addition of NaCl to *B. juncea* and *B. oleracea* led to a significant decline in CGR content, under *in vitro* salt stress. According to the salinity × genotype interaction, the salinity stress resulted in a decrease in RGR in all the studied genotypes, but its effect was not the same in all of them (Table 2). The genotypes of bra 2993 and bra 2828 genotypes showed more reduction in RGR at high concentrations of salinity (Table 2). The cultivar of cr3356 (*B. juncea*) did not show a significant decrease in RGR up to 200 mM (NaCl) (Table 2), which indicated high tolerance of this genotype to salinity stress at callus level.

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

Malondialdehide (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 genotypes of br2993 and cr113, respectively, averaged over all salinity levels (Fig. 2B). The mean comparison for MDA changes under different salinity levels, demonstrated an increase in its content parallel with an increase in the salinity levels (Fig. 3B). Its content showed variation from 0.00328 µg/g DW in control to 0.0045 µg/g DW under 200 mM NaCl (Fig. 3B). The significant differences observed among MDA values at different levels of salinity might be attributed to the non-supplementary effects of adequate osmolites accumulation as proline in these species at salinity conditions through detoxification of ROS and the subsequent protection of membrane integrity (Ashraf and Foolad 2007). On the other hand, this type of response might be associated with the increased content in H_2O_2 which could be drastically enhanced under salinity stress among the plant species that undergo higher lipid peroxidation (Khan and Panda 2008).

The increased MDA content in salinity–stressed calli has been reported in other species as safflower (Golkar and Taghizadeh 2018) *Nigella sativa* (Golkar et al. 2020) and Acanthophyllum (Niknam et al. 2011). In diploid genotypes, the amounts of MDA showed an increase with an increase in the salinity levels except for bra2828 and bra258 under 75 mM NaCl which had a significant decrease compared to the control (Table 2). Treatments

of 150 and 200 mM NaCl had the highest rates in all three genotypes which had significant differences with the control treatment (Table 2). This finding showed an increase in peroxidation and severe degradation of lipids in these genotypes under stress conditions similar to the report of *B. olerace* (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 2). The highest content of MAD (0.006 µg/g DW) for diploid genotypes was observed at 200 mM NaCl. This result could point to a selective advantage of species with amphidiploid levels to combat with deleterious effects of the salinity stress in Brassica species. Similar results in the Acanthophyllum calli show that callus of hexaploid species has a lower level of MDA than the callus of tetraploid species under the salinity stress (Niknam et al. 2011), which demonstrated a higher salinity tolerance in higher ploidy levels than that of lower ones.

H_2O_2 content

An increase in the content of hydrogen peroxide leads to lipid peroxidation, which ultimately leads to the destruction of cell membranes (Hossain et al. 2015). The mean comparison between different levels of salinity showed that it was ranged from 0.392 µmol/ g DW under 200 mM NaCl to 0.330 µmol/ g DW under control conditions (Fig. 3C).

The highest (0.45 µmol/ g DW) and the least (0.25 µmol/ g DW) content for H_2O_2 were observed at genotypes of *B. oleracea* br2993 and *B. juncea* cr113, respectively (Fig. 2C). According to the genotype × salinity interaction, H_2O_2 content was 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 2). Under the control condition, there was a significant difference between different genotypes (Table 2). However, this difference was not significant between *B. oleracea* br2828 and *B. oleracea* br2993 cultivars. (Table 2).

In three diploid genotypes, the amount of hydrogen peroxide was increased with increasing the salinity levels (Table 2). However, the genotype of *B. oleracea* br2993 had a significant reduction under 150 mM of NaCl compared to the control treatment. (Table 2). At 200 mM NaCl, three diploid genotypes showed a significant difference with the control treatment, which indicates the high sensitivity of these genotypes to salinity at high concentrations of NaCl (Table 2). Dissimilar to the diploid genotypes, amphiplipid ones (*B. juncea*) showed a decrease in H_2O_2 content with an increase in the salinity levels (Table 2). This result demonstrated at higher affinity of CAT and APX antioxidants in the amphidiploid genotypes rather than the diploid ones, in reduction of H_2O_2content under the salinity stress.

Protein content

Protein synthesis can be affected by different environmental stresses including salt stress (Muchate et al. 2016). Several salt-induced proteins have been identified, which belong to two distinct groups: salt stress proteins that accumulate only due to salt stress, and stress-associated proteins, which accumulate in response to various environmental stresses including salinity (Ashraf and Harris 2004). Proteins accumulated in plants grown under saline conditions may provide a storage form of nitrogen which could be reutilized when stress is over and they may play a role in osmotic adjustment (Muchate et al. 2016). Averaged over all salinity levels, the highest (1.05 µg/g DW) and the least (0.68 µg/g DW) contents of total protein were observed at *B. juncea* cr3356 and *B. oleracea* br258 genotypes, respectively (Fig. 2D)

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 (Fig. 3D).

The mean comparison for salinity × genotype interaction (Table 2) indicates an increase in the total protein content with an increase in the concentration of sodium chloride in all of the genotypes in the culture medium. According to Table 2, the highest content of protein (1.12 µg/g DW) was observed at 200 mM NaCl in *B. juncea* cr3356 genotype, which showed a significant difference with other treatments and cultivars (Table 2). The similar results were observed in terms of increase in protein content of *B. juncea* genotypes, but this increase was more in *B. juncea* genotypes rather than in *B. oleracea* ones. Similar to this finding, an increase in protein content was also reported in *B. oleracea* calli at 100 mM NaCl (Mukhtar and Hasnain 1994) and also 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 enzyme activities such as catalase, gayacol peroxidase, and peroxidases. On the other hand, in order to counteract the toxic effects of increasing the amount of ROS under salinity stress, various defense mechanisms including antioxidant enzyme activity are activated in the plant (Gupta and Huang 2014). Under these conditions, the enzymes inhibiting the production of ROS are increased to reduce the toxic effects of oxidative stress. In this study, a significant variation has been found regarding the anti-oxidant enzyme activities of APX and GPX. Amphidiploid genotypes generally showed higher antioxidant activity (APX and GPX) as compared to diploid ones. This suggests that high antioxidant enzyme activity has a significant role in imparting salt tolerance in these amphidiploid genotypes.

GPX activity

GPX participates in numerous physiological processes and characterized as an electron donor (Ahire, et al., 2013).The content of GPX for genotypes ranged from 0.32 µmol/mg protein in *B. juncea* (cr3356) to 0.007µmol/mg protein in genotype of *B. oleracea* br258 genotype (Fig. 4A). The content of GPX showed an increasing trend from control (0.084 µmol/mg protein) to 0.12 (µmol/mg protein) under 200 Mm NaCl (Fig. 5A). This increase under *in vitro* salinity culture was similar to previous reports on *Bacopa monnieri* (Ahire et al. 2013) and *Spinacia oleracea* (Muchate et al. 2019). Comparison of salinity × genotype interaction for GPX activity showed that the highest activity (0.39 µmol/mg protein) was observed at 200 mM NaCl for *B. juncea* cr3356 genotype (Table 2), but 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 2A in diploid cultivars (*B. oleracea* br2993 and *B. oleracea* br258) no significant difference was observed for 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 decreased sharply at 200 mM NaCl, which was not significantly different from the control treatment (Table 2). However, in the two amphiploid genotypes, GPX activity showed a significant increase with an increase in the concentration of NaCl (Table 2). The results of the previous reports show that in the amphiploid species of Brassica, the activity of GPX was higher than that of the diploid species under non-salinity stress (Menget al., 2011).

APX activity

Peroxidases are a set of glutathione reductase ascorbate cycle enzymes that can convert oxygenated water to water by removing H_2O_2 (Das and Roychoudhury 2014). APX is the most important reducing substrate that carries the dismutase of H_2O_2 to water. The mean comparison of genotypes showed a variation from 0.28 µmol/mg protein in *B. juncea* cr3356 to 0.07µmol/mg protein in *B. oleracea* br258 (Fi.g 4B). 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. 5B). Comparison of salinity× genotype interaction on the activity of APX showed that the highest activity of APX (0.432 µmol per minute per gram of protein) belonged to the treatment of 150 mM NaCl on *B. juncea* cr3356, which was not significantly different from 200 mM NaCl treatment (Table 2). Also, the lowest activity of APX was related to 150 and 200 mM NaCl on *B. oleracea* br258, which did not have significant difference with the control and 75 mM NaCl treatments (Table 2). With an increase in the salinity levels, the activity of APX significantly decreased in *B. oleracea* br258, but in other genotypes, this trend was not significantly observed (Table 2). As the duration of exposure to salinity stress increases, the activity of APX showed more increase in the amphiplipoid genotypes, rather than the diploid genotypes. This finding was similar to the finding in different ploidy levels of turnip (Meng et al. 2011) and the calli of eggplant (Yasar et al. 2006)and shoot culture in *Spinacia oleraces* (Muchate et al. 2019) under salinity stress.

Catalase activity

Plants are endowed with H_2O_2-metabolizing enzymes such as CAT and APX (Das and Roychoudhury 2014; Sofo et al. 2015). Averaged over all the salinity levels, the highest CAT activity (0.041 µmol/mg protein) was related to *B. juncea* cr3356 genotype which shows a significant difference with other cultivars (Fig. 4C). No significant difference was observed for catalase activity for diploid genotypes. The CAT activity showed an increase from the control (0.02 µmol/mg protein) to 0.031 µmol/mg protein under 150 mM NaCl (Fig. 5C) that was not significanly different with other salinity levels including 75 mM and 200 mM (Fig. 5C). The considerable increase in CAT activity observed in the calli of different Brassica species under salinity stress could 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 presence of catalase are peroxisomes which convert H_2O_2 to H_2O and O_2 (Sofo et al. 2015). According to the non-significant effect of salinity × genotype interaction for CAT activity, it can be concluded that trend responses of different genotypes was similar at different salinity levels.

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

Proline content

Proline, as an important buffer in maintenance of osmotic homeostasis, exhibited significant dose-dependent concentration increases upon salt treatment (Sofo et al. 2015). The mean comparison of genotypes averaged over all salinity levels for proline showed that it varied from 139.8 µg/g DW in *B. oleracea* br2993 to 503. 4 (µg/g DW) in *B. juncea* cr3356 (Fig. 4D). In Brassica calli, the proline content averaged over all the genotypes was found to be increased dramatically with increasing the salt from 175.5 µg/g DW in control to 406.7µg/g DW in 200 mM (NaCl) (Fig. 5D). Our findings indicating proline accumulation in response to increasing salt

concentration 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; Muchate et al. 2019). The mean interaction for genotypes × salinity showed that the highest amount of proline) 639.77µg/g DW) was related to *B. juncea* cr113 genotype under 200 mM NaCl that had no significant difference with *B. juncea* cr3356 genotype under 200 mM NaCl (Table 2), while the lowest one (47.70 µg/g DW) was observed at *B. oleracea* br2828 under 150 mM NaCl, (Table 2). No steady trend was observed for proline changes in different genotypes in response to an increase of the salinity levels (Table 2). So it could be compromised that the role of proline accumulation in tolerance to salt stress remains controversial, and implied that the enhanced proline levels are stress-effected rather than being a factor in stress tolerance. According to the findings, the increase of proline was greater in amphiploid genotypes rather than in diploid ones. Similar to this finding, Chandra and Dubey (2010) reported more efficient antioxidant system in hexaploids rather than diploids in Cenchrus. Higher chromosome number and gene expression caused an increase in the concentration of particular secondary metabolites and chemicals that are responsible for better defense mechanism in polyploids than in diploids (Yildiz 2013).

Finally it could be stated that understanding the physiological and biochemical factors that lead to salt tolerance is a very important issue in the selection of stress-tolerant plants (Gupta and Huang 2014). However, callus culture under salinity stress technique offers a means for focusing only on those biochemical and physiological indicators inherent to cells that contribute to adaptation to salt stress (Rai et al. 2011; Golkar and Taghizadeh 2018). It is possible that defense against oxidative stress is organized differently in Brassica species with differing ploidy levels. This study evaluated, for the first time, the effects of salinity stress on biochemichal changes in two different species of Brassica with various ploidy levels.

The findings obtained from this research confirmed that polyploid species in Brassica (Ashraf and McNeilly 2004) and other plant species as sugar cane (Yildiz 2013) could generally withstand salinity stress better than their respective diploid ones. But this research implied that this conclusion at callus level is a novelty finding. Moreover, with regard to the role of polyploidy in the evolution and speciation of the Brassica genus, the higher ability for the salt tolerance observed in *B. juncea*, might have been due to the greater polyploidy level in this species compared to *B. oleracea*. It could be suggested 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 Achanthyphyllus (Niknam et al. 2011)) were considerably more salt-tolerant than in lower ploidy levels.

Conclusion

This study showed a considerable interspecific and intraspecific variation for salt tolerance within Brassicas species that should be exploited by screening and subsequently identifying the opposing genotypes that could function as auspicious novel varieties or as superior genetic backgrounds for more enhancements for raising tolerant varieties. In conclusion, salinity tolerance of *B.juncea* is associated with a higher antioxidant activity of GPX and APX, more accumulation of proline rather than *B. oleracea*. Additionally, we noticed the higher antioxidant activity of machinery APX, GPX and CAT in the salt treated callus cultures, which indicates their role in stress tolerance in both Brassica species, including *B. oleracea and B. junceae*. According to our findings, it could be beneficial to choose amphidiploid species over diploid species since it seems that they show slightly higher resistance to salinity. Thus, they could be more tolerant in saline climates. Nevertheless, it is essential to conduct research on a more elaborate screening of antioxidant responses so that these conclusions can be

supported. Besides, the research paves the way for studies on the molecular events related to salinity in these species of Brassica that are different in terms of life span and ploidy.

Declarations

Acknowledgments

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Author contributions

E.S conceived and designed the research. S.J done all the experiments section under the supervision of E.S and AA.M. A-A.M prepared technical materials. P.PM analyzed the data. E.S wrote the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

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

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Tables

Table 1
Analysis of variance for different biochemical traits of Brassiceace genotypes under different levels of salinity through callus culture.

S.O.V	DF¥	RWC	RGC	MDA	H_2O_2	Protein	CAT	APX	GPX	Proline
Genotype (G)	4	NS	**	**	**	**	**	**	**	**
Salinity (S)	3	NS	**	**	**	**	*	*	**	**
G*S	12	NS	**	**	**	**	NS	**	**	**
• ¥; D.F: degree of freedom; RWC: relative water content; RGR: relative growth rate; GPX; Guaiacol peroxidase; CAT: catalase; APX: Ascorbate peroxidase; MDA: Malondialdehide.										
• NS,* and ** not-significant, significant at $P < 0.05$ and $P < 0.1$, respectively.										

Table 2
Mean of various Brassica genotypes for different physio-biochemical traits under *in vitro* salinity stress.

		GROW¥	MAD	H_2O_2	Protein	GPX	APX	CAT	Proline
Genotyps	NaCl	%	µg/g DW	µmol/g DW	µg/g DW	µmol/mg protein	µmol/mg protein	µmol/mg protein	µg/g DW
B. juncea cr113	0	16.32 ± 0.46	0.0015 ± 0.0003	0.267 ± 0.008	0.93 ± 0.006	0.059 ± 0.009	0.151 ± 0.026	0.018 ± 0.003	241.74 ± 5.29
	75	14.97 ± 0.17	0.0023 ± 0	0.296 ± 0.006	1.04 ± 0.012	0.068 ± 0.008	0.115 ± 0.018	0.021 ± 0.001	212.72 ± 1.73
	150	13.13 ± 0.54	0.0024 ± 0.0003	0.211 ± 0.009	1.02 ± 0.006	0.105 ± 0.003	0.151 ± 0.03	0.023 ± 0.003	507.18 ± 6.42
	200	11.46 ± 0.24	0.0024 ± 0.0001	0.244 ± 0.006	1.04 ± 0.006	0.176 ± 0	0.351 ± 0.073	0.044 ± 0.014	639.77 ± 3.85
B. juncea cr3356	0	19.22 ± 0.64	0.0015 ± 0.0001	0.372 ± 0.001	0.957 ± 0.009	0.285 ± 0.018	0.072 ± 0.018	0.025 ± 0.006	198.31 ± 1.1
	75	17.8 ± 0.34	0.0024 ± 0.0002	0.308 ± 0.003	1.05 ± 0.015	0.292 ± 0.024	0.229 ± 0.026	0.033 ± 0.019	586.32 ± 4.74
	150	16.64 ± 0.34	0.0021 ± 0.0001	0.278 ± 0.01	1.077 ± 0.018	0.312 ± 0.014	0.432 ± 0.02	0.05 ± 0.015	599.81 ± 0.37
	200	14.09 ± 0.5	0.003 ± 0.0001	0.261 ± 0.003	1.12 ± 0.006	0.39 ± 0.009	0.388 ± 0.057	0.053 ± 0.007	629.04 ± 4.53
B. oleracea br258	0	12.76 ± 0.92	0.0039 ± 0.0001	0.226 ± 0.002	0.627 ± 0.015	0.017 ± 0.003	0.102 ± 0.014	0.012 ± 0.001	89.67 ± 0.91
	75	10.99 ± 0.33	0.0036 ± 0.0001	0.34 ± 0.003	0.66 ± 0.012	0.002 ± 0.001	0.083 ± 0.02	0.023 ± 0.006	249.38 ± 2.65
	150	7.43 ± 0.37	0.0055 ± 0.0002	0.469 ± 0.003	0.73 ± 0.006	0.003 ± 0.001	0.041 ± 0.019	0.026 ± 0.005	527.55 ± 9.93
	200	4.32 ± 0.33	0.005 ± 0	0.425 ± 0.002	0.69 ± 0.006	0.006 ± 0.001	0.041 ± 0.015	0.02 ± 0.003	222.29 ± 1.33
B. oleracea br2828	0	11.07 ± 0.11	0.0048 ± 0.0002	0.309 ± 0.006	0.757 ± 0.029	0.008 ± 0.002	0.157 ± 0.022	0.015 ± 0.001	195.05 ± 2.89

¥;RWC: relative water content; RGR: relative growth rate; GPX; Guaiacol peroxidase; CAT: catalase; APX: Ascorbate peroxidase; MDA: Malondialdehide.

		GROW¥	MAD	H_2O_2	Protein	GPX	APX	CAT	Proline
	75	8.68 ± 0.41	0.0042 ± 0.0001	0.488 ± 0.018	0.84 ± 0.017	0.004 ± 0.001	0.101 ± 0.007	0.021 ± 0.001	133.85 ± 3.24
	150	5.77 ± 0.08	0.0053 ± 0.0003	0.462 ± 0.011	0.87 ± 0.012	0.026 ± 0.003	0.093 ± 0.012	0.037 ± 0.014	122.88 ± 1.59
	200	3.46 ± 0.44	0.006 ± 0.0001	0.52 ± 0.014	0.84 ± 0.035	0.019 ± 0.001	0.166 ± 0.013	0.01 ± 0.001	358.94 ± 7.11
B. oleracea br2993	0	10.91 ± 0.15	0.0048 ± 0.0001	0.479 ± 0.001	0.7 ± 0.012	0.006 ± 0.001	0.067 ± 0.011	0.027 ± 0.001	152.51 ± 10.61
	75	8.33 ± 0.28	0.0053 ± 0	0.488 ± 0.003	0.75 ± 0.012	0.049 ± 0	0.062 ± 0.001	0.011 ± 0.001	175.39 ± 4.92
	150	5.08 ± 0.33	0.0054 ± 0.0002	0.314 ± 0.014	0.74 ± 0.012	0.065 ± 0	0.081 ± 0.034	0.02 ± 0.01	47.71 ± 3.82
	200	2.82 ± 0.16	0.006 ± 0.0004	0.529 ± 0.001	0.8 ± 0.012	0.002 ± 0	0.117 ± 0.021	0.009 ± 0.001	183.42 ± 2.83

¥;RWC: relative water content; RGR: relative growth rate; GPX; Guaiacol peroxidase; CAT: catalase; APX: Ascorbate peroxidase; MDA: Malondialdehide.

Figures

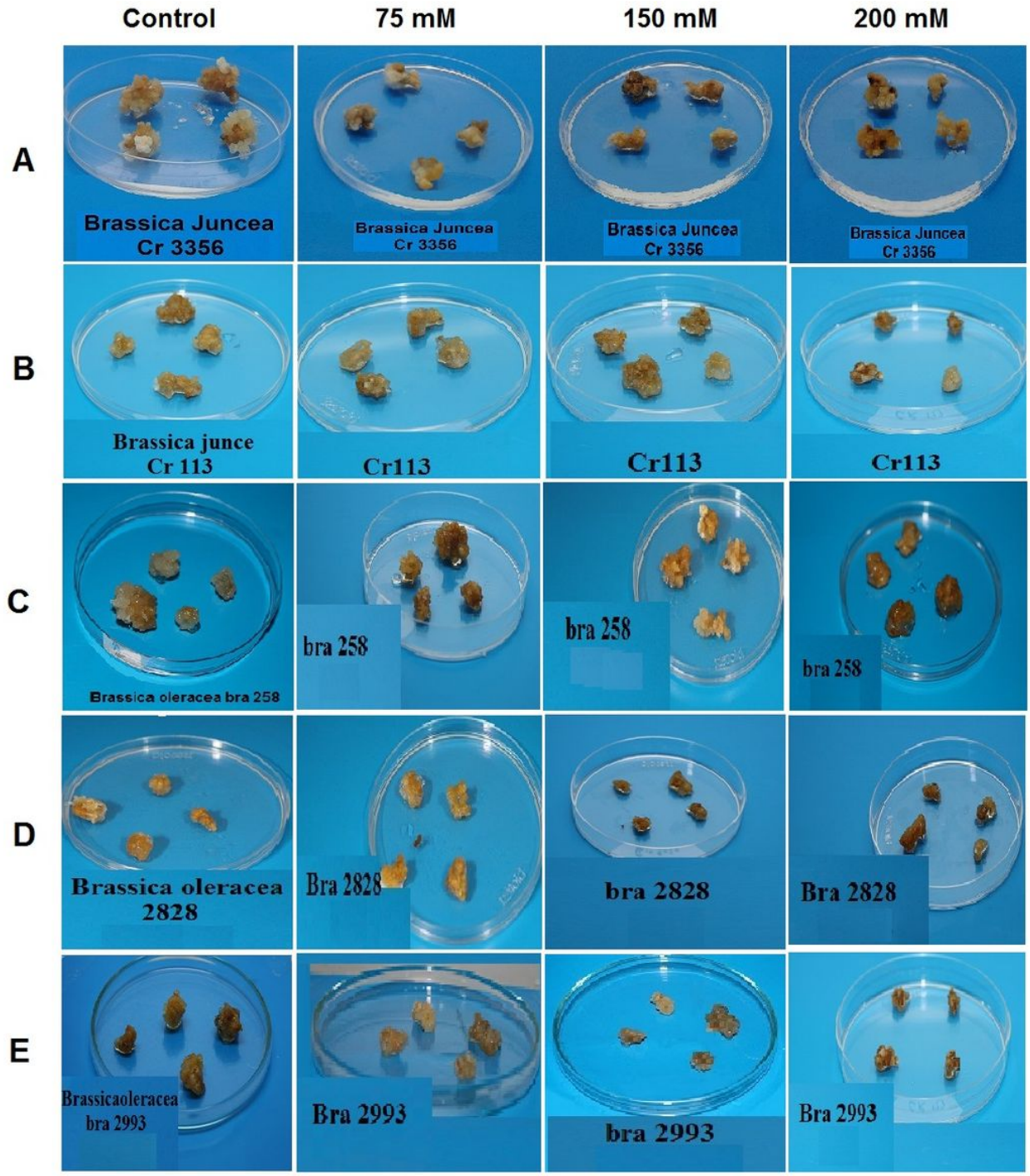

Figure 1

The effects of different salinity levels on callus texture and color of different Brassica genotypes including A (B. j uncea cr3356), B (B. j uncea cr113), C (B. oleracea bra 258), D (B. oleracea bra 2828) and E (B. oleracea bra 2993).

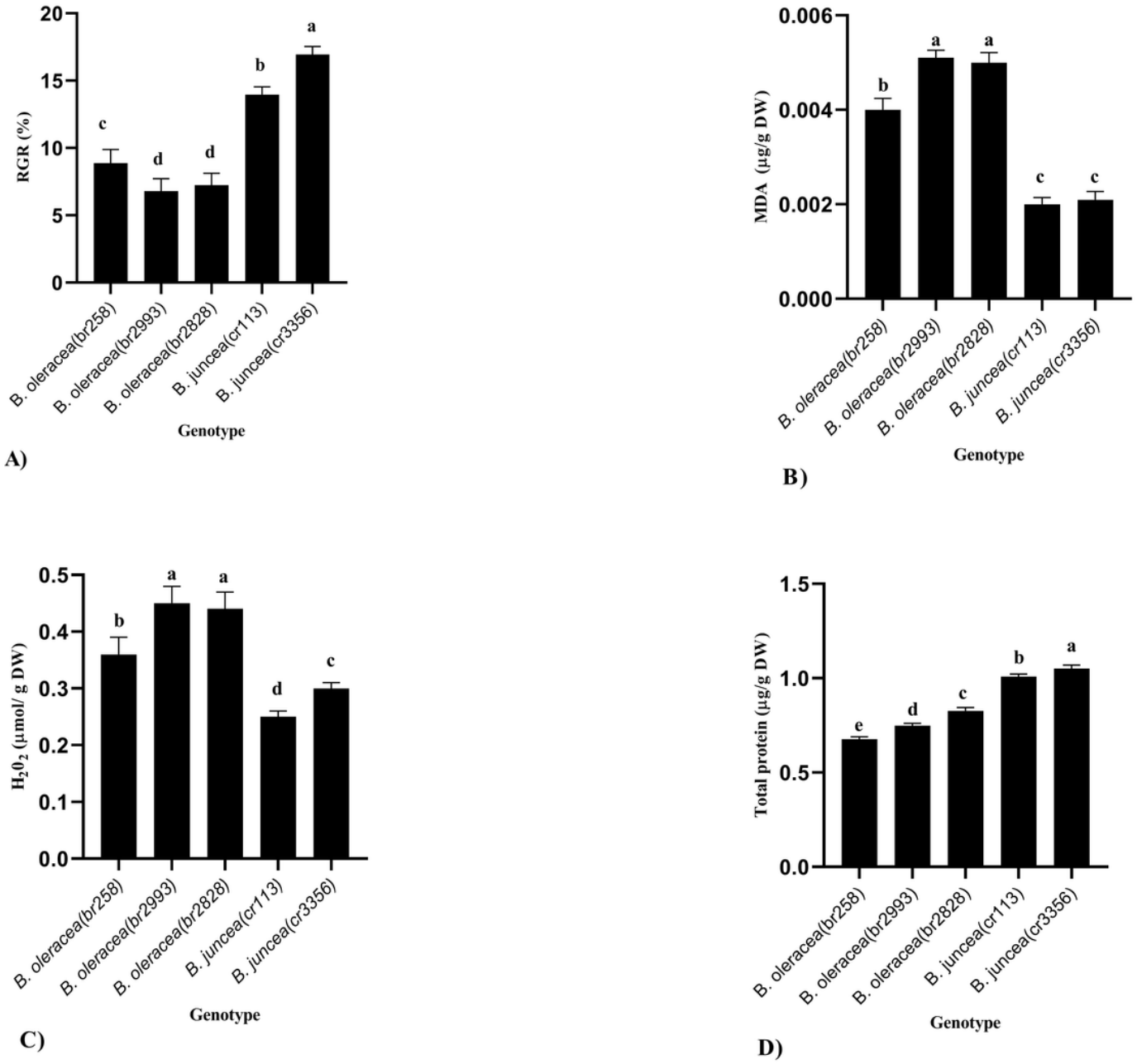

Figure 2

The content of relative growth rate (RGR) (A) malondialdehyde (B) H2O2 (C) and total protein (D) of different genotypes of B. oleraceae and B. junceae averaged over different salinity levels (0 to 200 mM NaCl). Error bars standard error (n = 3). Within each set of experiments, means with different letters are significantly different at $P<0.05$.

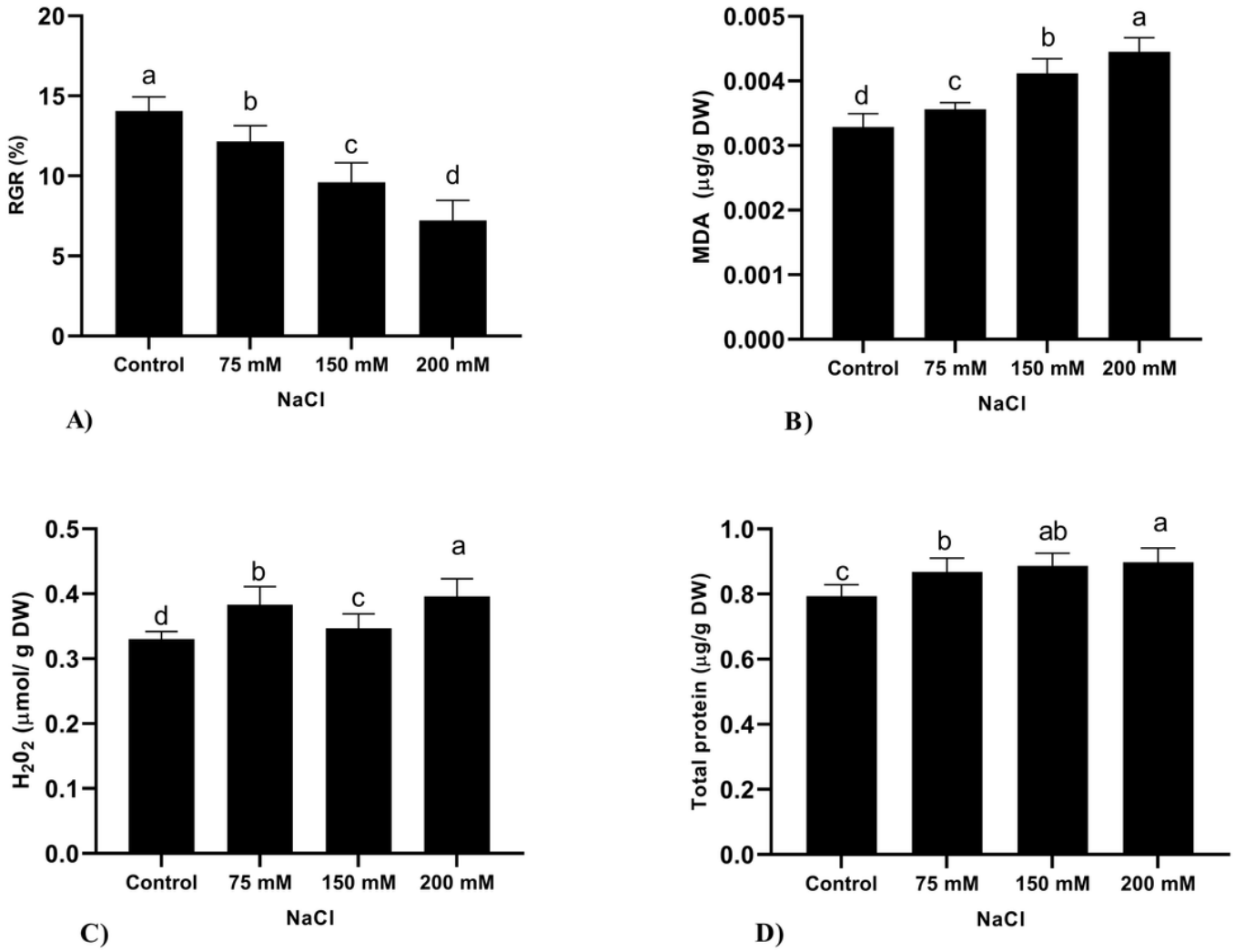

Figure 3

The content of relative growth rate (RGR) (A) malondialdehyde (B) H2O2 (C) and total protein (D) in B. oleraceae and B. junceae calli under different levels of salinity stress. Error bars standard error (n = 3). Within each set of experiments, means with different letters are significantly different at P<0.05.

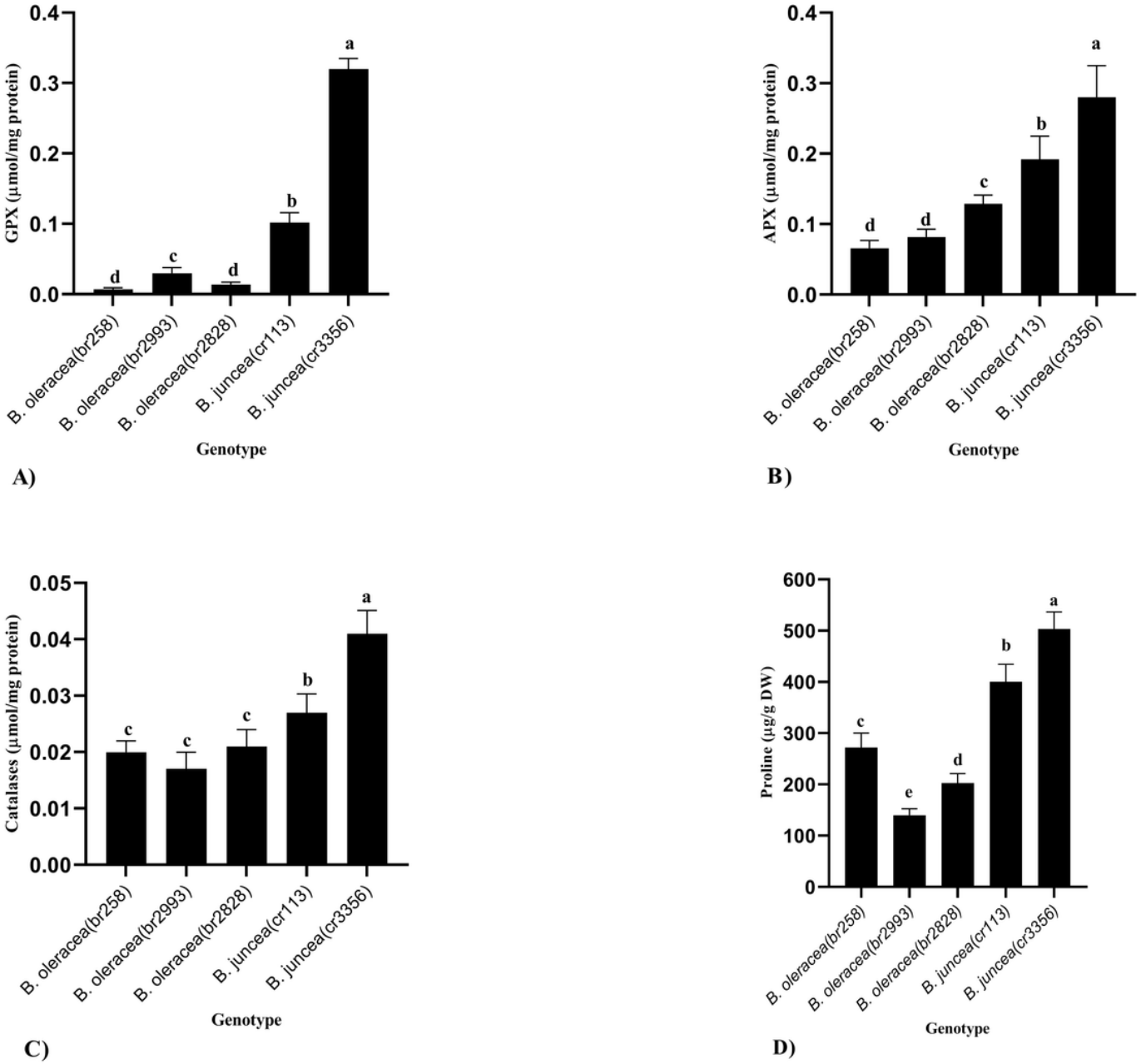

Figure 4

The content of gayacoul peroxidase (A) ascorbate peroxidase (B) catalase (C) and proline (D) of different genotypes of B. oleraceae and B. junceae averaged over different salinity levels (0 to 200 mM NaCl). Error bars standard error (n = 3). Within each set of experiments, means with different letters are significantly different at $P<0.05$.

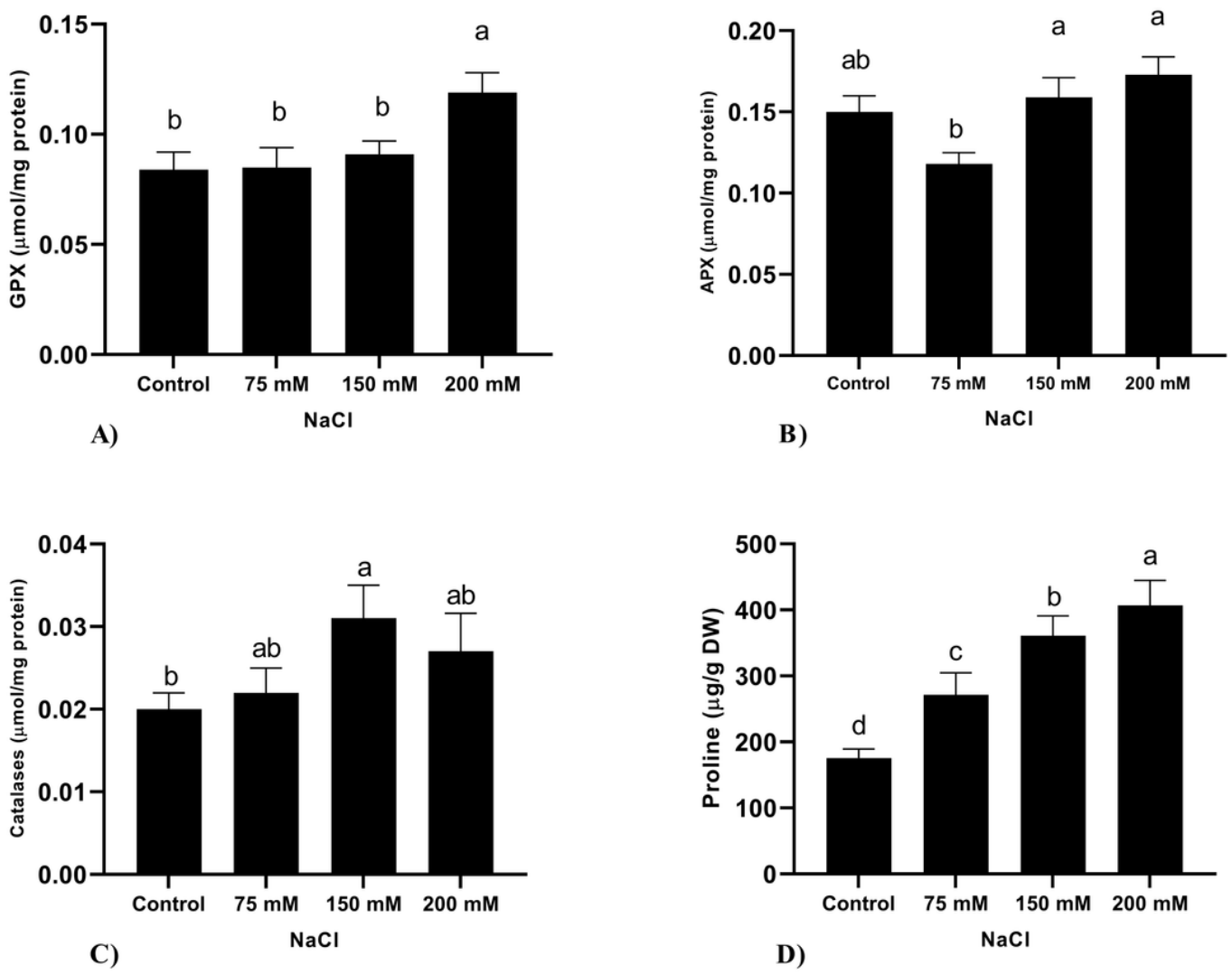

Figure 5

The content of guaiacol peroxidase (A) ascorbate peroxidase (B) catalase (C) and proline (D) in B. oleraceae and B. junceae calli under different levels of salinity stress. Error bars standard error (n = 3). Within each set of experiments, means with different letters are significantly different at P<0.05.