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RESEARCH ARTICLE

Evaluating Morpho-Physiological and Antioxidative Mechanisms of Salinity Tolerance in Contrasting Maize Varieties

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Abstract High soil salinity greatly impacts maize productivity, especially in regions with high salinity levels. This research examines how purple sweet (PS) maize and purple waxy (PW) maize react physically and biochemically to salty environments. Both maize varieties were grown hydroponically and subjected to salinity treatments (0-200 mM NaCl) under controlled growth conditions. We evaluated physiological parameters including growth, chlorophyll content, length, and stomatal conductance, along with their stress markers and antioxidant activity in leaf and root samples, in triplicate. PS maize showed a positive response to salinity by maintaining stable proline and hydrogen peroxide (H_2O_2) and non-significant changes in superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities particularly in roots. On the other hand, the PW genotype exhibited significant increases in antioxidant enzyme levels, indicating a strong reaction to oxidative stress, though this could suggest decreased natural tolerance. Meanwhile, PW showed better performance in physiological parameters compared to PS. Under 100 mM NaCl salt stress, the levels of flavonoids in both genotypes rose considerably, protecting them from oxidative damage. Strong positive correlations among proline, flavonoid content, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity were found using Pearson's correlation analysis, indicating that these markers are essential for salt tolerance. On the other hand, a negative relationship between proline and SOD activity indicated that osmoprotection and antioxidant processes may involve a trade-off. These results provide important information for breeding initiatives that aim to develop maize variants capable of withstanding salinity.

Keywords: Purple waxy maize, salt stress, oxidative stress, antioxidant, plant physiology.

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Introduction

Climate change is a major obstacle in combating food security. These unexpected extreme weather such as rises in temperatures, and shifts in precipitation patterns, will impact agricultural activities and production, thus actions are needed to overcome these problems [52]. A significant impact of climate change is the widespread occurrence of soil salinization, intensified by drought and increasing sea levels that lead to the infiltration of saltwater into freshwater and agricultural areas [19]. Salinity stress has become a significant abiotic constraint on crop production globally, especially in areas where irrigated farming prevails [31].

There is increasing pressure to develop salinity-resilient food crops that could supply the growing global population while also maintaining high productivity in this increasingly hostile environment on the crops [3].

Maize (*Zea mays*), a staple crop that provides a significant portion of the world's food and feed supply, is particularly sensitive to salinity [18]. When exposed to salt stress, maize experiences physiological and biochemical disruptions, including ionic imbalance, osmotic stress, and the generation of reactive oxygen species (ROS), resulting in oxidative damage and impaired growth [56] . These stress conditions reduce crop yield, particularly in maize-growing regions that are vulnerable to the effects of salinity [26]. These impacted both maize kernel yield and weight by 25% and 8% respectively, thus reducing grain yield production under salinity stress conditions [18]. Therefore, understanding the mechanisms by which maize varieties respond to salt stress is essential for developing more resilient genotypes [4].

The purple maize is a purple variant with versatile applications. The purple colour of its kernel is attributed to the high anthocyanin content than yellow maize, which confers a high antioxidative potential [44, 63]. The higher antioxidant abundance suggests its potential to protect against diabetes, cardiovascular diseases and cancer [13, 38]. Additionally, there is a waxy variant of the maize attributable to almost exclusive amylopectin content within its starch granules due to a mutation of the *WAXY* gene [16]. Foods rich in amylose are associated with lower glycaemic index and are more beneficial for diabetic patients, whereas foods high in amylopectin generally have higher glycaemic index values [42]. Thus, the purple waxy maize would potentially be a more economically lucrative crop than its conventional yellow sweet variety.

Abiotic stress, such as from high salinity conditions, can be mitigated by upregulating antioxidant enzymes and non-enzymatic activities, thus decreasing oxidative damage towards the plant [6]. These compounds and enzymes neutralize ROS, maintain cellular homeostasis, and protect plants from oxidative damage [23, 24] . Previous studies on maize varieties suggest a correlation between the abundance of ROS scavengers produced under salinity stress, with increased salinity stress tolerance [24, 27, 49]. Thus, the increased antioxidant enzyme activities observed in purple maize and in addition to the waxy trait reflects a typical stress response, which may contribute to salinity tolerance.

In this study, we investigated the physiological and biochemical responses of two maize genotypes, purple sweet (PS) and purple waxy (PW), to salinity stress. These genotypes were selected for their unique characteristics, PS maize for its potential salinity parameters [11] and PW maize for its high amylopectin content [15], a trait of economic importance in the starch industry. By examining physiological changes, stress markers, antioxidant activity and correlations between key traits, this study aims to identify the mechanisms that contribute to salinity tolerance in maize. These results are useful for breeders to develop new maize varieties that are better adapted to a particular region, more tolerant to abiotic stressors, and aligned with changing consumer preferences. Given the increasing threats posed by climate change and soil salinization, the development of salt-tolerant maize varieties is critical for ensuring agricultural sustainability and global food security [22].

Materials and Methods

Plants Material and Experimental Design

Two varieties of maize were used: PW maize (*Zea mays* var. *ceratina* cv. Raja Pulut WX300) and PS maize (*Zea mays* var. *saccharata* cv. Sweet Scarlet SCP1311) were used in this study. Both varieties were grown using a hydroponic system and supplied with AB leafy hydroponic fertilizer following the manufacturer's instructions. Seedlings were germinated in trays and after 4 days of germination, they were transferred to the hydroponic setup. The plants were grown in this system until they reached the three-leaf stage, which typically took around two weeks.

Once the seedlings reached the three-leaf stage, the salinity stress test was initiated. The seedlings were divided into two groups: control and salt-stressed. For the salt-stressed group, 100 mM NaCl was introduced into the water to induce salinity stress [24, 46]. Randomized complete block design (RCBD) was used for the study. The experiment was conducted in a plant incubator laboratory maintained at 24°C, under white fluorescent lighting (12-hour light/12-hour dark cycle). The static hydroponic solution was changed every 3 to 4 days to ensure a consistent nutrient supply. All apparatus used in the experiment were sanitized with bleach to prevent contamination. For morpho-physiological measurements, plants were subjected to four salinity levels (0, 50, 100, 200 mM NaCl), with eight replicates per treatment. For biochemical and antioxidative assays, only control (0 mM) and 100 mM NaCl treatment were analysed, as 100 mM represented the optimum stress level that induced significant physiological changes without causing lethality, while higher concentrations led to severe growth inhibition. The analyses were conducted in three replicates each.

Morpho-Physiological Measurement

The morpho-physiological measurements were conducted over eight days, following the initiation of salinity treatments. To balance workload while capturing representative stress dynamics, data were collected at different intervals depending on the parameter. Salinity treatments of 50 mM, 100 mM and 200 mM NaCl were applied after two weeks of planting. Measurements were recorded on the second leaf from the base of the plant for consistency.

Leaf and Root Length Measurement

Leaf and root lengths were measured using a standard sewing tape. For leaf length, the second leaf from the bottom was measured from the base to the tip. Root length was measured from the point where the root system started to its longest extension. Measurements were taken on days 1, 3, 5, and 7.

Chlorophyll Content Measurement

Chlorophyll content was measured using the SPAD-502Plus meter (Konica Minolta, Tokyo, Japan). Calibration was performed by using the calibration cap provided with the instrument. The second leaf from the bottom was clamped between the two jaws of the SPAD meter, and readings were taken by pressing the measurement button. Each leaf was measured three times, with the average value being recorded for further analysis. SPAD readings were taken under ambient light conditions as recommended by the manufacturer. SPAD measurements were taken at 2-days interval (days 0, 2, 4, 6, and 8).

Chlorophyll Fluorescence Measurement

Chlorophyll fluorescence (measured in Fv/Fm) was measured using the Hansatech Handy Plant Efficiency Analyzer (PEA) + chlorophyll fluorimeter (Hansatech Instruments, Pentney, UK). Before each measurement, the leaf clip was placed on the second leaf from the bottom for a dark adaptation period of at least 30 minutes. After the dark adaptation, chlorophyll fluorescence was recorded by using a PEA meter. Each measurement was performed on at least three randomly selected plants per tray. Measurements were taken at 2-days interval (days 0, 2, 4, 6, and 8).

Stomatal Conductance Measurement

Stomatal conductance was measured using SC-1 Leaf Porometer (Meter Group, Pullman, USA). Calibration was done according to the manual by verifying the sensor in a stable humidity environment. The second leaf from the bottom was clamped gently between the sensor head, ensuring proper contact for accurate measurements. The porometer recorded stomatal conductance in real-time as the leaf exchanged gases with the surrounding environment. Measurements were repeated for three plants per tray to ensure consistency. Between readings, the sensor was cleaned to prevent contamination and ensure accurate data collection. Measurements were taken only on day 0 and day 8.

Biochemical and Antioxidative Assays

Sample Homogenization

Leaf and root samples of both PW and PS maize plants were ground separately in liquid nitrogen using a mortar and pestle in triplicate. Extraction buffer used for stress marker assay, enzymatic antioxidant and non-enzymatic antioxidant were 0.1% Trichloroacetic acid (TCA), 50 mM potassium phosphate buffer (pH 7) and methanol respectively.

Stress Marker Assay

Malondialdehyde Content

Malondialdehyde (MDA) Colorimetric Assay Kit (Plant Samples) from Elabscience Biotechnology Inc., USA (Catalog No. E-BC-K027-M) was used to measure MDA content of leaf and roots samples for both maize varieties by following the manufacturer's protocol. The samples were prepared at 40 mg in Reagent 5 at a ratio of 9:1 on an ice-water bath, then continued with centrifugation at 1,000 g for 15 minutes. 50 µl of supernatant was collected and then mixed with 300 µl of working solution followed by incubation for 40 minutes at 97℃. The mixture was then cooled and centrifuged for 10 minutes at 2,000 g. 170 µl supernatant was measured at 532 nm absorbance by using Multiskan™ FC Microplate Photometer (Thermo Scientific™, Finland). Serial dilution of (0-30 nmol/mL) of a 200 nmol/mL standard solution was prepared for a standard curve. The MDA content was expressed nmol/g fresh weight (FW), and all samples were measured in triplicate.

Hydrogen Peroxide (H_2O_2) Assays

H_2O_2 levels in maize leaves and roots were quantified according to [28], based on the triiodide reaction method described by Junglee *et al*. (2014). 0.1% of TCA buffer was used to extract samples then treated with 1 M potassium iodide and 10 mM potassium phosphate buffer (pH 6.5). The mixture was incubated in the dark for 20 minutes and was measured at 390 nm at room temperature. A standard curve was prepared using known concentrations (5-20 nmol) of H_2O_2. The H_2O_2 content was expressed as nmol/g FW, where FW is the fresh weight of the sample.

Proline Content

Proline (Pro) Colorimetric Assay Kit from Elabscience Biotechnology Inc., USA (Catalog No. E-BC-K177-M) was used to determine the proline content in both leaves and roots samples. 50 mg of samples in 500 µl reagent 1 was homogenized and centrifuged for 15 minutes at 1,000 g, 4℃. Reagent 2, acetic acid and reagent 3 were mixed in a ratio of 1:24:16 and was prepared to make a reaction solution and heated until dissolved in shading light. The assay involved preparing blank, standard, and control solutions using acetic acids and reagent mixtures. At 100℃, the reaction mixture was incubated for about 30 minutes before being cooled and 200 µl of supernatant was taken to measure absorbance at 520 nm using Multiskan™ FC Microplate Photometer reader. Pro concentrations were expressed as µg/g wet weight.

Enzymatic Antioxidant

Superoxide Dismutase (SOD) Activity

SOD activity was assessed using a microplate-based method following [9]. About 50 mg of plant tissue was weighed and then homogenized into 50 mM potassium buffer (pH 7.0) containing 0.4% polyvinylpyrrolidone (PVP). The centrifugation was performed at 10,000 ×g for 30 minutes and maintained at 4℃. The supernatant was collected. The reaction solution was made by transferring weighed chemicals of 0.0307 g of Nitro Blue Tetrazolium (NBT), 0.00038 g of Riboflavin, 0.0019 g of EDTA-Na_2, and 0.9699 g of Methionine into 500 mL final total volume of distilled water. About 5 µL sample supernatant was added to 255 µL reaction solution and 25 µL of potassium phosphate buffer. Then, all samples were placed under light condition (4000 lux) and 100% dark conditions respectively for 20 minutes. SOD activity was measured at 560 nm as the sample under dark conditions serves as a blank for reading and was expressed in unit U/g FW.

Peroxidase (POD) Assay

POD activity is estimated using Zhang's method, with modifications by [55]. About 50 mg of plant sample was homogenized in 500 µL 0.1% TCA and centrifuged at 10,000 g for 30 minutes at 4℃. The reaction mixture contains 10 µL of plant extract, 10 µL of 1.5% guaiacol, and 250 µL of 50 mM potassium phosphate buffer. The reaction was initiated by adding 10 µL of 300 mM H_2O_2. Absorbance was measured at 470 nm immediately by using Multiskan™ FC Microplate Photometer reader. For control, 260 µL of phosphate buffer was added to 10 µL of guaiacol and 10 µL of H_2O_2. One unit of POD activity was defined as the amount of enzyme that oxidizes 1 nmol of guaiacol/minute as the activity constant, i.e., 25.5 mM/cm. The activity was expressed as mM/g FW, based on guaiacol oxidation.

Catalase (CAT) Assay

CAT activity was determined following [9]. The plant extract was also collected from previous peroxidase assay. The reaction was initiated by mixing 10 µL of plant extract, 250 µL of 50 mM phosphate buffer, and 10 µL of H_2O_2 and gently shaken. For blank, 10 µL of distilled water (dH_2O) was added instead of plant extract. The absorbance was read at 240 nm/minute after 2 minutes in triplicate. Changes in absorbance over time reflect CAT activity. CAT activity was expressed as the change in absorbance per minute (Δ240 nm/min).

Non-Enzymatic Antioxidants

Sample extraction was conducted with slight modification following the method by [37]. 1 mL methanol was added to 50 mg of leaves and roots powder in a microcentrifuge tube and gently shaken using a rotary shaker (Orbital Shaker 2 Tier. Model: 722-2T, Protech) at 120 rpm and at 22 °C for 12 hours. The supernatant was collected, and the sample residue was re-extracted three times. All the collected supernatant containing methanolic extract was evaporated using nitrogen gas to produce crude extract. The crude extract was then re-diluted in 500 µL methanol.

Total Phenolic Content (TPC)

TPC of the sample was determined using the Folin-Ciocalteu method followed by [17]. The standard and sample were prepared by adding 10 µl each into 120 µl Folin-Ciocalteu at room temperature for 5

minutes. The reaction occurred when mixing 120 µL of 60 gL^{-1} Na_2CO_3 solution together and incubated for 90 minutes under dark condition. Absorbance was read by using a microplate reader (Thermo Scientific™ Multiskan™ FC Microplate Photometer) at 725 nm. A standard curve was created using Gallic acid solution (2-40 µg mL−1). The result was expressed as µg of gallic acid equivalent per gram of sample (µg GAE g−1).

Total Flavonoid Content (TFC)

TFC was quantified using an aluminium chloride colorimetric assay followed by [50]. About 50 µL of sample aliquot was added into 150 µL methanol in a microplate in triplicate each. 20 µL of 10% $AlCl_3$ solution was then added into each well, followed by shaking for about 5 minutes, and subsequent 3 minutes incubation. 20 µL of 1M CH_3COON_2 was added, and absorbance measurement was taken at 430 nm. Standard for quercetin (50 – 250 µg/mL) was prepared. TFC was expressed as mg quercetin equivalent per gram of dry extract (mg QE/g).

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was measured using a microplate assay followed by Gabriela *et al*. (2019) with slight modification based on [54]. A total of 200 µL mixture consists of (100 µL) sample supernatant + (100 µL; 100 mM) DPPH solution into each well in triplicate. The mixture was then incubated for 30 minutes in a dark condition at room temperature. Trolox standard was prepared and read at 517 nm. The antioxidant activity was expressed as mmol Trolox equivalent per gram of dry weight (mmol TE /g FW).

Statistical Analysis

Student's t-test analysis was performed for all data collection and calculated using Excel 365 statistical tools (Microsoft) at $p < 0.05$. Pearson's correlation analysis was conducted to assess relationships between the traits using XL STAT 2021.

Results

Growth Parameters

Leaf Growth

Salinity stress promotes oxidative stress towards plants by interrupting K^+/Na^+ ratio in leaves and roots, thus inhibiting plant growth. The sensitivity of maize to salinity increases with higher salt concentrations. It has been reported that a salinity level of 250 mM NaCl can lead to plant stunted and severe wilting [2]. Our results showed visible physical changes in both PW and PS maize on day 8 under four different concentration (Figure 1). Both plant types exhibited healthy physical appearance under control condition after day 8. However, PW maize began to show leaf discoloration from green to brown under 50 mM NaCl, with more pronounced effects at 100 mM NaCl. In contrast, PS plant showed only slight changed in leaf color at both 50 mM and 100 mM NaCl. At 200 mM, most plant from both types exhibited severe wilting or had died, with this effect being especially evident in the PS plants.

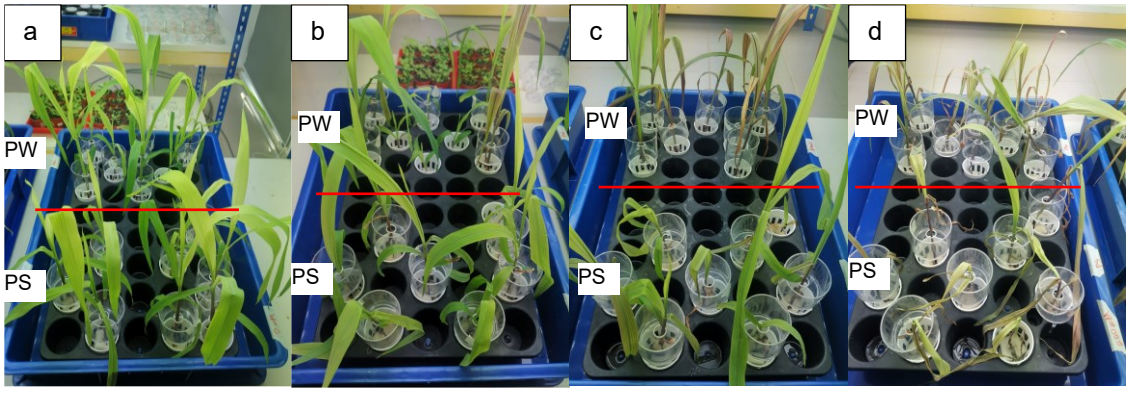

Figure 1. Physical changes on maize sample on different treatment concentrations on day 8 of planting for both PW and PS (a) 0 mM NaCl (b) 50 mM NaCl (c) 100 mM NaCl (d) 200 mM NaCl

At day 0, there was no significant changes to the leaf length between control and salinity-treated plants, which established the baseline for each variety (Figure 2). As salinity stress progressed, a significant reduction in leaf length became evident by day 4 in both PW and PS ($p < 0.05$) when compared to their respective controls. This trend continued, with further significant reductions noted on days 6 and 8 ($p < 0.01$) for PW. PS showed a similar trend, but an even more significant reduction was observed on day 8 ($p < 0.001$). The leaf growth thus suggested that the PS maize displayed a more significant growth retardation.

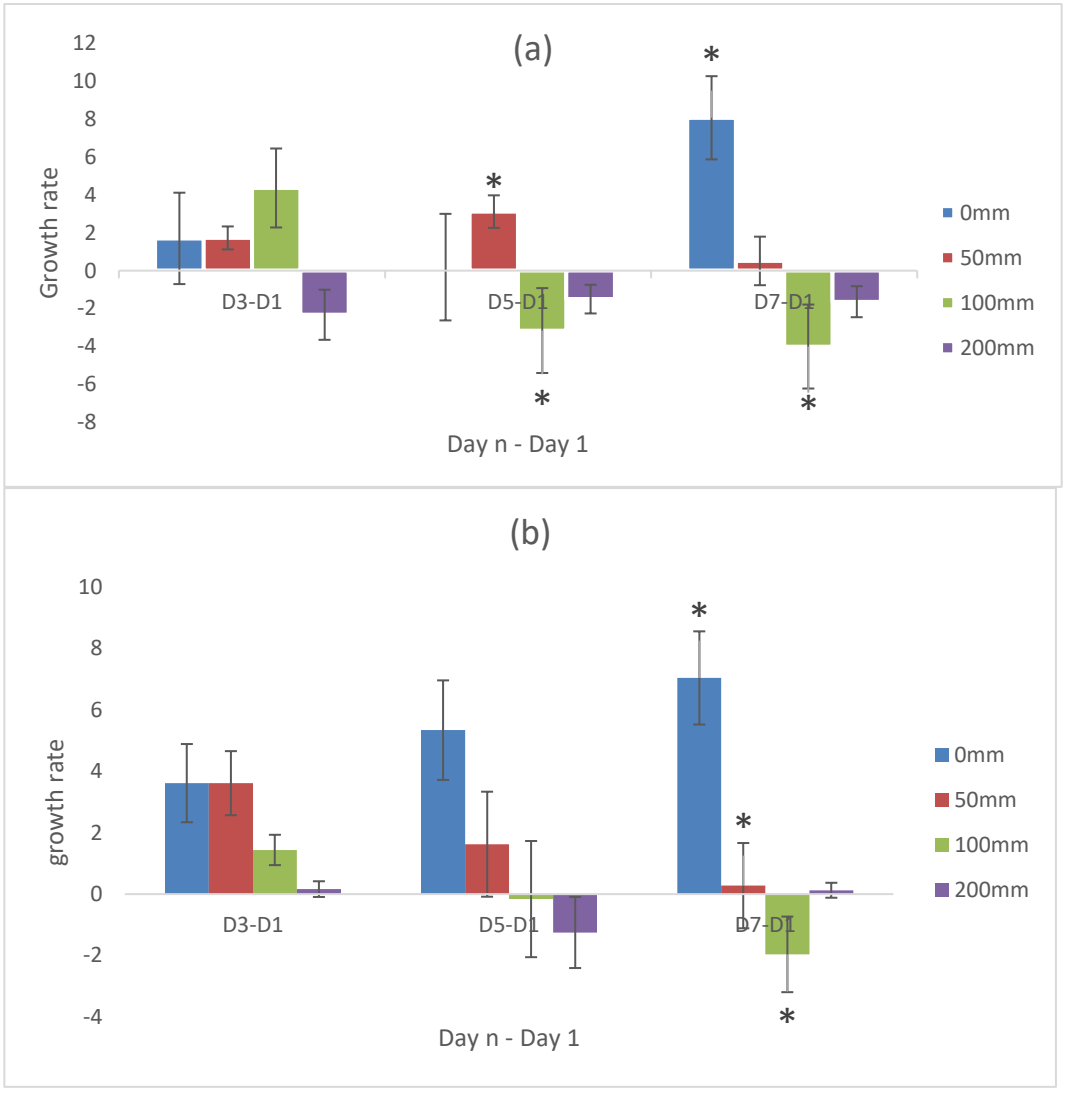

Figure 2. Changes in leaf length over eight days for both Purple Waxy and Purple Sweet maize. (a). Purple waxy (PW) maize. (b). Purple sweet (PS) maize. Statistical test was performed by comparing between leaf growth rates for each maize type. The error bars in all figures represent the standard error (SE) of the mean. *Significant at $p < 0.05$

Root Growth

Control plants of both PW and PS maize exhibited steady increases in root length (Figure 3). However, in salinity-treated plants, a significant reduction in root elongation was apparent from day 2 in PS maize ($p < 0.05$) and from day 4 in PW maize. The trend continued to be an increasingly significant reduction on day 6 ($p < 0.01$) and day 8 ($p < 0.001$). By day 8, both genotypes exhibited a highly significant reduction in roots length ($p < 0.001$), although the effect appeared earlier and was more pronounced in PS maize than in PW maize. These results suggest that salinity stress inhibits root development more severely in PS maize.

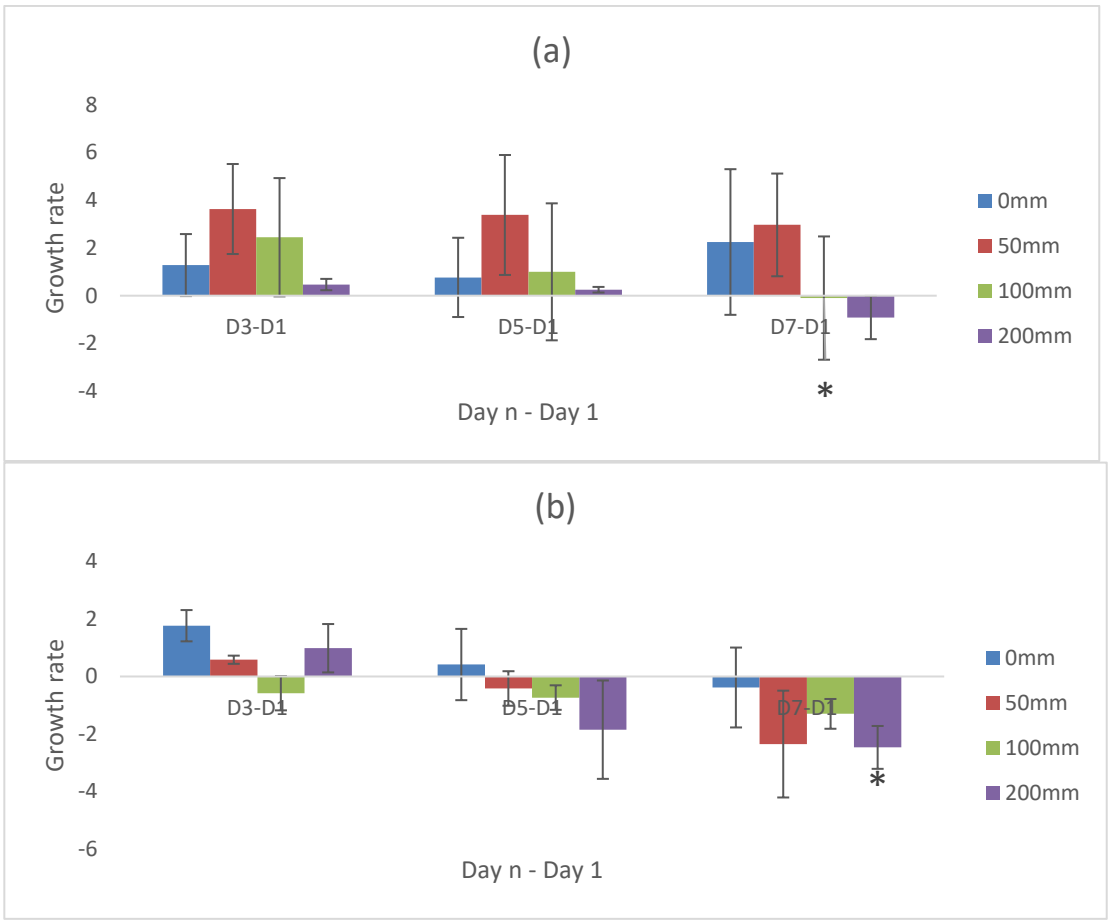

Figure 3. Change in root length for purple sweet and purple waxy maize varieties over the 8-day period. (a). Purple waxy (PW) maize. (b). Purple sweet (PS) maize. Statistical test was performed by comparing between the root growth rate for each maize type. *Significant at $p < 0.05$

Physiological Responses

SPAD Measurements (Chlorophyll Content)

No significant difference in chlorophyll content was observed for control groups over the experiment's duration. In PW, the earliest observation of a significant drop in chlorophyll content ($p < 0.05$) was in the 200 mM NaCl concentration on day 6 post-salinity stress (Figure 4a). For PS, we observed an increase in chlorophyll content in the control group, and a significant decline in the 200 mM NaCl group (Figure 4b). Then, we observed a consistent significant decline for 200 mM NaCl on day 6 and 8.

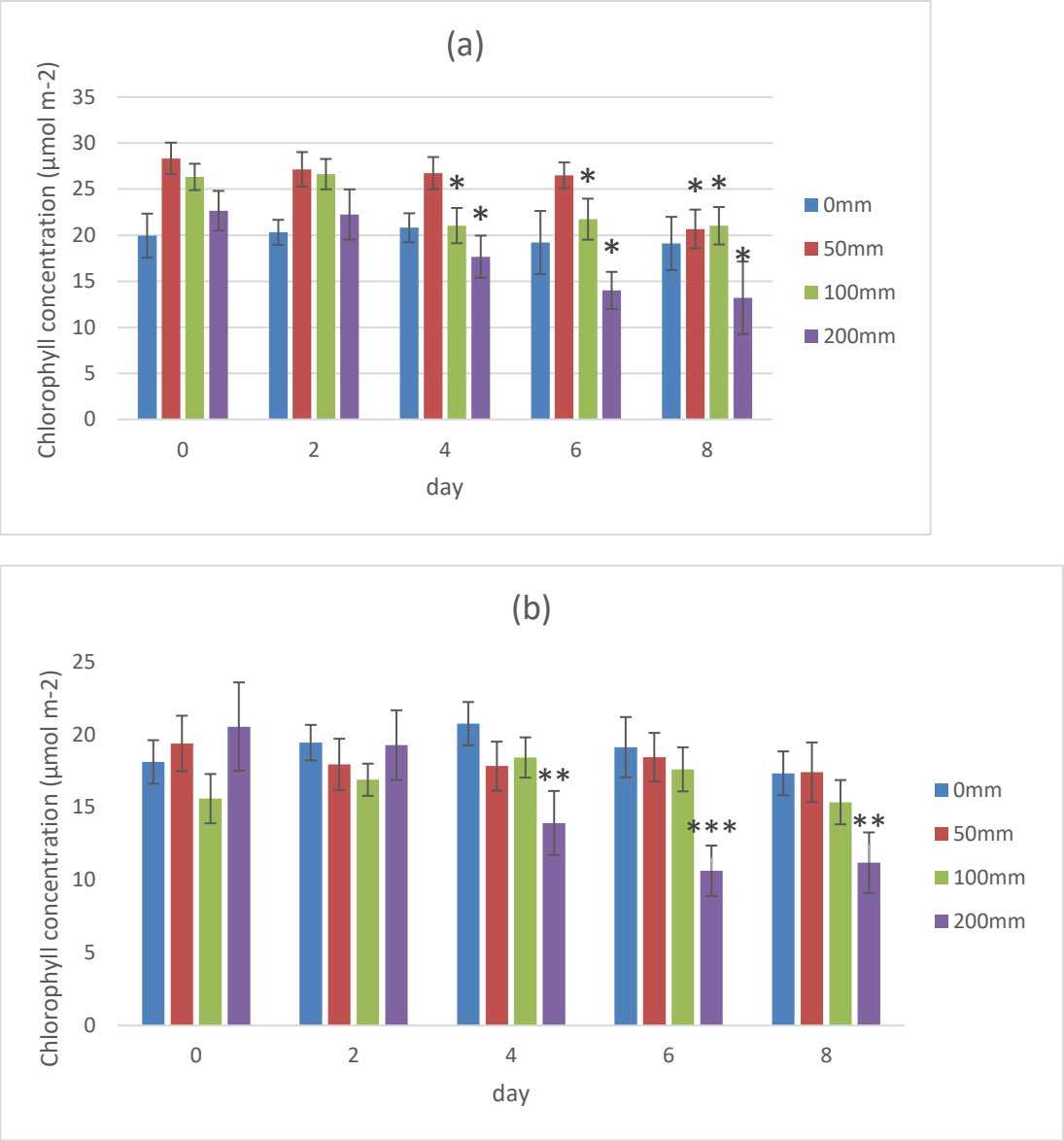

Figure 4. The chlorophyll content of PW and PS leaves. (a) PW (b) PS. Values represent means ± SE(n=8). Mean comparisons were performed for all salinity treatments against their respective controls at each time point (days 2, 4, 6, 8). *Significant at $P < 0.05$, **Significant at $p < 0.01$, ***significant at $p < 0.005$

Chlorophyll Fluorescence Analysis

Photosystem II (PSII) efficiency, represented by Fv/Fm ratios measures the efficiency of PSII in converting light energy into chemical energy (Park *et al.*, 1995). Both PS and PW control plants maintained a stable PSII efficiency, close to optimal levels (Fv/Fm ≈ 0.8) (Figure 5a, b). PW exhibited a progressive reduction under 200 mM NaCl, becoming increasingly significant from day 4 to day 8 after the onset of salinity stress (Figure 5a). In PS maize, PSII efficiency showed a marked reduction under 200 mM NaCl, and significantly reductions were also observed at 100 mM NaCl (Figure 5b).

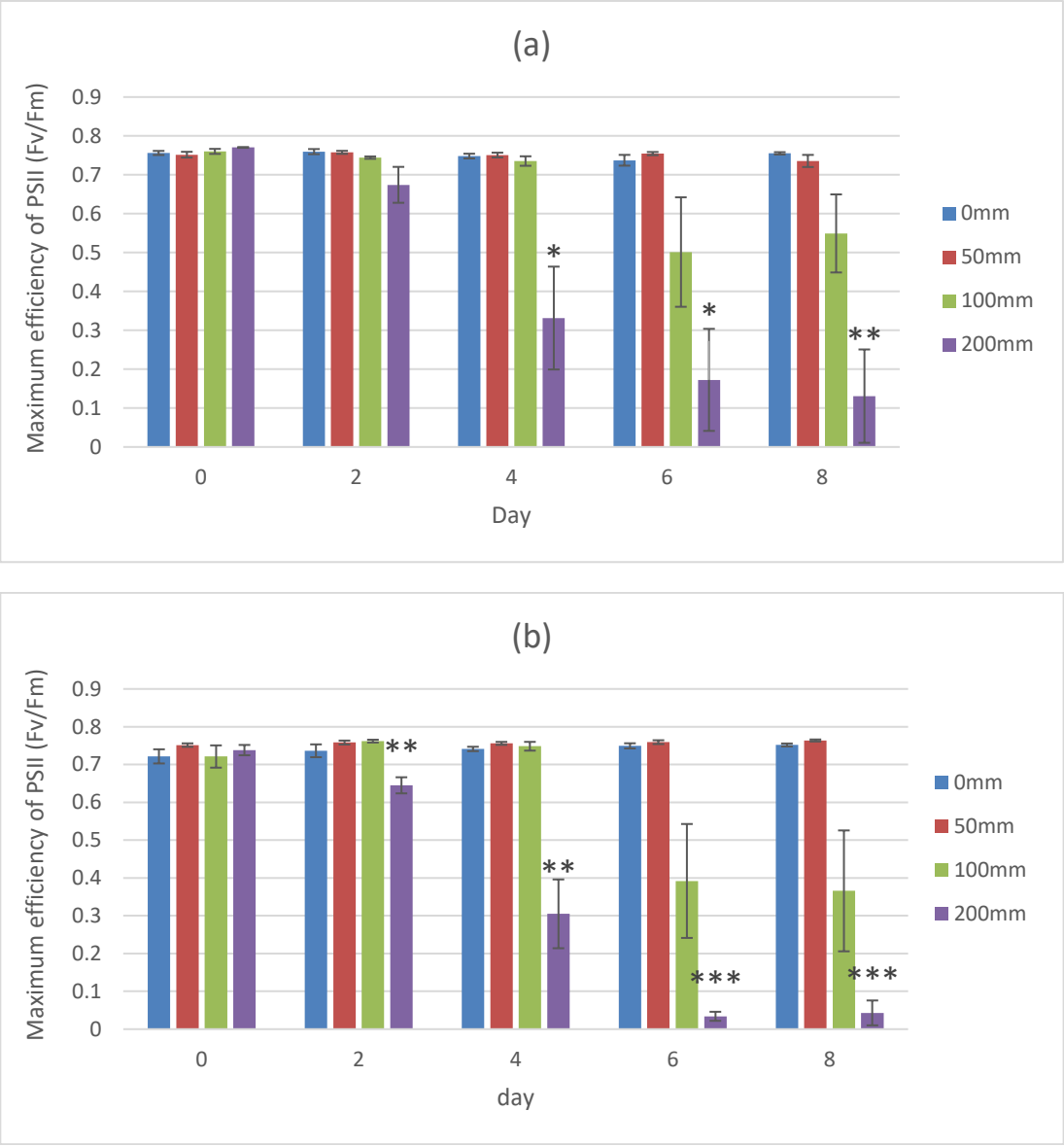

Figure 5. Photosystem II (PSII) efficiency of PW and PS. (a) PW (b) PS. *Significant at P < 0.05. Statistical test was performed by comparing [day n (2,4,6,8) to day 0 for each concentration]. **Significant at p < 0.01. ***Significant at p < 0.005

Stomatal Conductance

The stomatal conductance of the plants at day 0 already exhibited very different levels of conductance (Figure 6a, b). PW and PS maize exhibit different reactions to salinity stress at varying concentrations (0 mM, 50 mM, 100 mM, and 200 mM NaCl), according to the results of stomatal conductance measurements. Purple waxy maize showed no significant differences in stomatal conductance between treatments on day 0 but showed a significant decrease on day 8 for 100 mM NaCl treatment compared to 0 mM NaCl. No significant was observed for both 50 mM and 200 mM treatments. Like PW, PS showed no change on day 0 but did show a significant decrease in 50 mM and 100 mM NaCl compared to control. Since 200 mM does not show any changes for PS, PS maize exhibits notable sensitivity at both lower (50 mM) and moderate (100 mM) salinity levels, whereas PW maize is mainly impacted at moderate salinity levels (100 mM).

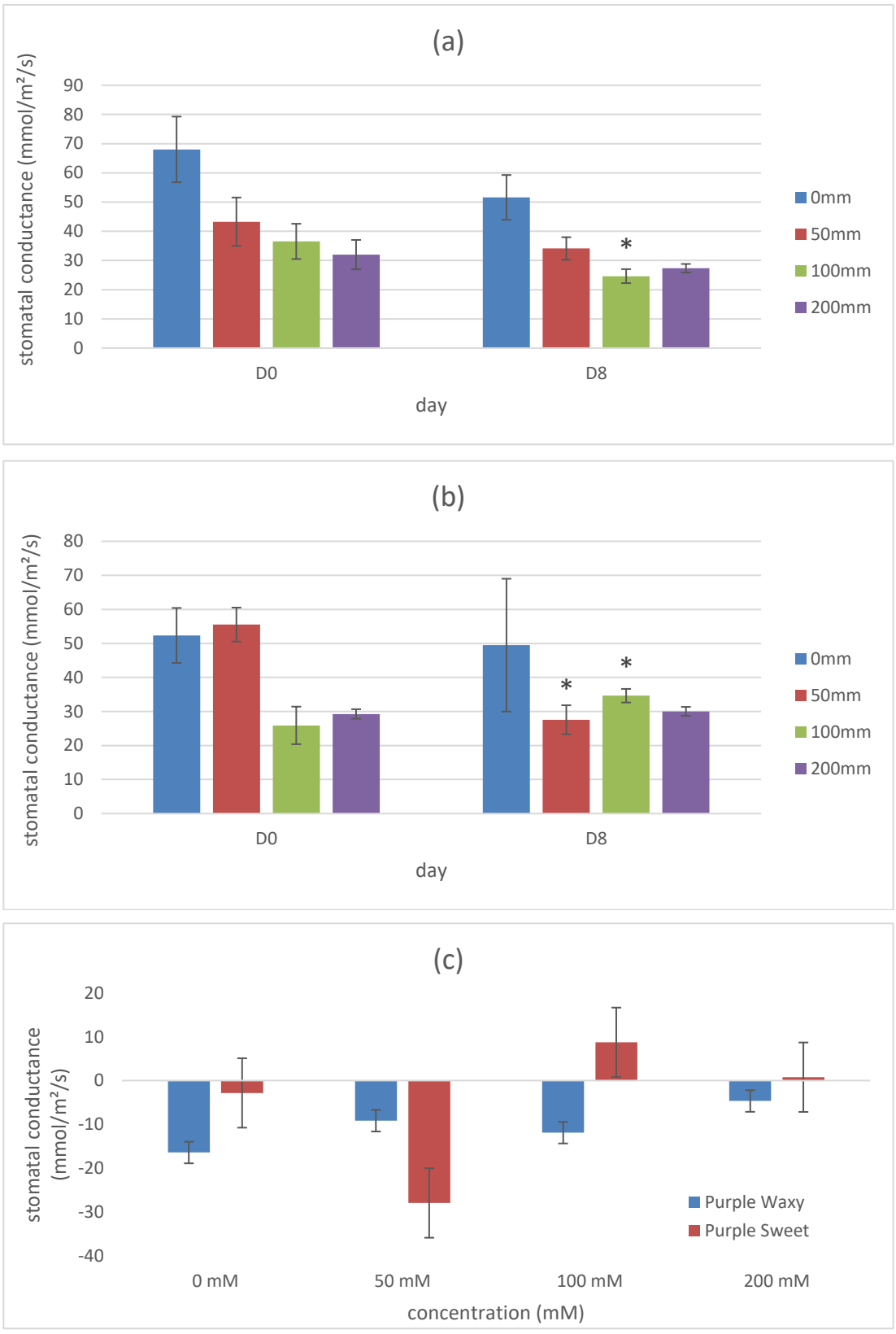

Figure 6. Stomatal conductance of PW and PW maize. (a) PW (b) PS. Statistical test was performed by comparing day 0 to day 8 for each type of maize. (c) change in stomatal conductance (D8 - D0) for PW and PS. Statistical test was performed by comparing the change in stomatal conductance (D8 - D0) to control of each concentration for PW and PS. *Significant at $p < 0.05$

This suggests that the two maize types have different tolerance levels for salinity. For PW, there were no significant differences between the day 0 and day 8 stomatal conductance (Figure 6a, c). For PS, there was a significant stomatal conductance decline in 50 mM NaCl (p-value < 0.05) but not at 100 mM and 200 mM NaCl (Figure 6b, c) as it contributes to physical changes in terms of a more severe wilting.

Stress Marker Assay

For the subsequent assays, we compared the control, PW and PS at 100 mM NaCl treatment. At this concentration, we observed significant physical changes as well as growth reduction in PW and PS (Figure 7). PW showed slightly wilting after 8 days in the control group (Figure 7c, a), while the PS plant remained unchanged (Figure 7g, e). Under 100 mM NaCl treatment, PW exhibited significant leaf discoloration compared to control (Figure 7d, b) whereas PS showed only slight changes (Figure 7h, f).

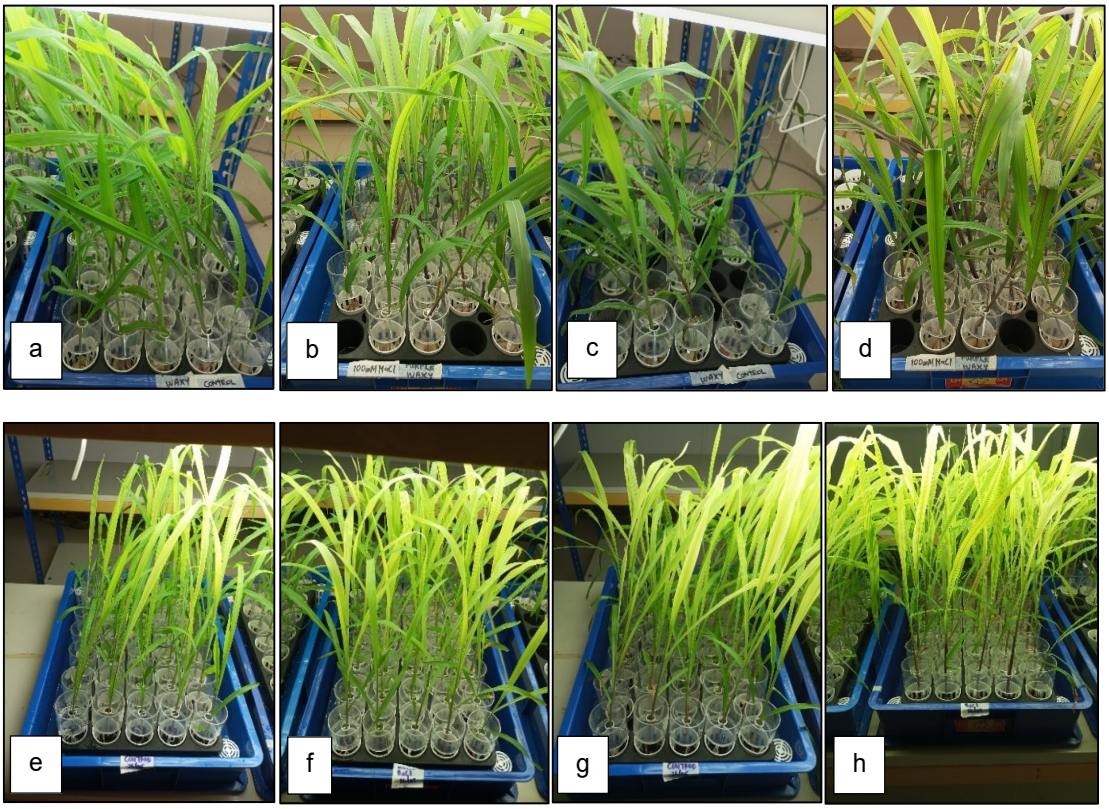

Figure 7. The PW and PS plants phenotype under 0 mM NaCl and 100 mM NaCl treatment (a) PW on day 0 treatment with 0 mM NaCl. (b) PW maize on day 0 treatment with 100 mM NaCl. (c) PW maize on day 8 treatment with 0 mM NaCl. (d) PW maize on day 8 treatment with 100 mM NaCl. (e) PS on day 0 treatment with 0 mM NaCl. (f) PS maize on day 0 treatment with 100 mM NaCl. (c) PS maize on day 8 treatment with 0 mM NaCl. (d) PS maize on day 8 treatment with 100 mM NaCl

MDA Content

The MDA content, indicative of lipid peroxidation, demonstrated a minor but non-significant increase in PW leaves under salt stress (100 mM NaCl). In contrast, the PS leaves exhibited a slight, non-significant decrease in MDA content when exposed to salinity (Figure 8a). No observable changes were detected in the MDA content of the roots for both PW and PS varieties under salt treatment. The statistical analysis confirmed that all variations in MDA content across different tissues and treatments were non-significant.

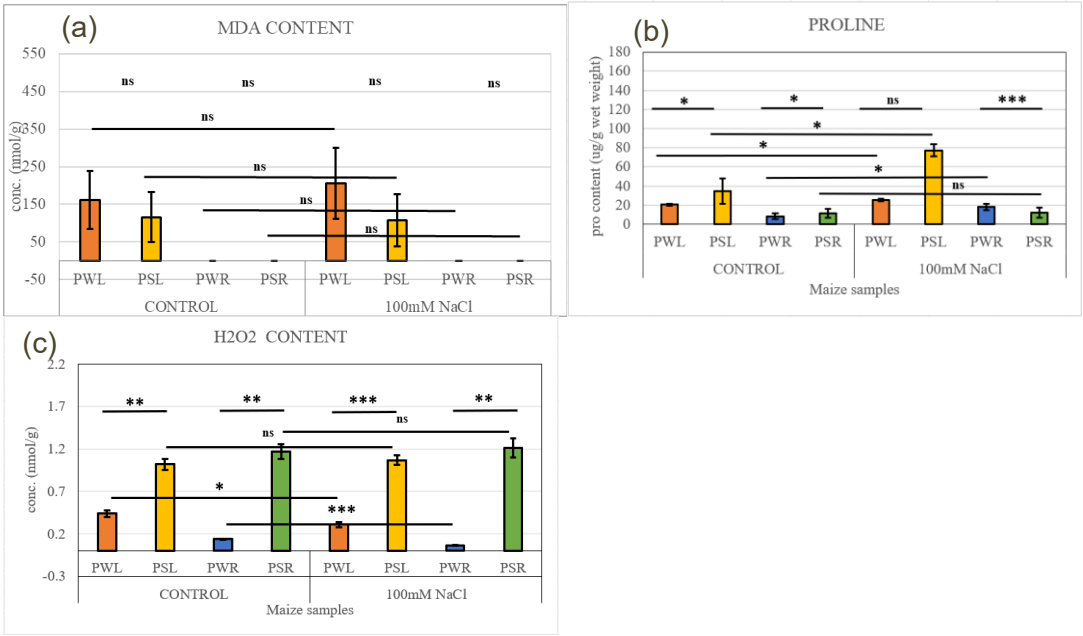

Fig. 8 Comparison of Maize Plant Samples (Leaves and Roots) on (a) MDA Content (nmol/g) (b) Proline Content (µg/g Wet Weight) (c) Hydrogen Peroxide (H_2O_2) Content (nmol/g FW) as Shown in Bar Graphs Between Control (0 mM NaCl) and Treatment (100 mM NaCl) for Two Maize Varieties. ns – non-significant; *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); PWL – Purple Waxy Leaves; PSL – Purple Sweet Leaves; PWR – Purple Waxy Roots; PSR – Purple Sweet Roots

H_2O_2 Assays

H_2O_2 content, an indicator of oxidative stress, showed a significant increase in most samples under salt stress (Figure 8b). PW leaves and roots exhibited significantly higher H_2O_2 levels under 100 mM NaCl compared to the control. In contrast, PS leaves and roots displayed non-significant changes in H_2O_2 levels between the control and salt treatments, indicating less oxidative stress in this variety. Overall, significant differences were found between the two varieties for most of the tissues and treatments, except for the PS maize samples under salt stress.

Proline Content

Proline, an important osmoprotectant, showed significant increases in both the leaves of PW and PS varieties under salt treatment, as well as in the roots of PW (Figure 8c). In contrast, PS roots exhibited a non-significant increase in Pro accumulation observed between the two varieties, particularly in root samples, while Pro levels in leaves were only significantly different between the control and treatment groups, with no significant differences noted between varieties under salt stress.

Antioxidant Enzyme Activity

Superoxide Dismutase (SOD) Activity

SOD activity, an essential antioxidant enzyme involved in the detoxification of superoxide radicals, showed a significant increase in PW leaves under salt stress compared to the control (Figure 9a). In contrast, the SOD activity in PS leaves, as well as in both varieties' roots, did not exhibit significant changes between treatments. When comparing the two varieties, a significant difference was observed in leaf samples, where PW leaves had higher SOD activity under salt stress, while no significant differences were noted in root samples.

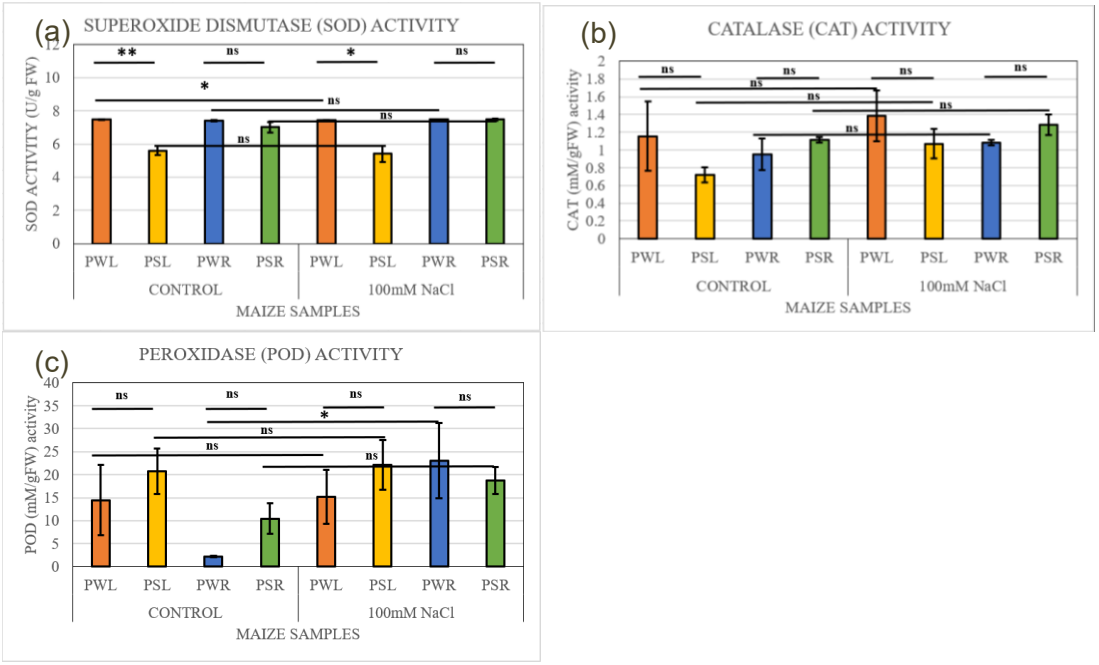

Figure 9. Comparison of Antioxidative Activity of Maize Plant Samples (Leaves and Roots) on (a) SOD Activity (U/g FW) (b) POD Activity (mM/gFW) (c) CAT Activity (mM/gFW), Shown in Bar Graphs Between Control (0 mM NaCl) and Treatment (100 mM NaCl) for Two Maize Varieties. ns – non-significant; *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); PWL; PSL; PWR; PSR

Peroxidase (POD) Activity

POD activity, which contributes to scavenging hydrogen peroxide and protecting cells from oxidative damage, showed a non-significant increase across both varieties and treatments (Figure 9b). However, a notable exception was the significant increase in POD activity observed in PW roots under salt stress, indicating a potential role in the adaptive response of this tissue to salinity. All other tissues, including the PS roots and leaves, exhibited non-significant changes in POD activity across treatments and between varieties.

Catalase (CAT) Activity

CAT activity, another key antioxidant enzyme that mitigates oxidative stress by breaking down H_2O_2 into water and oxygen, showed non-significant changes between control and salt-treated samples in either variety (Figure 9c). Similarly, there were no significant differences in CAT activity between the two maize varieties, regardless of treatment, in both leaves and roots. This suggests that CAT may not play a prominent role in the specific antioxidant defence responses of these maize varieties under salt stress conditions.

Non-Enzymatic Antioxidants

Total Phenolic Content (TPC)

TPC, an indicator of the antioxidant potential of plant tissues, showed differential responses under salt stress across the maize varieties and tissues (Figure 10a). PS leaves and PW roots exhibited significant increases in TPC when exposed to 100 mM NaCl, suggesting a stronger accumulation of phenolic compounds in these tissues under salt stress. In contrast, PW leaves and PS roots showed a non-significant increase between treatments. When comparing the varieties, a significant difference was observed in the roots of the control samples, where PS roots had higher TPC levels compared to PW roots, while other tissue samples showed non-significant differences between varieties.

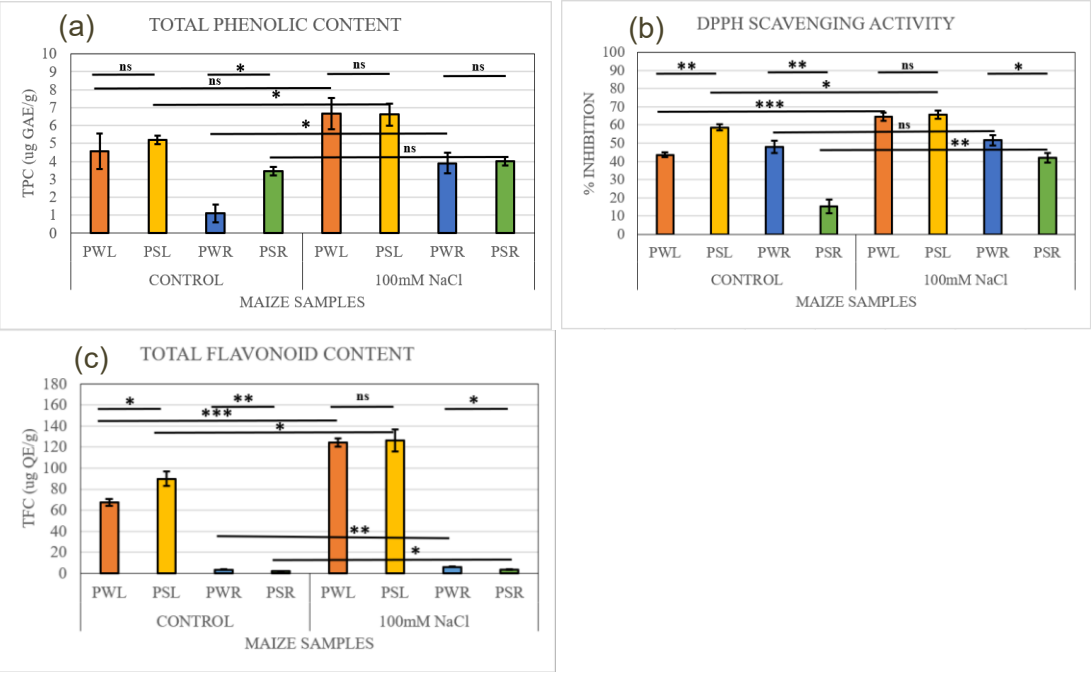

Figure 10. Comparison of Non-Enzymatic Antioxidants Activity in Maize Plant Samples (Leaves and Roots) on (a) Total Phenolic Content (TPC, µg GAE/g), (b) Total Flavonoid Content (TFC, µg QE/g), and (c) DPPH Radical Scavenging Activity (% Inhibition), Shown in Bar Graphs Between Control (0 mM NaCl) and Treatment (100 mM NaCl) for Two Maize Varieties. ns – non-significant; *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); PWL; PSL; PWR; PSR

Total Flavonoid Content (TFC)

TFC, another measure of antioxidant capacity, significantly increased across both maize varieties and treatments, indicating that flavonoid biosynthesis was activated under salt stress (Figure 10b). The only exception was found in the leaves under salinity conditions, where the comparison between varieties did not show a significant difference. This suggests that while salt stress enhances flavonoid accumulation in most tissues, varietal differences in leaves under salt treatment are minimal.

DPPH Radical Scavenging Activity

DPPH radical scavenging activity, which reflects the overall antioxidant capacity of the samples, showed significant differences between varieties and treatments in most tissues (Figure 10c). However, no significant changes were observed in PW roots between treatments, indicating that the antioxidant defence mechanisms in these roots were less responsive to salt stress in terms of DPPH scavenging. Additionally, the comparison between varieties in leaf samples under salt stress conditions showed non-significant differences, suggesting a similar antioxidant response in both PW and PS leaves when exposed to 100 mM NaCl.

Correlation Analysis

Pearson's correlation analysis revealed several key relationships between the oxidative stress markers and antioxidant assays. Notably, there was a strong positive correlation observed between Pro and TFC, indicating that higher Pro levels were associated with increased flavonoid accumulation under salt stress (Table 1). Additionally, DPPH and TFC exhibited a strong positive correlation, suggesting that the flavonoid content significantly contributed to antioxidant capacity. Similarly, a strong positive correlation was found between TPC and TFC, reinforcing the role of phenolics and flavonoids in antioxidative responses. Conversely, a negative correlation was observed between Pro and SOD activity, implying an inverse relationship between these two stress-response mechanisms. This suggests that as Pro accumulation increased, SOD activity decreased, potentially indicating a shift in the plant's strategy to cope with oxidative stress under different conditions.

Table 1. Pearson's Correlation Analysis Between Oxidative Stress Assays for Both Varieties and Treatments. PRO-proline content, DPPH-Diphenylpicrylhydrazyl, H_2O_2-hydrogen peroxide, MDA-malondialdehyde, POD-Peroxidase, TPC-total phenolics content, TPC-total flavonoid content, SOD-superoxide dismutase, CAT-catalase

VARIABLES	PRO	H_2O_2	MDA	SOD	POD	CAT	DPPH	TPC	TFC
PRO	**1**	0.318	-0.230	**-0.821**	0.543	-0.165	0.607	0.703	**0.758**
H_2O_2	0.318	**1**	0.073	-0.543	0.276	-0.094	-0.288	0.271	0.081
MDA	-0.230	0.073	**1**	-0.190	-0.382	-0.680	-0.293	-0.332	-0.069
SOD	**-0.821**	-0.543	-0.190	**1**	-0.429	0.600	-0.388	-0.471	-0.568
POD	0.543	0.276	-0.382	-0.429	**1**	0.002	0.434	0.694	0.384
CAT	-0.165	-0.094	-0.680	0.600	0.002	**1**	-0.075	0.272	0.039
DPPH	0.607	-0.288	-0.293	-0.388	0.434	-0.075	**1**	0.567	**0.731**
TPC	0.703	0.271	-0.332	-0.471	0.694	0.272	0.567	**1**	**0.866**
TFC	**0.758**	0.081	-0.069	-0.568	0.384	0.039	**0.731**	**0.866**	**1**

Values in bold are different from 0 with α=0.05

Discussion

The current study highlights the critical role of antioxidants in managing salinity stress, which is largely influenced by the ionic imbalance caused by salt presence. Leaf and growth reduction was observed in both PS and PW with a minimum concentration of 50 mM for PS and 100 mM NaCl for PS. We observed a decline in chlorophyll content and chlorophyll fluorescence but no significant changes in the stomatal conductance for both varieties. The ROS assays showed a differential abundance of stress markers, antioxidative enzymes and non-enzymatic antioxidants, highlighting some degree of difference between PW and PS in responding to oxidative stress. Overall, the PW showed greater tolerance to salinity stress.

The reduction in leaf and root growth observed in PW and PS maize under salinity stress, with less observable effects in PW, aligns with the established impacts of salinity on growth due to osmotic imbalance. Excessive salinity disrupts water uptake, inflicting osmotic stress, which limits cell turgor and inhibits growth. This is particularly detrimental for root systems as roots are the first to encounter soil salinity, affecting their ability to absorb water and nutrients, and limiting overall plant growth [20, 40]. In addition to compartmentalization, osmotic adjustment through the accumulation of compatible solutes like proline, glycine and betaine helps maintain cellular turgor and supports continued growth under stress [5]. PW maize's comparatively stable root growth under saline conditions suggests a higher accumulation of osmolytes. This is consistent with PW exhibiting elevated proline levels in this study.

The decline in chlorophyll content in response to salinity stress suggests chlorophyll degradation due to oxidative stress. Under high salinity, chloroplasts are exposed to elevated oxidative stress due to excessive ROS generation, which damages chloroplast membranes and degrades chlorophyll molecules [39]. Recent studies have shown that varieties capable of maintaining chlorophyll under salinity stress often exhibit higher antioxidant capacities [30, 47]. Additionally, salt-tolerant varieties tend to maintain photosynthetic efficiency by sustaining chlorophyll synthesis, even under stress [25]. PS exhibiting a greater chlorophyll retention capacity but a more significant chlorophyll reduction at 200 mM NaCl over the 8-day experiment period suggests a different means of regulating chlorophyll abundance, which reflects upon the other photosynthesis parameters.

The observed decrease in PSII efficiency (Fv/Fm) under salinity stress, particularly in PW maize, suggests photoinhibition and impaired photochemical function due to ionic and osmotic stress. Under salinity, excess light energy absorbed by chlorophyll cannot be efficiently used, resulting in ROS accumulation, which can damage PSII reaction centers [61]. PSII efficiency is closely linked to the plant's capacity for non-photochemical quenching (NPQ), a mechanism that dissipates excess energy as heat, protecting PSII from photooxidative damage [34]. PS exhibiting a greater reduction of Fv/Fm ratio suggests limited NPQ and PSII repair capability, leading to photoinhibition and decreased photosynthetic performance [8, 25]. The less divergent Fv/Fm ratio observed in PW maize suggests greater photoprotective capacity, possibly through enhanced NPQ mechanisms or increased xanthophyll cycle activity, which helps dissipate excess energy safely [62].

Stomatal conductance was largely unchanged under salinity stress in both maize varieties over the 8-day period but showed an early significant reduction among the 100 Mm and 200 mM NaCl. The difference between the different concentration groups among the PW and PS at day 0 was probably attributed to the sampling differences, as the stomatal conductance was not significantly different for 100 mM and 200 mM NaCl after 8 days of salinity exposure. This data suggested that in PS and PW, the salinity stress impairs the chlorophyll integrity but does not impede upon the stomatal opening. Stomatal regulation is primarily mediated by ABA, which accumulates under osmotic stress, triggering stomatal closure to conserve water. In salt-tolerant plants, a balanced ABA response through hormonal crosstalk can modulate stomatal opening to allow gas exchange, supporting photosynthesis while minimizing water loss [12, 57]. Taken together, ROS accumulation may incur damage to the chlorophyll structure, causing a decline in its abundance and efficiency, but the ABA levels may have been modulated by a series of hormonal crosstalk to preserve the stomatal opening. Based on the above physiological data, among these salt-stressed treatment concentrations, 100 mM NaCl suits the most for experimenting on the effect towards salinity as maize plants were slightly impacted at 50 mM NaCl and were at a detrimental stage when reaching 200 mM NaCl. Therefore, only control and 100 mM NaCl salt concentration was then continued for evaluating stress marker and antioxidative assays.

Salinity noticeably impacted the maize with respect to H_2O_2 and proline accumulation, whereas MDA levels did not show significant changes [1, 14, 58]. Previous investigations also indicate minimal or insignificant alterations in MDA content, which varied by maize variety [1, 10, 14, 24, 58]. It is proposed that stable MDA levels suggest a tolerant genotype, whereas increased MDA indicates a more susceptible one [14]. In our findings, the rise in H_2O_2 levels suggests that the maize is actively reacting to salt stress, with the PW genotype exhibiting minimal variations in H_2O_2 levels, potentially indicating its adaptability to endure salt stress [1, 14].

As mentioned previously, the Pro acts as an osmoprotectant. However, it also serves as a marker for abiotic stress. The Pro levels remained relatively constant in the PS roots, further affirming their salinity tolerance [1]. On the other hand, the PW roots showed a considerable increase in Pro, indicating a stress response [58]. Earlier studies have documented mixed results, with some noting increases while others report slight decreases or no changes in Pro levels under salt stress [1, 58]. It is anticipated that oxidative stress will rise when plants face salinity, making the involvement of both enzymatic and non-enzymatic antioxidants essential [58].

Our research revealed PW leaves displayed a notable increase in SOD activity, indicating a heightened oxidative response to salinity [24]. POD activity showed minimal changes, apart from a significant increase in the purple waxy roots as a response to salt stress. Generally, POD activity tends to rise under salt stress, but its reaction can differ among maize varieties [1, 10, 14, 24]. CAT activity indicated a slight increase in PS and PW, and though both were not statistically significant, both seem to correspond with previous findings that associate elevated catalase activity with salinity tolerance [10, 14] . Similar non-significant changes in CAT activity have also been reported in other improved maize varieties in past studies [10].

The TPC demonstrated varied responses, with increases noted in the roots and mature leave, while younger leaves exhibited no changes, aligning with earlier research [1, 7, 36] . Our findings revealed both significant and non-significant variations, depending on the organ assessed. Unlike previous studies that showed no significant changes in TFC across different organs under salinity stress [1, 7] , our study indicated significant increases in TFC under salinity conditions. DPPH scavenging activity, which indicates antioxidant capability, revealed substantial changes in response to salt stress, apart from the PW roots [7, 36]. The strong positive associations among DPPH, proline, phenolic content, and flavonoid content emphasize the interrelatedness of these antioxidant mechanisms [6, 26] . In contrast, a significant negative correlation between proline and SOD activity implies that as proline levels increase, SOD activity declines, perhaps reflecting a change in the plant's strategy for tackling oxidative stress in saline conditions.

Similar findings were observed in other multi-coloured crops. Purple sweet potato was found to increase cyanidin-producing gene expressions under higher salinity stress [64]. However, higher cyanidin levels may not be the determining factor for salinity tolerance as other antioxidants that confers other colors such as orange pigmentation of β-carotene could enhance salinity tolerance as well. In a large scale study conducted in Bangladesh [59] and Turkey [60], the orange sweet potato performed better under salinity stress than the purple variety. Thus, it may be of interest to compare the salinity tolerance of PW with other coloured varieties of maize.

The waxy trait appears to contribute to PW resilience. The amylopectin structure is highly branched, which was theorised to be able to retain more water and be more accessible for enzymatic action than the linear amylose. A study assessing the performance of hydrogels reported that increasing the amylopectin to be higher than amylose, enhances the water absorption and retention performance [35]. Likewise, amylopectin is more easily digestible than amylose, allowing amylopectin to quickly fuel the metabolic processes needed to retain ionic homeostasis and respond to ROS accumulation under salinity stress [29].

By concentrating breeding efforts on enhancing both its salinity adaptability and amylopectin-rich starch content, breeders can cultivate varieties that not only endure saline conditions but also provide significant economic benefits in food production [53]. Furthermore, non-enzymatic antioxidants such as total phenolic and flavonoid content contributed to salinity tolerance. The considerable changes in flavonoid content across both genotypes suggest that non-enzymatic defences against oxidative stress could serve as additional targets for breeding [41]. Selecting higher flavonoid and phenolic levels in maize varieties could further improve salinity tolerance by enhancing the plant's natural resistance to ROS accumulation [32]. The positive correlations among DPPH scavenging activity, proline, and flavonoid contents highlight a synergistic role for these compounds in promoting salinity tolerance [6]. The differential physiological responses between PW and PS maize highlight the importance of selecting and breeding for traits like efficient ion compartmentalization, chlorophyll stability, enhanced NPQ capacity and regulated stomatal behaviour for salinity tolerance. CRISPR/Cas9 technology offers powerful tools to edit genes responsible for these traits, enabling the development of maize lines with enhanced salt tolerance [48].

The outcomes of this study provide crucial insights into the physiological and biochemical responses of diverse maize varieties to salinity stress, emphasizing key antioxidant mechanisms and stress markers that could be utilized in breeding programs to enhance salinity tolerance [2]. The growth parameter of PW showed better adaptability towards salinity stress compared to PS which was more sensitive. However, both PW and PS genotypes exhibited features slightly different towards other parameters such as stable proline levels, low hydrogen peroxide accumulation, and consistent antioxidant enzyme activities, indicating its clear salinity tolerance [1, 14, 15] . These characteristics render the PW maize a promising option for breeding salt-resistant varieties capable of prospering in salinity-prone agricultural areas [3]. Integrating these traits into maize breeding efforts, through both traditional and molecular approaches, can enhance the resilience of maize crops under salt stress, thereby preserving productivity in challenging growing conditions [21, 33, 45, 51].

Conclusions

The morpho-physiological analysis concluded that maize exhibited adaptability under 100 mM NaCl, while no significant effects were observed at 50 mM and severe lethality at 200 mM NaCl. Further investigations focused on identifying stress markers and antioxidant enzyme activities, which are crucial for understanding salt-tolerant varieties. Both PW and PS demonstrated good adaptability to salt stress, with PW exhibiting superior performance compared to PS, indicating a stronger inherent trait. This makes PW an ideal candidate for breeding resilient maize varieties. Leveraging these findings, maize breeding programs can develop varieties that are not only better suited to saline environments but also more economically viable, thus addressing both agronomic and industrial needs.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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