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Morpho-Physiological and Anatomical Alterations of Salt-Affected Thompson Seedless Grapevine (*Vitis vinifera* L.) to Brassinolide Spraying

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Abstract: Salinity is one of the most critical crises worldwide that ultimately compromises future food security. Brassinosteroids including brassinolide (BL) are a class of polyhydroxy steroids phytohormones, that play a crucial role in several plant metabolic pathways and boost plants' stress tolerance, but less data is accessible on its function in salt-affected grapevine. The experiment was conducted throughout the 2019 and 2020 experimental seasons at EL-Baramon experimental farm, Horticulture Research Institute, Mansoura, Egypt, to recognize the remediation potential of BL (1 and 2 mg L^{-1}) in lightening salinity (NaCl at 1000, 2000, and 3000 mg L^{-1}) injury on Thompson seedless grapevine seedlings (H4 strain) growth and physio-anatomical attributes. Data advocated that while salinity reduced growth attributes, BL applications substantially improved the overall salt-affected plant performance. Salinity stress significantly decreased photosynthetic pigment, relative water content, and ions percentage (nitrogen, phosphorus, potassium, potassium/sodium ratio). Alternatively, BL spraying significantly ($p \leq 0.05$) increased the photosynthetic pigment, maintaining a favorable potassium/sodium ratio and increasing the ions percentage. Additionally, increasing salinity levels significantly boost plant sodium percentage and induce a membrane malfunction associated with increased membrane permeability; conversely, the application of BL decreased the sodium percentage associated with decreasing membrane permeability relative to non-treated salinized plants. Moreover, salinity and/or BL significantly improved the antioxidant capacity associated with rising proline accumulation and antioxidant enzyme activities. Anatomically, salinity stress considerably modified leaf structure; meanwhile, the spraying with BL drastically mitigates the harmful effects of salinity on leaf anatomy. Additionally, salt-affected plant cells explained various obvious organelles ultrastructural modifications and cellular damage; meanwhile, BL spraying to salt-affected plants repealed the ultrastructural modifications of cell organelles. Taken together, BL, especially 2 mg L^{-1}, has a great potential to boost the salt tolerance of Thompson seedless grapevine seedlings (H4 strain). It improves salt tolerance by sustaining higher photosynthetic pigment concentrations, maintaining ion homeostasis, regulating water status, and stimulating antioxidant capacity as well as maintaining leaf anatomical attributes.

Keywords: antioxidant systems; brassinolide; grapevine; ion accumulation; leaf anatomy; salt stress; ultrastructure

1. Introduction

Grapevine (*Vitis vinifera* L.), which has both monetary significance and positive effect on human health, is considered one of the most tasty, stimulating, and healthful fruits worldwide. The berries are an excellent supply of sugars, minerals, and vitamins [1]. Owing to its wealthy phenolic compounds, the grapevine is extensively consumed in diverse shapes, i.e., fresh, raisins, wine, vinegar, molasses, grapevine juice, etc.; additionally, it is utilized in food additives, pharmaceutical production, and natural cosmetic products [2]. Customer attentiveness to the connection between foods and health, alongside environmental concerns, has improved the requirement for foods with elevated nutritional qualities [3]. Thompson seedless grapevine is the most imperative table grapevine cultivar in Egypt, particularly in the Delta region for local consumption and exportation. Recently, H4 is a promising strain of Thompson seedless grapevine introduced to Egypt in 2012, which has been cultivated extensively owing to its high vigor and fertility, superior yield and high cluster weight [4]. Yet, a huge acreage is situated at the newly reclaimed soils that have several troubles such as salinity. Grapevines are considered moderately sensitive to salinity, and the injury is primarily originating from chloride ions [5].

Salinity is considered one of the prime exigent environmental threats against sustainable food production [6–8]. About 33% of irrigated croplands are classified as salt-affected soil, which may exceed 50% by 2050 [9]. The undesirable impacts of excess salinity on crop development are possibly attributed to osmotic stress, cytotoxicity provoked by excess sodium (Na^+) and chloride (Cl^-), nutritional imbalances, decreased turgor, and leaf anatomical modifications [6,8,10,11]. Likewise, excess Na^+ evoked the excess generation of toxic reactive oxygen species (ROS) that may interrupt cellular functions and negatively affect metabolic processes. ROS generation usually impedes the redox homeostasis, resulting in loosening photosynthetic effectiveness [12], modifying nitrogen and osmolytes assimilation, and decreasing nutrient absorption, changing phytohormones profile and genes expression [13]. The studies by Farouk et al. [6], Farouk and Al-Huqail [8], and Kaur et al. [11] showed that excess salinity activates the antioxidant enzymes in plant tissues. In this regard, superoxide dismutase (SOD) accelerates the conversion of superoxide radicals (O^{-1}) to hydrogen peroxide (H_2O_2), while peroxidase (POD) and catalase (CAT) decompose H_2O_2 into water (H_2O) and O_2 [14,15]. Additionally, salinity induces necrosis of adult leaves, and increasing Na^+ influx and potassium (K^+) leakage leads to a superior Na^+/K^+ ratio in plant tissues [6,8,16]. In this regard, salinity normally disturbs the growth and yield of grapevine as well as induces physiological processes [5]. Additionally, Hatami and Pourakbar [17] found that irrigation grapevines with saline water (50 and 100 mM NaCl) significantly decreased shoot length, shoot fresh and dry weight, chlorophyll concentration, and potassium%, while increasing Na^+ and Cl^-. Crops possess multiple strategies to mitigate salinity injuries, including up-regulation of the antioxidant capacity, osmotic adjustment, and anatomical alteration [17–19].

There are various methods to minimize the destructive impacts of salinity on plants, i.e., scraping, flushing, and leaching to draw off the extra salt from the plant's rhizosphere [20], exploitation of different irrigation practices [21], and enhancement of plant salt tolerance [22]. Nevertheless, owing to their elevated cost and employment prerequisites, these approaches can be ineffective in alleviating the salinization threats. Consequently, developing novel techniques associated with the modulation of plants' own physiological and metabolic adaptive routes for combating the destructive effects of salinity could be decisive for cultivating salt-affected soil or utilizing saline water for irrigation. In this regard, eco-friendly phytohormones occupy energetic functions in regulating numerous biochemical pathways and enhancing plants' stress tolerance [23,24]. Amongst phytohormones, brassinosteroids (BRs) are ubiquitous steroid plant growth substances that occupy prominent functions in various biochemical pathways leading to accelerating plant stress responses [25,26]. BRs regulated stress response owing to a complex progression of biochemical reactions such as activation or repression of key enzymatic reactions, stimulation of protein assimilation, and the assembly of diverse chemical defense materials [23,26–28].

Additionally, BRs application regulates the ROS metabolism and the rise in the antioxidant enzyme activity, plus a superior concentration of ascorbic acid, carotenoids, etc. [26,29]. Moreover, Ali et al. [30] established that BRs also modified the plasma membrane, improved ion absorption, and facilitated the translocation of photosynthesis to the sink, in addition to enhancing metabolic activities within stress environment. Additionally, exogenous application of BRs under salinity conditions, maintained cell organs ultrastructure including nucleus and chloroplast [31]. There are some reports designating that BRs application mitigates the harmful effects of salinity on several crops [32–34]. All of these outcomes designated the magnitude of BRs in defense within stress-induced injury without bad effects on human health [35]. BRs are commonly classified into three groups depending on the number of carbon atoms in their structure, i.e., C27, C28, and C29 [36]. Vardhini et al. [37] stated that brassinolide (BL), 28-homobrassinolide (28-HomoBL) and 24-epibrassinolide (24-EpiBL) are the three bioactive BRs being extensively utilized in agriculture.

Although recent reports have shown that BL will possibly lessen salinity toxicity [26,28], the mechanisms of BL on inducing grapevine salt tolerance are still far from being implicit. Therefore, the experiment aimed to evaluate the role of BL spraying on the growth, several physio-anatomical trials of grapevine seedlings under salinity. We anticipate that the data acquired from the current study will present a reliable hypothetical basis for the expansion of Thompson seedless grapevine (H4 strain) production in the regions that irrigated with salinized water up-to 3000 mg L^{-1}.

2. Materials and Methods

The current experiments were conducted throughout 2019 and 2020 seasons at EL-Baramon experimental farm (31.1195° N, 31.4487° E), Horticulture Research Institute, Mansoura, Egypt, to evaluate the nullifying effect of BL on salt-affected Thompson seedless grapevines (H4 strain) seedlings. The experimental site is distinguished as the arid environment with cool and low rainfall winter and hot dry summer. Average monthly temperature and relative humidity within experimental periods are available in Figure 1.

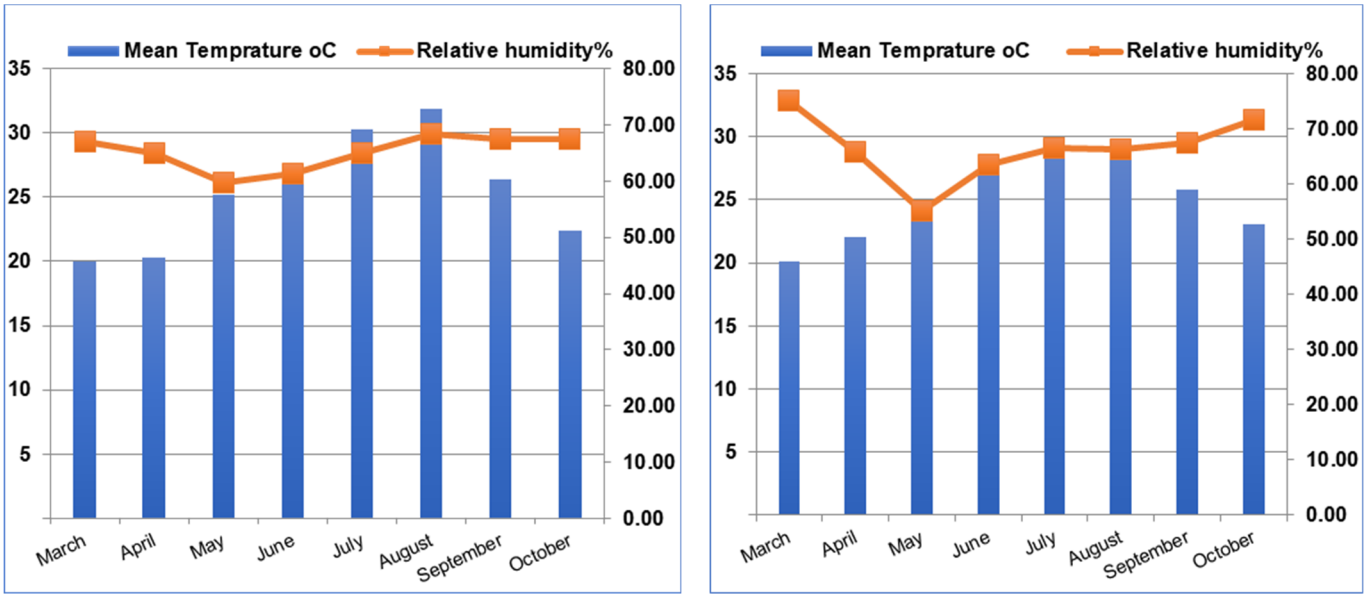

Figure 1. Average temperature and relative humidity of experimental site throughout 2019 and 2020 seasons.

2.1. Experimental Treatments and Design

An open field experiment was conducted in a completely randomized design with three replicates (each replicate included ten plants; in total, the experiment contained 300 plastic bags). In total, there were 10 treatments as indicated in Table 1.

Table 1. Experimental treatments and their abbreviation.

Code	Treatment
T1	Control, irrigated with tap water, 0 salinity (NaCl) without BL application
T2	Irrigated with saline water (1000 mg L^{-1} NaCl) without BL application
T3	Irrigated with saline water (1000 mg L^{-1} NaCl) plus 1 mg L^{-1} BL foliar application
T4	Irrigated with saline water (1000 mg L^{-1} NaCl) plus 2 mg L^{-1} BL foliar application
T5	Irrigated with saline water (2000 mg L^{-1} NaCl) without BL application
T6	Irrigated with saline water (2000 mg L^{-1} NaCl) plus 1 mg L^{-1} BL foliar application
T7	Irrigated with saline water (2000 mg L^{-1} NaCl) plus 2 mg L^{-1} BL foliar application
T8	Irrigated with saline water (3000 mg L^{-1} NaCl) without BL application
T9	Irrigated with saline water (3000 mg L^{-1} NaCl) plus 1 mg L^{-1} BL foliar application
T10	Irrigated with saline water (3000 mg L^{-1} NaCl) plus 2 mg L^{-1} BL foliar application

The concentration used was selected upon the pilot study utilizing 1000, 2000, 3000, 4000, 5000 mg L^{-1} irrigation water for 20 days, and wilting was observed at 4000 and 5000 mg L^{-1}; conversely, there was no visible wilting under 1000, 2000, and 3000 mg L^{-1}. The proper BL concentrations were designated based on earlier investigation [33].

The uniform and healthy cuttings of Thompson seedless grapevine (H4 strain) were taken from one-year-old matured canes (5 years old, grown in the vineyard at EL-Baramon experimental farm, Horticulture Research Institute, Mansoura, Dakahlia Governorate, Egypt). The cuttings were planted on 1st March in bottom holes polyethylene bags (17 × 30 cm) containing 5 kg clay soil (sand 27.16%, silt 24.69%, clay 48.15%, cation exchange capacity 36.5 Cmolc kg^{-1}, pH, 7.8, electric conductivity 0.62 mmose cm^{-1}, organic matter 2.1%, nitrogen 38 mg kg^{-1} soil, phosphorous 11 mg kg^{-1} soil, potassium 282 mg kg^{-1}, calcium 1.88%). The cuttings were irrigated with tap water two times each week for two months in both seasons. After that, the successive seedlings were irrigated using tap water and/or NaCl saline solution (1000, 2000, and 3000 mg L^{-1}) from 1 May till the end of September during the two growing seasons (twice a week with 2 L, in the morning for each irrigation). BL (as a commercial product named © Blank, European group for agricultural development, Alexandria, Egypt, active ingredient, BL 1%, phosphor 20%, and nitrogen 8%) with Tween 20 (0.05%) was sprayed at a rate (1 and 2 mg L^{-1}) on Thompson seedless grape seedlings three times (60, 90 and 120 days from planting). All plastic bags were irrigated with tap water monthly to prevent salinity accumulation. Each plastic bag was given nitrogen (N) in 3 g of ammonium sulfate (20.5% N), phosphorus (P) in 2 g of calcium superphosphate (15.4% P_2O_5), and potassium (K) in 1 g of potassium sulfate (48% K_2O) each month.

2.2. Analyses of Plant Samples

The plant samples were collected after 15 days from the last BL spraying (135 days from planting) for morpho-anatomical and biochemical determinations.

2.3. Ion Determination

For ion estimation, oven-dried plant samples (0.1 g) were entirely digested with H_2SO_4 (98%, 5 mL), at 200 °C, supplemented with a few drops of H_2O_2 (30%, *v*/*v*). Once digestion was completed, the sample was brought up to 25 mL with distilled-deionized water. P, N, K, Na^+ were measured as described in Cooper [38], and Motsara and Roy [39], by micro-Kjeldahl technique (N), ammonium molybdate and ascorbic acid protocol (P), and flame photometer (K^+ and Na^+), and then the K^+/Na^+ ratio was calculated.

2.4. The Photosynthetic Pigments

The concentration of chlorophylls and carotenoids was determined following Lichtenthaler and Wellburn [40] protocol, using methanol, and expressed as mg g^{-1} fresh weight.

2.5. Leaf Relative Water Content (LRWC)

The LRWC was estimated by Shams et al. [22] protocol. Leaf pieces (10 mm) were directly weighed for fresh mass (FM) assessment. Afterward, pieces were floated in bi-distilled water at lab. temperature for 24 h to assess the turgor mass (TM). Lastly, leaf pieces were oven-dried at 70 °C for 48 h then recorded as the dry mass (DM). LRWC (%) was designed by the subsequent equation:

$$\text{LRWC (\%)} = \frac{\text{FM} - \text{DM}}{\text{TM} - \text{DM}} \times 100.$$

2.6. Membrane Permeability (MP)

The leaf pieces were rinsed in bi-distilled water to eliminate surface-adhered electrolytes, and afterward they were put in Petri dishes containing deionized water (25 mL) at the lab temperature for 3 h. Electrical conductivity (EC1) in the bath solution was recorded. Subsequently, the leaf pieces were killed by boiling for 60 min, and the conductivity of the bath solution was recorded again (EC2), then calculating MP% following this equation [41],

$$\text{MP\%} = \frac{\text{EC1}}{\text{EC2}} \times 100$$

2.7. Proline Estimation

Proline concentrations (mg g FW^{-1}) were assessed spectrophotometrically following the procedure of Bates et al. [42] using ninhydrin reagent and standard curve.

2.8. Antioxidant Enzymes and Phenols Concentration

Fresh leaf samples were homogenized with 50 mM sodium phosphate buffer (pH 7.8) including 0.2 mM EDTA and 2% insoluble polyvinylpyrrolidone in a cooled mortar and pestle, then centrifuged at 12,000× *g* for 20 min, the supernatant was utilized in enzymatic activities assessment. Peroxidase (POD, EC 1.11.1.7) activity was measured by the increase in absorbance at 470 nm owing to guaiacol oxidation [43]. Polyphenol oxidase (PPO, EC 1.10.3.1) activity was determined according to Augustin et al. [44]. Catalase (CAT, EC 1.11.1.6) activity was deliberate as the decrease in absorbance at 240 nm following the technique of Tian et al. [45].

For phenols (mg equivalents of gallic acid g^{-1} dry weight) determination, 0.5 g oven-dried leaf samples were extracted with 80% ethanol. An aliquot of plant extract was mixed with 1 N Folin–Ciocalteu reagent and Na_2CO_3 and then incubated for 60 min at the lab. temperature, subsequently the absorbance was recorded at 765 nm [46].

2.9. Anatomical Study

Specimens (5 × 5 mm) from the 5th upper leaf including the main midvein were taken in the 2nd year. The specimens were put in formalin aceto alcohol for 48 h, afterward washed and dehydrated in ethanol succession, and embedded in paraffin wax (52–54 °C melting points). Cross-sections were prepared at 12–15 µm by a rotary microtome, stained in toluidine blue, cleared in toluene, and then mounted in Canada balsam. The randomly selected slides were examined with a light microscope (Olympus CX41, Philippines, Tokyo, Japan) outfitted with a digital camera (TUCSEN, USB2, H serial) to visualize the microscopic images.

2.10. Transmission Electron Microscopy (TEM)

Selected leaf blade samples (5 mm^2) (control 'T1′, severe salinity 'T8′, severe salinity with 2 mg L^{-1} BL 'T10′) were double fixed immediately in cold glutaraldehyde (2.5%) and

postfixed in osmium tetroxide (1%) for 3 hr. The samples were then dehydrated in a graded alcohol series and embedded in Spurr's resin. The ultrathin sections (50–100 μM) were performed by a Reichert ultramicrotome (Germany). Ultrathin sections were mounted on copper grids (400 mishes), and double-stained for 10 min., with uranyl acetate and Reynolds' lead citrate for 15 min each. Ten stained sections were examined and photographed by using a JEOL 100s transmission electron microscope (Electron Microscope Unit, Mansoura University, Mansoura, Egypt).

2.11. Growth Parameters

Seedling survival percentage, plant height (cm), stem thickness (mm), leaves number plant^{-1}, and mean of leaf surface area (cm^2) of the growing shoot were deliberated using Leaf Area Meter, AM 300 (ADC Bioscientific Ltd., Hoddesdon, UK). Shoot and root dry weights were recorded in g. The coefficient of wood ripening (CWR) was deliberate according to Rizk and Rizk [47]:

$$\text{CWR} = \frac{\text{length of the ripened part of the shoot}}{\text{total length of the shoot}}$$

2.12. Statistical Analysis

Homogeneity of error variance for all variables was determined before the analysis of variance (ANOVA). The outputs displayed that all data fulfilled the homogeneity required to achieve additional ANOVA tests. The data were statistically analyzed using COSTATC statistical package (CoHort software, 2006; Cary, NC, USA). A one-way ANOVA was achieved to examine the impacts of salinity and BL on grapevine plant growth and physiological parameters. Means were separated using Tukey's honestly significant difference (HSD) test at the $p < 0.05$ level of significance, and significant differences were indicated by different letters. Data existed as means ± standard error (SE) of five independent biological samples.

3. Results

3.1. Mineral Nutrient Concentration

Table 2 shows that irrigation with saline water from 0 to 3000 mg L^{-1} provoked a depressing impact on ion percentage except sodium, which was increased with salinity. Specifically, N% was decreased from 2.53% and 2.51% to 1.14% and 1.11%, P% was decreased from 0.364% and 0.362% to 0.209% and 0.201%, and K^+% was also decreased from 0.94% and 0.96% to 0.34% and 0.33% in the 1st and 2nd season, respectively, while Na^+% was increased from 0.29% and 0.31% to 0.94% and 0.96%, respectively, relative to untreated non-salinized plants. Under salinity, BL spraying displayed a greater impact on improving nutrient accumulation over non-treated salt-affected seedlings (Table 2). The Table also indicates that 2 mg L^{-1} BL was more effective than 1 mg L^{-1} BL in increasing ion percentage (N, P, and K) and decreasing Na^+%. The K^+/Na^+ ratio significantly decreased with salinity (Table 2). Conversely, BL spraying improved the K^+/Na^+ ratio in leaves, especially at 2 mg L^{-1} above non-treated plants under such salinity levels.

3.2. Photosynthetic Pigments

Relative to control, the concentration of chlorophyll a declined by 32.11% and 22.91%, 44.95% and 41.98%, and 50.45% and 48.47% under T1, T5, and T8 alone, in the first and second seasons, respectively (Table 3). Likewise, concentrations of chlorophyll b declined by 33.33% and 29.17%, 46.25% and 43.45%, and 51.70% and 51.19%, respectively (Table 3). Accordingly, the total chlorophyll concentration decreased by 32.87% and 25.52%, 45.47% and 42.69%, and 50.95 and 49.41%, while carotenoid concentration decreased by 27.02% and 20.93%, 34.05% and 35.81%, and 23.78% and 40.46% (Table 3) under T1, T5, and T8, respectively. Spraying salt-affected plants with BL concentrations drastically ($p < 0.05$) enhanced the leaves' chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids relative to untreated plants under such salinity levels. The concentration of 2 mg L^{-1} BL was more

effective than 1 mg L^{-1} on increasing the concentrations of photosynthetic pigments under salinity (Table 3).

Table 2. Ions percentage and potassium/sodium ratio of grapevine seedlings as affected by salinity (NaCl) and brassinolide (BL) during both growing seasons.

Treatments	Nitrogen%		Phosphorous%		Potassium%		Sodium%		Potassium/Sodium Ratio	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
T1	2.53 ± 0.03 a	2.51 ± 0.02 a	0.364 ± 0.002 a	0.362 ± 0.002 a	0.94 ± 0.01 a	0.96 ± 0.01 a	0.29 ± 0.01 f	0.31 ± 0.01 e	3.25 ± 0.11 a	3.11 ± 0.21 a
T2	1.61 ± 0.03 d,e	1.60 ± 0.02 d,e	0.285 ± 0.003 d	0.280 ± 0.002 d	0.63 ± 0.01 c	0.62 ± 0.01 c,d	0.64 ± 0.01 d	0.66 ± 0.01 c	0.98 ± 0.03 c	0.93 ± 0.01 d
T3	1.90 ± 0.03 c	1.92 ± 0.02 c	0.301 ± 0.002 c	0.305 ± 0.001 c	0.88 ± 0.01 a	0.89 ± 0.01 a	0.57 ± 0.01 e	0.65 ± 0.02 c	1.54 ± 0.02 b	1.37 ± 0.08 b,c
T4	2.14 ± 0.03 b	2.15 ± 0.01 b	0.324 ± 0.002 b	0.331 ± 0.001 b	0.89 ± 0.02 a	0.89 ± 0.01 a	0.54 ± 0.01 e	0.55 ± 0.01 d	1.65 ± 0.06 b	1.61 ± 0.03 b
T5	1.49 ± 0.02 e	1.52 ± 0.03 e	0.227 ± 0.001 g	0.225 ± 0.001 g	0.49 ± 0.01 d	0.47 ± 0.02 e	0.87 ± 0.01 b	0.89 ± 0.01 a	0.56 ± 0.02 d	0.52 ± 0.01 e,f
T6	1.80 ± 0.03 c	1.82 ± 0.02 c	0.243 ± 0.002 f	0.249 ± 0.001 e,f	0.67 ± 0.01 b,c	0.65 ± 0.02 b,c	0.78 ± 0.01 c	0.75 ± 0.02 b	0.85 ± 0.01 c	0.86 ± 0.02 d,e
T7	1.89 ± 0.01 c	1.91 ± 0.02 c	0.277 ± 0.002 d	0.281 ± 0.003 d	0.71 ± 0.01 b	0.72 ± 0.01 b	0.66 ± 0.01 d	0.67 ± 0.01 c	1.07 ± 0.01 c	1.07 ± 0.02 c,d
T8	1.14 ± 0.01 f	1.11 ± 0.01 f	0.209 ± 0.001 h	0.201 ± 0.002 h	0.34 ± 0.01 e	0.33 ± 0.01 f	0.94 ± 0.01 a	0.96 ± 0.01 a	0.36 ± 0.01 d	0.34 ± 0.01 f
T9	1.64 ± 0.02 d	1.70 ± 0.01 d	0.239 ± 0.001 f	0.241 ± 0.001 f	0.48 ± 0.01 d	0.56 ± 0.01 d	0.81 ± 0.01 b	0.76 ± 0.01 b	0.59 ± 0.02 d	0.73 ± 0.01 d,e
T10	1.84 ± 0.02 c	1.85 ± 0.02 c	0.259 ± 0.001 e	0.255 ± 0.002 e	0.69 ± 0.01 b,c	0.70 ± 0.02 b	0.79 ± 0.01 b,c	0.71 ± 0.01 b,c	0.87 ± 0.03 c	0.98 ± 0.02 d
p value	***	***	***	***	***	***	***	***	***	***

Data represent the average of five replicates ± standard error. Different letters in each column indicate significant ($p \leq 0.05$) differences at $p \leq 0.05$ according to Tukey's HSD range at $p \leq 0.05$. Levels of significance are represented *** $p < 0.001$. (T1, 0 NaCl without BL application; T2, 1000 mg L^{-1} NaCl without BL application; T3, 1000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T4, 1000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T5, 2000 mg L^{-1} NaCl without BL application; T6, 2000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T7, 2000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T8, 3000 mg L^{-1} NaCl without BL application; T9, 3000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T10, 3000 mg L^{-1} NaCl with 2 mg L^{-1} BL application).

3.3. Physiological Parameters

Physiological trials, i.e., leaf relative water content (LRWC), membrane permeability (MP), and proline concentration were statistically affected by salinity and BL application Figure 2a–c. Salinity levels (1000, 2000, and 3000 mg L^{-1}) caused a significant reduction of 6%, 8%, and 13% in LRWC in the 1st season and 5%, 8%, and 13% in the 2nd season, respectively, compared to the non-saline conditions (Figure 2a). BL application successfully alleviated this impact and improved the LRWC in stressed plants. Under salinity, BL application (1 and 2 mg L^{-1}) proficiently boosted the LRWC over the salt-affected plants with no application of BL. Relative to non-treated salt-affected plants, application of 2 mg L^{-1} BL significantly increased LRWC by 3% in both seasons under low salinity level, and by 4% under moderate salinity level in the second season, as well as by 4% and 11% under high salinity level (Figure 2a) in the 1st and 2nd seasons, respectively. The data presented in Figure 2b indicate that increasing salinity levels significantly increased MP% in plants relative to control seedlings. The maximum increase was recorded under high salinity level, reaching 138% and 150% above control plants in the 1st and 2nd seasons, respectively. BL spraying mitigates the harmful effect of salinity on MP%, relative to untreated plants under such salinity level, and the most effective concentration in this regard was 2 mg L^{-1} BL. The level of proline exhibited an increase of 80%, 143%, and 194% in the 1st season and 84%, 154%, and 199% in the 2nd season, respectively, in response to the salinity levels (1000, 2000, and 3000 mg L^{-1}) of grapevine seedlings, compared to control plants (Figure 2c). Conversely, the influence was more definite under salinity, where BL profes-

sionally decreased the liberate of proline by mitigating salinity. Under high salinity level, the application of 2 mg L^{-1} BL caused a 14% and 12% decrease in the 1st and 2nd season, respectively, whereas the application of 1 mg L^{-1} resulted in a 10% and 8% reduction in proline concentration in the second season, relative to untreated salt-affected plants.

Table 3. Photosynthetic pigment concentrations (mg g FW^{-1}) of grapevine leave as affected by salinity (NaCl) and brassinolide (BL) during both growing seasons.

Treatments	Chlorophyll a		Chlorophyll b		Total Chlorophyll		Carotenoids	
	2019	2020	2019	2020	2019	2020	2019	2020
T1	2.18 ± 0.18 [a]	2.62 ± 0.12 [a]	1.47 ± 0.19 [a]	1.68 ± 0.10 [a]	3.65 ± 0.37 [a]	4.31 ± 0.22 [a]	0.185 ± 0.01	0.215 ± 0.01 [a]
T2	1.47 ± 0.13 [a–c]	2.02 ± 0.18 [a–c]	0.98 ± 0.09 [a,b]	1.19 ± 0.13 [b–d]	2.45 ± 0.23 [a,b]	3.21 ± 0.30 [a–d]	0.135 ± 0.01	0.170 ± 0.01 [a–c]
T3	1.76 ± 0.19 [a–c]	2.36 ± 0.14 [a,b]	1.12 ± 0.10 [a,b]	1.42 ± 0.17 [a–c]	2.88 ± 0.29 [a,b]	3.78 ± 0.31 [a,b]	0.154 ± 0.01	0.192 ± 0.01 [a,b]
T4	1.88 ± 0.24 [a,b]	2.44 ± 0.09 [a,b]	1.24 ± 0.16 [a,b]	1.53 ± 0.09 [a,b]	3.12 ± 0.40 [a,b]	3.97 ± 0.19 [a,b]	0.164 ± 0.01	0.198 ± 0.01 [a,b]
T5	1.20 ± 0.16 [b,c]	1.52 ± 0.12 [c,d]	0.79 ± 0.09 [b]	0.95 ± 0.06 [c,d]	1.99 ± 0.26 [a,b]	2.47 ± 0.18 [c,d]	0.122 ± 0.01	0.138 ± 0.01 [c]
T6	1.40 ± 0.12 [a–c]	1.88 ± 0.11 [b–d]	0.94 ± 0.09 [a,b]	1.16 ± 0.07 [b–d]	2.34 ± 0.22 [a,b]	3.04 ± 0.18 [b–d]	0.134 ± 0.01	0.163 ± 0.01 [b,c]
T7	1.52 ± 0.10 [a–c]	2.07 ± 0.16 [a–c]	0.99 ± 0.07 [a,b]	1.23 ± 0.09 [a–d]	2.51 ± 0.18 [a,b]	3.31 ± 0.25 [a,c]	0.141 ± 0.01	0.176 ± 0.01 [a–c]
T8	1.08 ± 0.10 [c]	1.35 ± 0.11 [d]	0.71 ± 0.08 [b]	0.82 ± 0.09 [d]	1.79 ± 0.19 [a,b]	2.18 ± 0.21 [d]	0.141 ± 0.02	0.128 ± 0.01 [c]
T9	1.18 ± 0.10 [b,c]	1.47 ± 0.07 [c,d]	0.78 ± 0.07 [b]	0.91 ± 0.03 [d]	1.96 ± 0.17 [a,b]	2.39 ± 0.10 [c,d]	0.121 ± 0.01	0.140 ± 0.01 [c]
T10	1.26 ± 0.13 [b,c]	1.57 ± 0.06 [c,d]	0.84 ± 0.08 [b]	0.98 ± 0.03 [c,d]	2.11 ± 0.21 [a,b]	2.55 ± 0.10 [c,d]	0.127 ± 0.01	0.147 ± 0.01 [b,c]
p value	**	***	**	***	**	***	NS	***

Data represent the average of five replicates ± standard error. Different letters in each column indicate significant ($p \leq 0.05$) differences at $p \leq 0.05$ according to Tukey's HSD range at $p \leq 0.05$. Levels of significance are represented by ** $p < 0.01$ and *** $p < 0.001$; NS, nonsignificant. (T1, 0 NaCl without BL application; T2, 1000 mg L^{-1} NaCl without BL application; T3, 1000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T4, 1000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T5, 2000 mg L^{-1} NaCl without BL application; T6, 2000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T7, 2000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T8, 3000 mg L^{-1} NaCl without BL application; T9, 3000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T10, 3000 mg L^{-1} NaCl with 2 mg L^{-1} BL application).

3.4. Antioxidant Enzyme Activities and Phenol Concentration

Salinity was established to have a considerable impact on POD, PPO, and CAT activities, and phenol concentration (Figure 3a–d). The present data recognize that BL spraying lessened the depressing effects of salinity on antioxidant enzyme activities and phenol concentration in grapevine seedlings (Figure 3a–d). A considerable enhancement in the activity of POD was recorded in salt-affected plants (Figure 3a). The POD activity was considerably superior (66%, 58%, 137% in the first season and 66%, 89%, 114% in the second season) in salt treatment (1000, 2000, 3000 mg L^{-1}), respectively, relative to the control. The application of BL significantly lessened POD activity in salt-affected plants without BL application. Under high salinity level, BL applications resulted in a decline by 10% and 13% in the 1st season as well as 5% and 7% in the 2nd season in POD activity at 1 mg L^{-1} BL and 2 mg L^{-1} BL, respectively, compared to salt-affected seedlings only. The PPO activity was established to be drastically influenced by salinity and BL (Figure 3b). Salinity stress increased the PPO activity in both seasons compared to the control. The maximum activity was recorded under a high salinity level (3000 mg L^{-1}) that amplified the activity by 296% and 281% in the 1st and 2nd seasons, respectively, relative to non-salinized control seedlings. BL (1 and 2 mg L^{-1}) application drastically reduced PPO activity relative to untreated

stressed seedlings. Figure 3c revealed that CAT activity was drastically influenced by salinity and BL. High salinity level (3000 mg L^{-1}) resulted in a 77% and 52% enhancement compared with the control in the 1st and 2nd seasons, respectively. Application of BL at both rates was established to be efficient in decreasing the CAT activity under salinity. Under severe salinity, 1 mg L^{-1} BL lowered the CAT activity by 5% and 5%, while a 10% and 8% reduction was noted with 2 mg L^{-1} BL application in the 1st and 2nd seasons, respectively, above untreated severe salinity stressed seedlings.

The concentration of phenols in grapevine leaves was significantly ($p < 0.05$) affected by salinity (Figure 3d). Relative to control, increasing salinity level increased the concentration of phenol by 66% and 61%, 107% and 97%, 137% and 122% in T2, T5, and T8 treatments in the 1st and 2nd season, respectively (Figure 3d). Under different saline conditions, the application of BL mitigated the adverse effect of saline stress on the concentration of phenol.

3.5. Leaf Anatomy

Data in Table 4 and Figure 4a–j indicate the anatomical modification of grapevine leaf under salinity and/or BL application. The data clearly show that low salinity level have a stimulation influence on leaf structure that increased the thickness of midrib (TM), width of the midrib (WM), thickness of leaf blade (LB), thickness of the upper epidermis (UE), spongy parenchyma thickness (SP), lower epidermis thickness (LE), thickness of compound vascular bundle (TVB) and width of the compound vascular bundle (WVB) by 8%, 15%, 11%, 43%, 24%, 36%, 34%, and 29%, respectively, while palisade parenchyma thickness (PP) decreased by 19% as compared with non-salinized control plants. On the other hand, severe salinity levels decreased TM, WM, LB, UE, PP, SP, TVB, and WVB, by 14%, 5%, 9%, 6%, 27%, 2%, 6%, and 23%, but they increased LE by 21% relative to non-salinized control plants.

Table 4. Anatomical modification of grapevine leaves as affected by salinity (NaCl) and brassinolide (BL) during the 2nd season.

Treatments	Dimension of Midrib (µm)		Thickness of Leaf Blade (µm)	Upper Epidermis Thickness (µm)	Palisade Tissue Thickness (µm)	Spongy Tissue Thickness (µm)	Lower Epidermis Thickness (µm)	Dimension of Compound Vascular Bundle (µm)	
	Thickness	Width						Thickness	Width
T1	75.83 ± 1.11 ^{d}	61.57 ± 0.57 ^{d}	15.84 ± 0.20 ^{e,f}	1.62 ± 0.10 ^{b}	5.87 ± 0.09 ^{d,e}	6.53 ± 0.09 ^{d}	1.82 ± 0.09 ^{d}	45.04 ± 0.22 ^{d}	42.76 ± 0.57 ^{d}
T2	82.41 ± 0.90 ^{b,c}	70.88 ± 1.19 ^{b,c}	17.71 ± 0.21 ^{e}	2.32 ± 0.09 ^{a}	4.75 ± 0.21 ^{e,f}	8.14 ± 0.24 ^{b,c}	2.48 ± 0.09 ^{a,b}	60.58 ± 1.00 ^{a,b}	55.24 ± 1.22 ^{b,c}
T3	85.88 ± 0.65 ^{a,b}	75.48 ± 0.79 ^{b}	30.75 ± 0.35 ^{b}	1.62 ± 0.1 ^{b}	19.42 ± 0.32 ^{a}	7.48 ± 0.12 $^{b–d}$	2.22 ± 0.09 $^{b–d}$	60.88 ± 0.91 ^{a,b}	58.60 ± 0.14 ^{a,b}
T4	89.34 ± 1.63 ^{a}	76.47 ± 0.79 ^{b}	33.76 ± 1.01 ^{a}	2.214 ± 0.25 ^{a}	19.35 ± 0.52 ^{a}	9.91 ± 0.32 ^{a}	2.27 ± 0.11 ^{b,c}	66.82 ± 1.01 ^{a}	57.81 ± 0.91 ^{a,b}
T5	75.48 ± 0.79 ^{d}	62.12 ± 0.85 ^{c,d}	21.64 ± 0.40 ^{d}	2.43 ± 0.06 ^{a}	6.45 ± 0.05 ^{d}	9.93 ± 0.32 ^{a}	2.83 ± 0.09 ^{a}	53.70 ± 2.45 ^{c}	47.27 ± 0.85 ^{c,d}
T6	68.80 ± 0.79 ^{e}	79.59 ± 0.91 ^{a,b}	27.21 ± 0.79 ^{c}	1.42 ± 0.06 ^{b}	16.13 ± 0.37 ^{b}	7.28 ± 0.38 ^{c,d}	2.38 ± 0.06 ^{b}	46.03 ± 0.91 ^{d}	55.24 ± 0.65 ^{b,c}
T7	79.00 ± 1.51 ^{c,d}	71.87 ± 1.94 ^{b}	28.18 ± 0.91 ^{b,c}	1.62 ± 0.1 ^{b}	16.64 ± 0.50 ^{b}	7.64 ± 0.38 $^{b–d}$	2.27 ± 0.08 ^{b,c}	56.03 ± 1.84 ^{b,c}	53.06 ± 1.23 ^{b,c}
T8	65.09 ± 0.48 ^{e,f}	58.41 ± 0.70 ^{d}	14.37 ± 0.16 ^{f}	1.52 ± 0.11 ^{b}	4.25 ± 0.20 ^{f}	6.37 ± 0.21 ^{d}	2.22 ± 0.09 $^{b–d}$	41.97 ± 0.91 ^{d}	32.86 ± 0.57 ^{e}
T9	61.62 ± 0.65 ^{f}	56.18 ± 1.18 ^{d}	23.63 ± 0.12 ^{d}	1.45 ± 0.04 ^{b}	14.04 ± 0.12 ^{c}	6.26 ± 0.09 ^{d}	1.87 ± 0.06 ^{c,d}	43.56 ± 0.70 ^{d}	41.33 ± 0.95 ^{d,e}
T10	82.66 ± 2.47 ^{b,c}	87.36 ± 5.26 ^{a}	20.98 ± 0.63 ^{d}	2.53 ± 0.11 ^{a}	6.76 ± 0.26 ^{d}	8.85 ± 0.52 ^{a,b}	2.83 ± 0.09 ^{a}	58.16 ± 2.56 ^{b,c}	67.56 ± 5.89 ^{a}
p value	***	***	***	***	***	***	***	***	***

Data represent the average of five replicates ± standard error. Different letters in each column indicate significant ($p \leq 0.05$) differences at $p \leq 0.05$ according to Tukey's HSD range at $p \leq 0.05$. Levels of significance are represented *** $p < 0.001$. (T1, 0 NaCl without BL application; T2, 1000 mg L^{-1} NaCl without BL application; T3, 1000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T4, 1000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T5, 2000 mg L^{-1} NaCl without BL application; T6, 2000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T7, 2000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T8, 3000 mg L^{-1} NaCl without BL application; T9, 3000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T10, 3000 mg L^{-1} NaCl with 2 mg L^{-1} BL application).

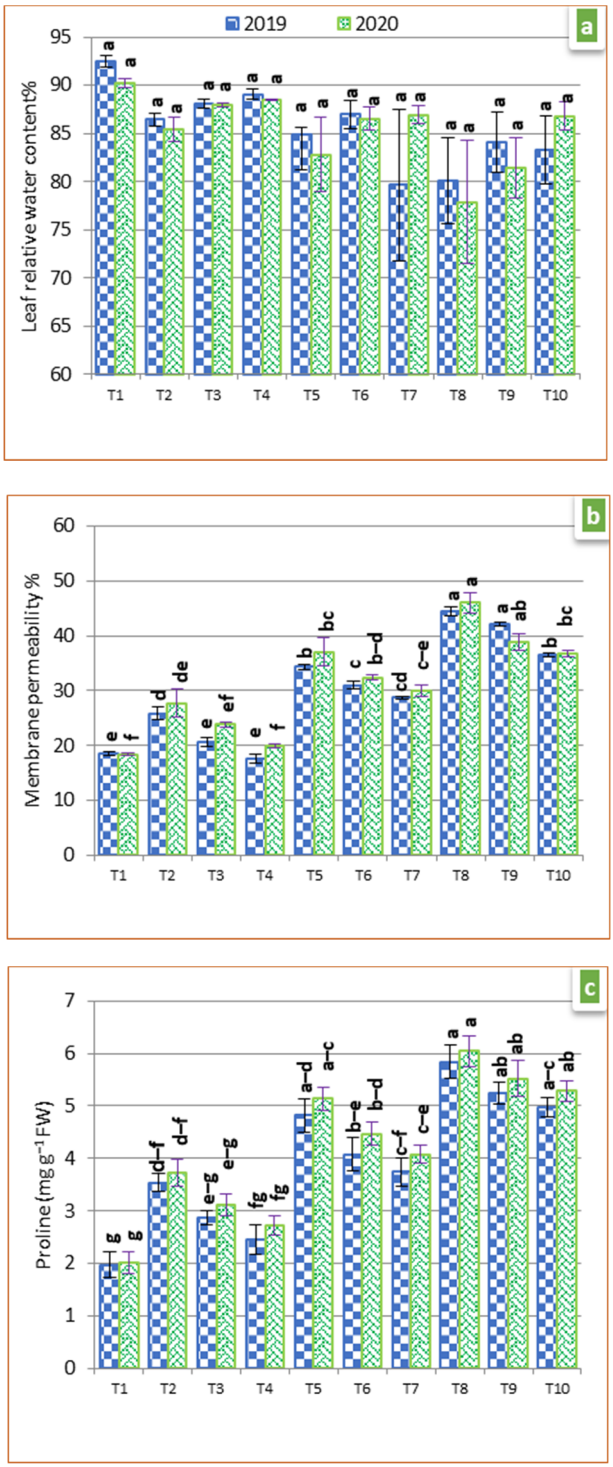

Figure 2. Leaf relative water content (**a**), membrane permeability percentage (**b**), and proline concentration (**c**) of grapevine seedling as affected by salinity (NaCl) and brassinolide (BL) during both growing seasons. Data represent the average of five replicates ± standard error. Different letters in each column indicate significant ($p \leq 0.05$) differences at $p \leq 0.05$ according to Tukey's HSD range at $p \leq 0.05$. (T1, 0 NaCl without BL application; T2, 1000 mg L^{-1} NaCl without BL application; T3, 1000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T4, 1000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T5, 2000 mg L^{-1} NaCl without BL application; T6, 2000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T7, 2000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T8, 3000 mg L^{-1} NaCl without BL application; T9, 3000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T10, 3000 mg L^{-1} NaCl with 2 mg L^{-1} BL application).

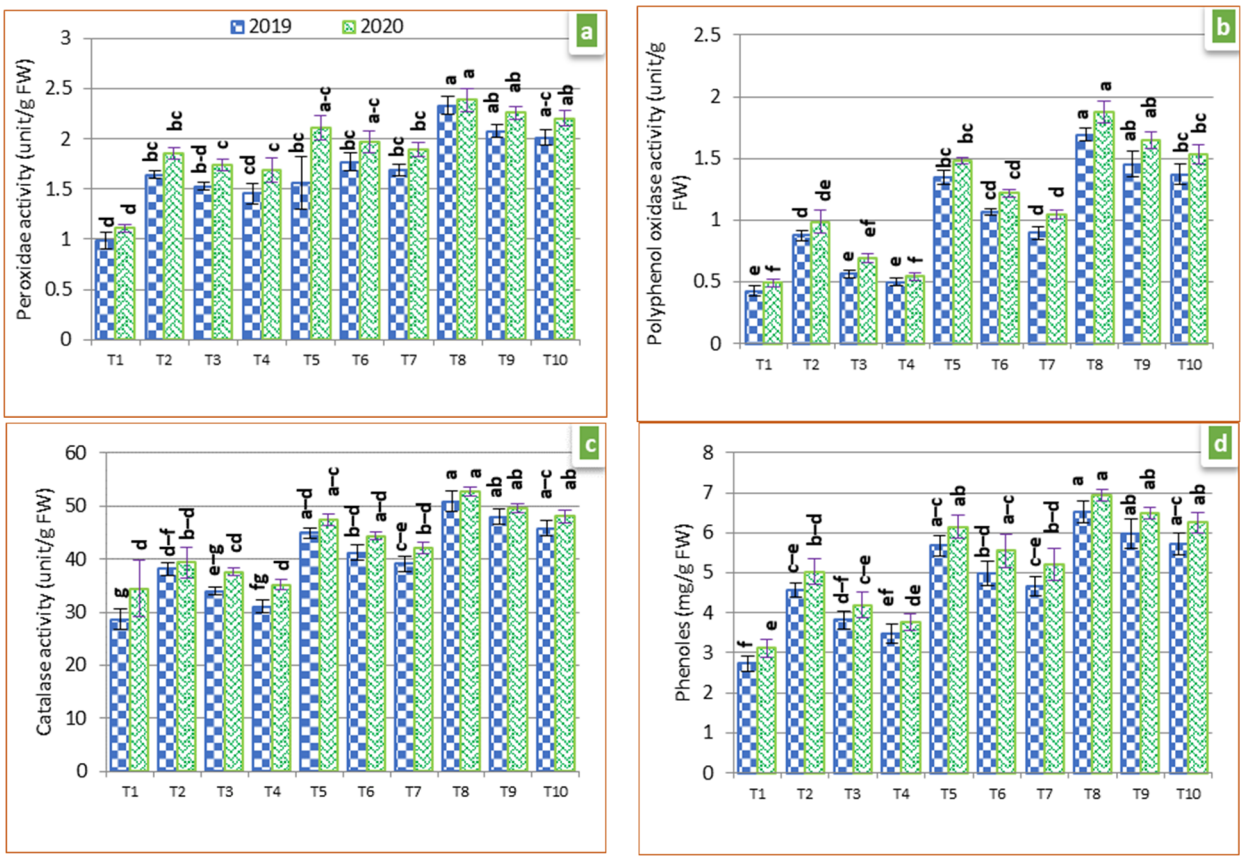

Figure 3. Antioxidant enzyme activities (**a**–**c**) and phenol concentration (**d**) of grapevine leave as affected by salinity (NaCl) and brassinolide (BL) during both growing seasons. Data represent the average of five replicates ± standard error. Different letters in each column indicate significant ($p \leq 0.05$) differences at $p \leq 0.05$ according to Tukey's HSD range at $p \leq 0.05$. (FW, Fresh weight; mg, milligram; T1, 0 NaCl without BL application; T2, 1000 mg L^{-1} NaCl without BL application; T3, 1000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T4, 1000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T5, 2000 mg L^{-1} NaCl without BL application; T6, 2000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T7, 2000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T8, 3000 mg L^{-1} NaCl without BL application; T9, 3000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T10, 3000 mg L^{-1} NaCl with 2 mg L^{-1} BL application).

Foliar application of salt-affected grapevine seedlings by BL levels in special 2 mg L^{-1} enhanced the anatomical feature of the leaves over the control plants. Under low salinity level, the application of 1 mg L^{-1} BL increased TM, WM, LB, PP, SP, LE, TVB, and WVB by 13%, 22%, 94%, 230%, 14%, 21%, 48%, and 37%, respectively, above non-salinized control plants, while spraying 2 mg L^{-1} BL led to an increase of 17%, 24%, 113%, 36%, 229%, 51%, 24%, 48%, and 35%, respectively, relative to non-salinized control plants (Table 4). The same direction was recorded under moderate and severe salinity levels. In this regard, application of 2 mg L^{-1} BL under severe salinity significantly increased TM, MW, LB, UE, PP, SP, LE, TVB, and WVB, by 9%, 41%, 32%, 56%, 15%, 35%, 55%, 29%, and 57%, respectively, above control (Table 4).

3.6. Ultrastructural Characterization of Leaf Mesophyll Cells by TEM

The ultrastructural study demonstrated grapevine leaf mesophyll cells with a bordered cell wall, and unbroken cell membranes, having a granular cytoplasm with many organelles (Figure 5a–o). Salt-affected plant cells illustrated various visible ultrastructural modifications of the organelles and cellular injuries (Figure 5a–o), i.e., nucleus condensation, protoplasm deterioration, and lesser organelles.

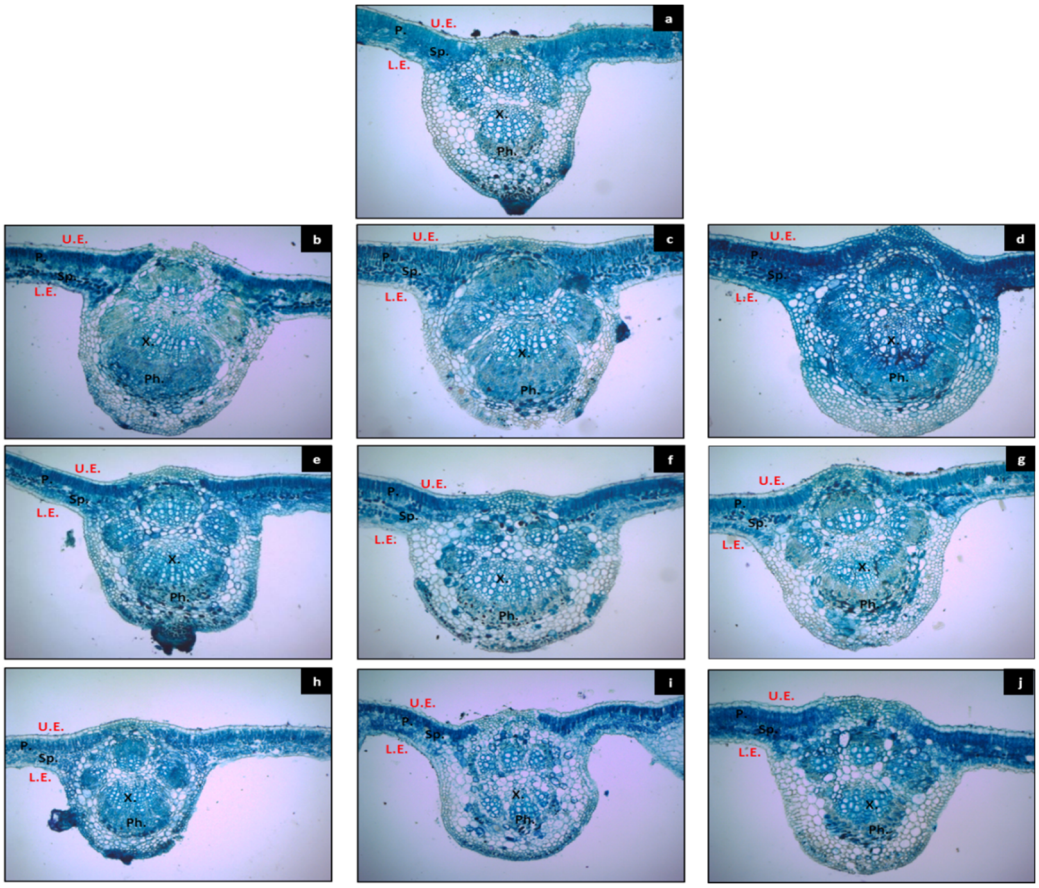

Figure 4. Microphotographs of cross-sections through the blade of leaves on the median portion of grapevine aged 135 days as affected by salinity (NaCl) and brassioloide (BL) (LE, Lower epidermis; P, Palisade parenchyma; SP, Spongy parenchyma; UE, Upper epidermis; X, xylem; Ph, phloem; (**a**), 0 NaCl without BL application; (**b**), 1000 mg L^{-1} NaCl without BL application; (**c**), 1000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; (**d**), 1000 mg L^{-1} NaCl with2 mg L^{-1} BL application; (**e**), 2000 mg L^{-1} NaCl without BL application; (**f**), 2000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; (**g**), 2000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; (**h**), 3000 mg L^{-1} NaCl without BL application; (**i**), 3000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; (**j**), 3000 mg L^{-1} NaCl with 2 mg L^{-1} BL application).

In T1, Figure 5d,e showed that in the control plant the cells having the distinctive chloroplast structure, an ellipsoidal form with well-arranged granum, compactly arranged thylakoid membranes, jointly with several starch grains (1–2 chloroplast^{-1}), without plastoglobules. Grana lamellae are completed thylakoids generally oriented parallel to the chloroplast's long axis. Under severe salinity, Figure 5h,i showed that salinity stress-induced clear alternations in chloroplasts, i.e., a decrease in the size of chloroplasts per cell, with the chloroplast becoming rounded and swelling of thylakoids,; Figure 5h,i also showed that the internal membranes were a disoriented lamellar system, a wavy configuration with starch grains was observed from 1–2 in T1 to 2–4 in T8, and the shape of starch grains were converted from the ellipsoidal shape in T1 to the rounded shape in T8; moreover, the number of plastoglobuli was increased. The chloroplasts became misshapen, grana stacking were less regular, and consequently, the thylakoids were loosened and imprecise (Figure 5i). Plants treated with BL demonstrated a distinctive chloroplast ultrastructure with no considerable alterations; the chloroplast was less than control (Figure 5n). BL application maintains the internal structure and grana staking, the number and size of starch grains, and fewer plastoglobule as compared to T8 (Figure 5n).

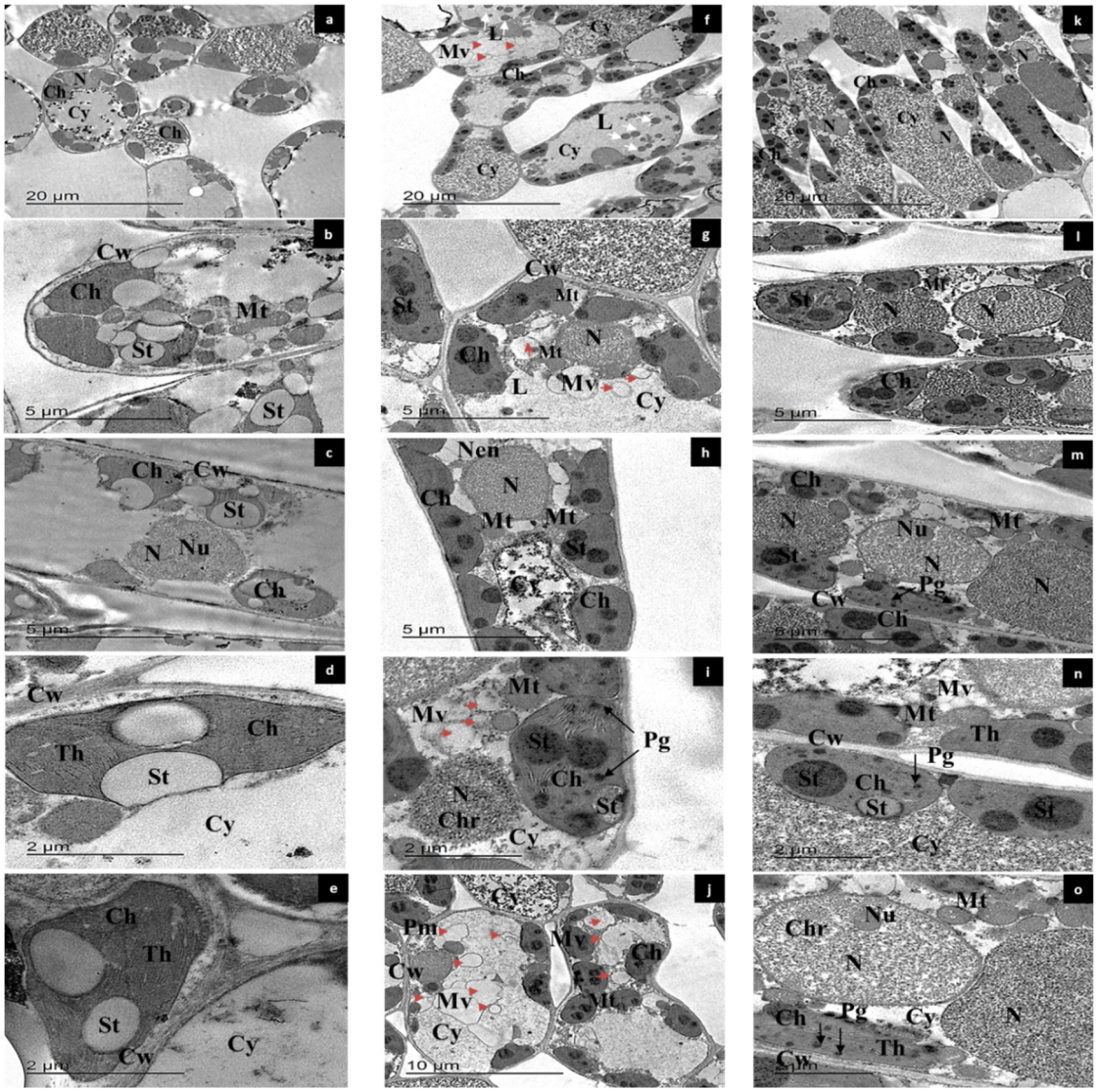

Figure 5. TEM micrograph of mesophyll cells of grapevine leaf, showing alternation in the ultrastructure of cell organelles including chloroplast, nucleus, mitochondria, and cell wall as well as the plasma membrane. (**a**–**e**) (control, T1); (**f**–**j**) (severe salinity, T8), (**k**–**o**) (severe salinity plus 2 mg L^{-1} BL foliar spraying, T10). An overview and details of control cells: (**d**,**e**) showing well-organized chloroplasts with smooth cell walls; (**c**) dispersion of nucleolus and chromatin in nucleus matrix and continuous nuclear envelope; (**b**) soft and thin cell wall with numerous mitochondria; (**f**,**j**) an overview of salt-affected cells indicating devastation of cell organelles, and a decrease in the size of chloroplast and accumulation of lipid droplets in the cytoplasm (white arrows) as well as an increase in the membrane vesicles (red arrows); (**g**) showing membrane vesicles (red arrows), and nucleus appeared without nucleolus; (**h**,**i**) swelling chloroplast with dilations of the thylakoid granum, and an increase in starch grains and plastogloubouline; (**k**) an overview of mesophyll cells in T10; (**i**,**m**,**o**) mesophyll cell having a well-organized nucleus, smooth nuclear envelop, mitochondria; (**n**), well-organized chloroplasts. (TEM, transmission electron microscopy; Ch, chloroplast; N, nucleus; Cy, cytoplasm; Cw, Cell wall; Mt, mitochondria; St, starch grain; Nu, nucleolus; Th, thylakoid; L, lipid droplets; Mv, membrane vesicles; Chr, chromatin; Pg, plastogloubouline; Nen, nuclear envelope; Pm, plasma membrane).

Under normal conditions, the nucleus appeared regular, with a distinctive nuclear envelope, nuclear chromatin, and nucleolus (Figure 5c). On the contrary, severe salinity induced a clear change in the nucleus; i.e., there was a decrease in the size and irregularity

in shape, the nuclear envelope was unclear in some cells, the nuclear chromatin was aggregated or condensed as well as the nucleolus vanishing or being absent (Figure 5g–i). However, in T10, spraying salt-affected plants with 2 mg L^{-1} BL nullifies the harmful effect of salinity on nucleus structure, and accordingly, the nucleus appears normal with nucleolus clear (Figure 5l,m,o).

In non-salinized control plants, mitochondria showed regularly with apparent double membranes with a typical distribution of cristae (Figure 5b). In T8 compared to T1, we observed a variation in the number, size, and shape of mitochondria which improved the number of mitochondria, and its size was reduced and the distribution of cristae was indistinct or abnormal (Figure 5g,h). In T10, BL spraying boosted the size and number of mitochondria as compared to T1 and T8 (Figure 5m,o) and appeared normal with a normal distribution of cristae (Figure 5n).

In T1, the cell wall was slim (Figure 5b), while in T8 it was thick (Figure 5g), while the plasma membrane appeared partially separated from the cell wall and not adjacent to it in some cells (Figure 5j). In addition, augmented the plasmolysis of plasma membranes that will increase the number of membrane vesicles (cytoplasmic vesiculation) and disintegration of tonoplast (Figure 5g,j). Additionally, there was an increase in the accumulation of lipid droplets in the cytoplasm (Figure 5f). In contrast, in T10, the treatment with BL led to maintaining the cell wall and plasma membrane structure, and the number of membrane vesicles was decreased as compared to salinity treatment alone (Figure 5n).

3.7. Plant Growth

Salinity levels (1000, 2000, and 3000 mg L^{-1} NaCl) drastically ($p \leq 0.05$) repressed all morphological attributes of grapevine seedlings. Conversely, when BL was sprayed, the undesirable impacts of salinity on morphological attributes were decreased (Figure 6a–h). The undesirable impacts of salinity increased gradually with increasing salinity levels. The severe salinity level (3000 mg L^{-1}) without BL application significantly ($p \leq 0.05$) reduced the survival percentage (by 39% and 40%), plant height (by 36% and 37%), stem thickness (by 36% and 36%), the number of leaves plant^{-1} (by 40% and 38%), leaf area plant^{-1} (by 39% and 38%), shoot DW (by 47% and 48%), root DW (by 39% and 40%), and coefficient of wood ripping (by 38% and 36%) in the 1st and 2nd season, respectively, when relative to the control (Figure 6a–h).

BL spraying at both rates (1 and 2 mg L^{-1}) with salinity resulted in improvement in all growth parameters compared to the untreated samples. The high level of BL (2 mg L^{-1}) was more effective than the low level (1 mg L^{-1}) for increasing morphological trials. Under high salinity level (3000 mg L^{-1}), spraying with 1 mg L^{-1} BL and 2 mg L^{-1} BL significantly increased the survival% (by 19% and 26%), plant height (by 7% and 9%), stem thickness (by 9 and 13%), number of leaves plant^{-1} (by 11% and 18%), leaf area plant^{-1} (by 10% and 17%), shoot DW (by 10% and 14%), root DW (by 10% and 15%), and coefficient of wood ripping (by 15% and 18%) in the first season, in addition to increase the survival% (by 22% and 28%); plant height (by 12% and 17%); stem thickness (by 12% and 12%); number of leaves plant^{-1} (by 13% and 17%); leaf surface area plant^{-1} (by 12% and 14%); shoot DW (by 13% and 19%); root DW (by 13% and 19%) and coefficient of wood ripping (by 12% and 14%), respectively, in the second season, when compared to the untreated treatments (Figure 6). Relative to control, all the treatments with BL provoked a considerable enhancement in plant growth and demonstrated the ability of BL to alleviate saline-related stress on plant growth.

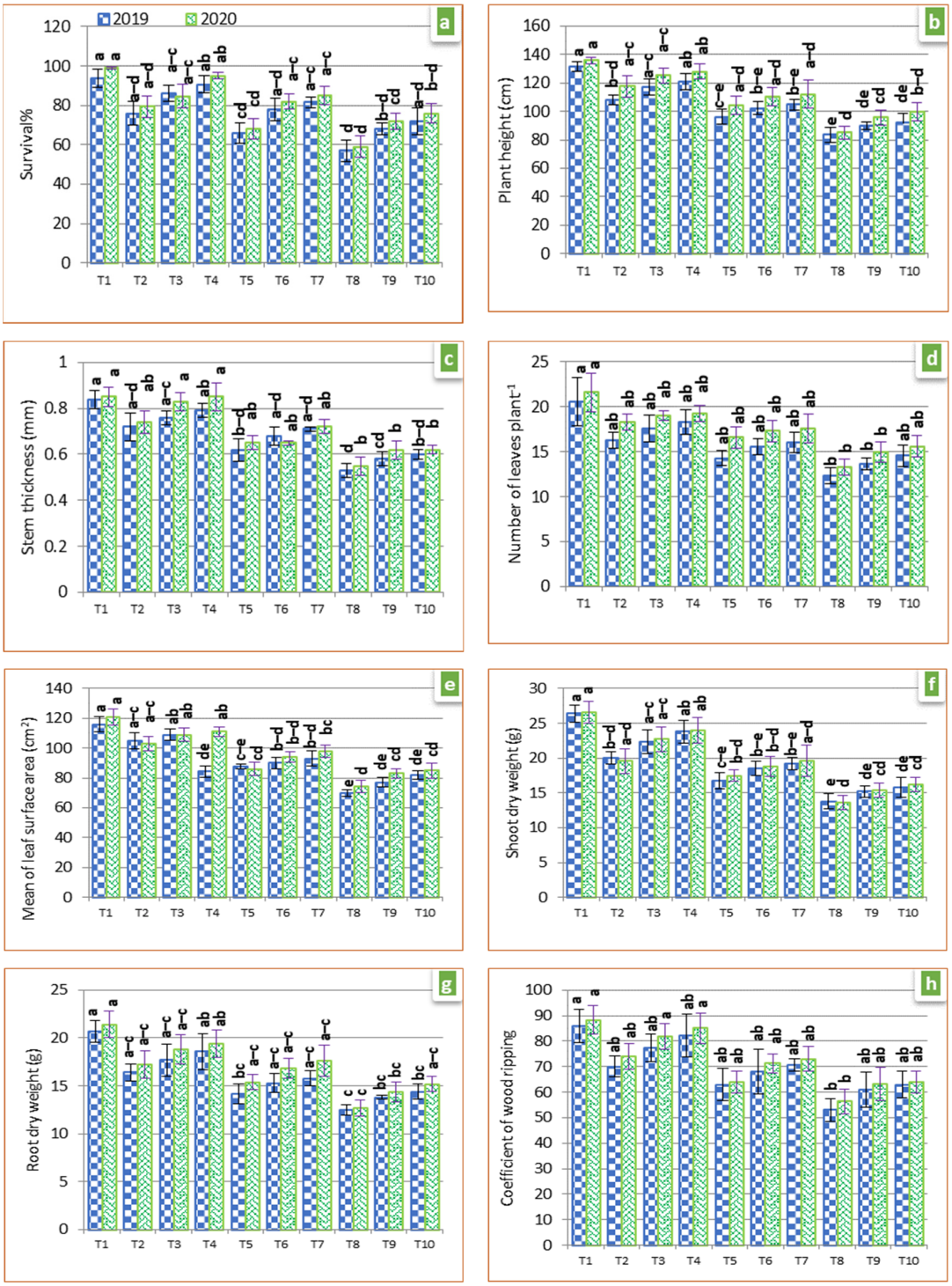

Figure 6. Plant growth trials (**a**–**h**) of grapevine seedling as affected by salinity (NaCl) and brassinolide (BL) during both growing seasons. Data represent the average of five replicates ± standard error. Different letters in each column indicate significant ($p \leq 0.05$) differences at $p \leq 0.05$ according to Tukey's HSD range at $p \leq 0.05$. (T1, 0 NaCl without BL application; T2, 1000 mg L^{-1} NaCl without BL application; T3, 1000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T4, 1000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T5, 2000 mg L^{-1} NaCl without BL application; T6, 2000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T7, 2000 mg L^{-1} NaCl with 2 mg L^{-1} BL application; T8, 3000 mg L^{-1} NaCl without BL application; T9, 3000 mg L^{-1} NaCl with 1 mg L^{-1} BL application; T10, 3000 mg L^{-1} NaCl with 2 mg L^{-1} BL application).

4. Discussion

Plants undergo salinity critically once they are grown in saline conditions. The drastic impact of salinity as recorded in the current investigation on plant growth was provided with earlier findings for numerous plants [6–8,11,16,17]. The overall decline under salinity might be caused by the negative impact of salinity on different metabolic processes and molecular alterations, i.e., photosynthesis, nutrient homeostasis, stomatal resistance, and ROS production in different plants [6,8,16]. In this concern, the decline in water absorption may result from decreasing hydraulic conductivity and the expression of aquaporins such as plasma membrane intrinsic protein (PIP) and tonoplast intrinsic protein (TIP) [48]. Salinity induced the buildup of salts inside the leaves that caused irreversible injury to the chloroplasts as recorded in the present experiment, resulting in metabolic restrictions of photosynthesis [49]. Additionally, the nutritional imbalance evoked the production of ROS that would induce oxidative stress and decreased cell division, proliferation, and elongation, and finally declined plant growth [14]. Moreover, growth reduction with salinity might partly be owing to the lack of energy, since processes occupied in salt uptake are energy-consuming [45]. The decline in growth-related parameters is possibly attributable to damaged cell development resulting from growth hormone efficiency, leading to a lessening in cell turgor, cell volume, and eventually cell growth and it may also be owing to the blocking up of conductive tissue vessels, thus blocking all translocation that passes throughout these tissues [50]. In the current study, the application of BL in special 2 mg L^{-1} considerably moderates the injury of salinity on plant growth. Comparable trends were recorded in different plant species [26,51]. However, the actual signaling mechanisms are largely unknown. The work with BL biosynthetic mutants in *Arabidopsis thaliana* [52] and *Pisum sativum* [53] have offered strong confirmation that BL signaling plays a vital for plant growth. Friedrichsen et al. [54] also stated that three redundant BL genes encode transcription factors that are necessary for typical growth, demonstrating the requirement of BRs for typical growth. BL controls the transcription of CycD3 (a D-type cyclin gene) throughout which cytokinin activates cell division, and BL mediated CycD3 induction has been recorded to control the de novo synthesis of proteins [55]. The motivating impact of BL on plant growth may result from its effect on physiological processes, including enhancing photoprotection and improving photosynthetic efficiency, improving antioxidant capacity and reducing ROS production, and improving mineral assimilation [56,57]. Accordingly, Anwar et al. [58,59] reported that BL application improved CAT, POD, and SOD activities in tomato and cucumber, respectively. Additionally, BL application improved photosynthetic efficiency by enhancing photochemical quenching coefficient, Rubisco enzyme activity, and over-expression of the large and small subunit genes with increasing CO_2 assimilation rate [27,60]. BL up-regulates water uptake and preservation of plant water potential, which leads to improving RWC as reported in the present study and earlier report [61], and/or reduced Na^+ accumulation and improved K^+ uptake resulted in the avoidance of osmotic and ionic upset to the plant [62]. Additionally, BL accelerates cell division and expansion in the apical meristem, which leads to improving leaf expansion [63].

Nutrient concentrations of grapevine seedlings except Na^+ drastically decreased under salinity; however, BL application mitigated salinity injuries via dropping Na^+ and increased N, P and K (Table 2). The outcomes were compatible with El-Taher et al. [7], Sarwar et al. [10], Hatami and Pourakbar [17] for salinity, Kolomeichuk et al. [27], and Karlidag et al. [64] for BL. Accordingly, Miao et al. [65] proved that BRs application improved root nodulation capacity and nitrogenase activity, resulting in increasing N% in plant tissues. It has been shown that severe salinity could confuse nutrient-ion activities, resulting in ionic imbalance, nutrients shortage, and specific ion toxicity [6,8,66]. Up-regulation of K^+ uptake with evading of Na^+ absorption, efflux of Na^+, and development for osmotic adjustment is an approach typically possessed by the plant for preserving an optimal K^+/Na^+ ratio that is an imperative decisive factor describing plant salinity tolerance. The competition between K^+ and Na^+ resulted in the aggressive uptake as the K^+ transporter lacks discrimination between K^+ and Na^+ ions [67,68]. Currently, salinity enlarged the

accumulation of Na^+, which is connected with lessened K^+ concentration, leading to a decline in K^+/Na^+ ratio. Earlier research has stated that under salinity, Na^+ accumulation is associated with the declined in K^+ [6,8,16,17,69–71]. This lessening is connected to the antagonistic routes, since Na^+ uptake by root cells takes place throughout non-selective K^+ channels and high-affinity K^+ transporters caused by physicochemical similarity among Na^+ and K^+ [72]. The preservation of ionic homeostasis under salinity is the requirement to defend the plants alongside the accumulation of lethal ions, with K^+ buildup and Na^+ realization the lowest concentration in grapevine seedlings. As a result, the organization of Na^+ accumulation and therefore a superior K^+/Na^+ ratio might maintain salinity tolerance [73]. Under salinity, BL application can revise the plasma membrane function and boost ion uptake [24]. The ability of BL to maintain plasma membrane structure may have been associated with the considerable reduction in Na^+ and the enhancement of K^+ ions [74]. Salinity tolerance accomplished by BRs amendment is possibly ascribed to its capability to enhance K^+ uptake and restrict Na^+ concentration into xylem, while sustaining an elevated K^+/Na^+ ratio in plant tissues [27,75]. This is possibly owing to the overexpression of salt overly sensitive 1 (SOS1, Na^+/H^+ antiporter), which shifts extra Na^+ outside the cytosol and assists preserve small cytosolic Na^+ levels in tissues, particularly in root epidermal cells and root tips [76]. SOS1 retrieves salinity tolerance mostly by facilitating Na^+ efflux from the cytosol to the rhizosphere [77] through (i) rising Na^+ storage time in vacuoles and dropping Na^+ accumulation in the cytoplasm, and (ii) controlling long-distance Na^+ transport throughout Na^+ repossession between roots and shoots. The elevated shoot K^+/Na^+ ratio might have been implicated in enhancing the plant development with BL application under salinized circumstances.

The current data indicated that grapevine photosynthetic pigments declined with salinity (Table 3). Alternatively, the application of BL causes a significant increase in the concentration of photosynthetic pigments. Similarly, Farouk et al. [6], El-Taher et al. [7], Farouk and Al-Huqail [8], Sarwar et al. [10], and Hatami and Pourakbar [17] indicated that salinity induced a considerable lessening in the chlorophyll level. The decline in chlorophyll under salinity was linked to the activation of chlorophyll degrading enzyme chlorophyllase and ROS production [45,78,79], restricted N absorption [80], and amplified susceptibility of pigment–protein complexes to deprivation [81], plus chloroplasts' ultrastructure [49]. Additionally, there is a reduction in chlorophyll biosynthesis intermediation levels [82] and the expression of ChlD, Chl H, and Chl I-1 genes encoding subunits of Mg-chelatase [83]. Under salinity, the over-production of ROS in cells induces oxidation and, therefore, the deprivation of photosynthetic pigments with the breakdown of the thylakoid membranes and changes in chlorophyll protein complexes [81,84]. However, the application of BL under salt stress restores imprecise chlorophyll accumulation caused by salinity (Table 3). These outcomes were compatible with former research [26,85]. This attenuating effect of BL can be reasoned from the possibility of BRs-induced impact on transcription and/or translation in the synthesis of pigments [26,86]. Additionally, BRs maintain thylakoid membrane, stability and regulate chlorophyll molecules by upregulating chlorophyllase activity. BRs regulate the protection scheme by controlling transcription of defense-related genes and alleviating the difficulty of diverse stresses and by regulating activated Rubisco genes [87]. Consistent with Deng et al. [88], BRs boost the activity of alternative oxidases (AOX) in a respiratory burst oxidase homolog (RBOH)-dependent way. So, a superior activity of AOX controls chloroplast and mitochondria's electron flow through dissipating the extra energy, thus lessening the ROS accretion and increasing the defense of photosynthetic apparatus. Additionally, as recorded in the current research, BL enhanced K^+ absorption, enlarged chloroplast number $cell^{-1}$, preserved chloroplast ultrastructure, or sustained chlorophyll stability by hastened ROS-mitigating activity. Furthermore, carotenoid assimilation was enhanced in grapevine under salinity upon BL spraying, probably by acting as an antioxidant, thus decreasing salinity-accelerated oxidative stress.

Sustaining crop water status-associated trials at an elevated level improves the metabolic pathways that are sustained by osmotic adjustment. Leaf RWC has been considered as a

substitute evaluation of plant water status, reflecting the plant's metabolic activity [45]. A reduction in LRWC under salinity has been previously recorded [6,10,71]. This decline can be ascribed to less water accessibility, or to defeat of the plant roots' aptitude to catch up on the water throughout a lessening of the absorbing surface [89]. On the other hand, the LRWC of BL-treated seedlings under salinity were preserved at high altitudes equivalent to salt-affected seedlings that did not receive BL, which consents with the outcomes of earlier [30,90]. This increase may result from the over-accumulation of osmolytes as proline [91] that will preservative tissue LRWC and rapid eradication of ROS [92]. Furthermore, BL application enhanced root development, reinforced water uptake, and controlled the expression of aquaporin-synthesizing genes [93]. This designates that BL application sustained cell membrane stability and sustained water status in salt-affected grapevine seedlings. The cell membrane represents the main cellular targets to diverse stresses. Salinity accelerates lipid peroxidation that was boosts the MP of grapevine seedlings. These results follow those recorded by Sarwar et al. [10], Abdelaal et al. [79], Dong et al. [94] in several crops. Salt stress evoked the over-production of ROS that consecutively aggravated the cell membrane damage and alternation of plasma membrane permeability [95]. The preservation of small MP% proved that the BRs-treated seedlings sustained plasma membrane integrity under salinity. The capability of the BL-treated plants to preserve plasma membrane integrity might connect to the valuable function of BL to either (i) alleviate the harmful effects of ROS, (ii) maintain membrane lipid and protein compositions, or (iii) decrease activities of lipid peroxidation and protein oxidation [57].

Proline represents the principally widespread defensive molecules within stressful conditions, i.e., salinity [7,10] as well as BL application [25,55,96]. In the present investigation, we recorded a speedy many-fold increase in the proline concentration in grapevine under salinity, with or without BL, that designated the key function of proline as a defensive substance under salinity conditions, probably to offer superior protection alongside salinity. These osmolytes, can control of the plant physio-biochemical pathways, i.e., sustaining membrane integrity, reducing the cellular water potential, facilitating continuous water uptake, preserving plant water status and cell turgidity maintaining the finest redox state, controlling salt-stress-responsive gene expression [97,98], and preserving plant water status [99]. Proline has also been recorded to participate in alleviating ROS's harmful impacts [100] and alleviating cytoplasmic pH [97]. Moreover, encouraging activation of proline assimilation in chloroplasts is a vital sink to ATP and NADPH, produced throughout the primary photosynthetic processes, thus encouraging the preservation of the electron flow among photosynthetic excitation centers, stabilization of redox equilibrium by maintaining NADPH/NADP hence preventing photoinhibition [27,101]. The hyper-assimilation of proline takes place chiefly through the motivated assimilation, the inhibition of proline oxidation, and the plant's capability to preserve the mitochondria membranes' permeability [102,103]. Several kinds of research have established that the over-expression of proline biosynthetic pathways genes Δ1-pyrroline-5-carboxylate synthetase (P5CS) exhibits improved tolerance to oxidative stress [104].

Salinity stress induces the repression of plant's metabolic pathways, including the hyper-accumulation of ROS that evoked oxidative burst [6,8,11]. Plant detoxification pathways involve the activation of antioxidant enzymes and the accumulation of antioxidant solutes [6,8,11]. The enzymatic antioxidant systems are composed of SOD, ascorbate peroxidase (APX), POD, and CAT that deactivate stress-provoked ROS production [16,79,105]. In the current study, NaCl and/or BL caused an increase in POD, PPO, and CAT activities of grapevine seedlings in both seasons. Antioxidant enzymes are part of proficient schemes for mitigating ROS and protecting plants from negative oxidative bursts [8]. Accordingly, Kaur et al. [11] also stated that salinity stress amplified antioxidant enzyme activity in chickpea genotypes. BL drastically eradicated ROS production via activation and strengthening of the antioxidant system, i.e., SOD and CAT, during salinity. Similarly, Lone et al. [26] and Arora et al. [106] found that BRs application increased antioxidant enzymes SOD, CAT, and POD.

Numerous phenolic compounds are stress-induced metabolites in plants [10,16]. It has been recorded that hyper-accumulation of phenols imparts superior radical scavenging activity so avoiding cellular oxidative rupture [91]. Soluble phenols provide an antioxidant since they have electron-donating mediators and, consequently, lessen extra ROS accumulation [107]. This production was probably provoked by eliciting the phenylpropanoid pathway and enhancing phenyl-aminolyase (PAL) gene expression [108]. In addition, a raise in PAL activity under salinity has been recorded by and is a key enzyme affecting the assimilation of plants' secondary metabolites [92].

Leaves are imperative places of essential biochemical pathways. El-Taher et al. [7], El-Banna and Abdelaal [109], and Nassar et al., [110] found that salinity decreased all leaf anatomical attributes including the thickness of the leaf blade and midvein of the mature leaf of strawberry, sweet basil, and cowpea plants, respectively. Ordinary, salt-affected plants were characterized by small cell size and declined in vascular tissue and cell wall thickness [111]. This reduction possibly resulted from the restriction of cell division and expansion plus a lessening in mesophyll parenchyma layer thickness as well as hampering procambial activity [112,113]. On the other hand, there are very few investigations related to the effect of BRs on plant anatomy. In this regard, Kulaeva et al. [114] recorded that the application of 24-EpiBL had a defensive role on cell ultra-structure in salt-affected leaves, which additionally prohibited nuclei and chloroplast deprivation, paving a way for better photosynthesis. Moreover, Ibrahim and Abo-ELwafa [115] on Thompson grape found that a high salinity level decreased the thickness of lamina and midvein of blades; the decline was more noticeable than that induced by a low salinity level, being 52.24% less than the control for thickness of lamina and 54.01% less than the control for thickness of midvein. Additionally, the thinner blade under 3000 ppm salinity could be attributed to the declines in thickness of palisade and spongy parenchyma and thickness of upper and lower epidermis by 54.62 and 56.44%, and 40.00 and 39.13%, respectively, compared with those of the control. The same authors revealed that vascular bundles of midvein displayed noticeable reduction in length by 61.29% and in width by 61.87% less than the control.

Studying the plant cell ultrastructural under salinity is possibly a practical implementation for understanding the deep strategies implicated in conferring salt tolerance. Salinity evoked the chief alterations in chloroplasts, i.e., swollen thylakoids, loose profiles of the piece of interior lamellae thylakoids, though mainly granal thylakoids were shattered. These outcomes were corroborated by Farouk and Arafa [49] and El-Banna and Abdelaal [109]. In this study, the deformations of grana stacking and swelling of thylakoids caused by salinity were possibly due to a modification in the ionic composition of the stroma. The degradation of the plastids is related to salt stress possibly provoked by ROS extra-accumulation causing oxidative anxiety [116]. The increase in the plastoglobule number evaluated in the current investigation is possibly a proper sign of ecological stress disorder [117,118]. The physical coupling among the plastoglobules and thylakoid membranes permits the free exchange of lipid molecules along with the plastoglobules and thylakoids [117]. The huge plastoglobule size and number recorded in the salt-affected plants are possibly one of the adaptive methods that avoid the oxidative injuries caused by high salinity. Conversely, boost the number of starch grains in the chloroplast (Figure 5h,i). Rahman et al. [119] reported that the raise of starch grains in chloroplast under salinity is owing to the injury of enzymes occupied in starch metabolism by alterations in ionic composition and/or the damage of the sucrose phosphate pathway biosynthesis in the cytosol leading to the triose phosphate pathway toward starch metabolism. Alternatively, BL lightened this structural injury by defending the chloroplasts from oxidative stress. Large chloroplasts with no swelling and only slight dilations of the thylakoids in BL and salt-affected plants are the existing signs of less oxidative anxiety. The relatively fewer number of plastoglobuli in chloroplasts of plants treated with salinity and BL alongside is another signal of smaller oxidative anxiety [120].

Within normal conditions, the mitochondria had well-organized cristae and an intact structure and were of similar size and appearance. Conversely, salt-affected cells had an

extremely small and the largest number of mitochondria with a defeat of the integrity of the outer mitochondrial membranes. Comparable outcomes have been a statement by Zhang et al. [120]. The injury elicited by salinity in mitochondria is probably a signal of salt-associated changes in mitochondria energy status resulting in decline ATP levels [121]. Nevertheless, BL spraying enhanced the dimensions and number of mitochondria under stressed circumstances. The superior mitochondrial number and size meet the increased needs of ATP under unfavorable circumstances when photosynthesis is commonly suppressed, and these organelles respond to stress by assimilation of different precise mitochondrial stress proteins [122].

The incidence of the membrane vesicles in the grapevine mesophyll cells is believed as an adaptive mechanism for sodium ions sequestration to ease their dangerous impact on cell organelles and cytoplasm [123,124]. Moreover, the accretion of lipid droplets in the cytoplasm may be considered as a preserve of energy to be used by the cell to cover the increased requirements in metabolic energy requisite to salinity tolerance and/or effect of ROS which fast the peroxidation of membrane lipids leading to loss membrane integrity [119].

5. Conclusions

Salinity declined grapevine seedling's growth, relative water content, and mineral concentrations. Conversely, with a spray of BL, the harmful impacts of salinity were mitigated. The possible strategies consist of the following: (1) BL could boost the concentration of chlorophyll and free proline; (2) BL could control activities of key antioxidant enzymes to eradicate ROS; (3) BL enhances cell membrane stability and nutrient uptake, as well as water status; (4) BL maintains the ultrastructure of cell organelles and leaf anatomy. Hence, BL could increase grapevine seedling growth under salinity, and the most favorable concentration appears to be 2 mg L^{-1} concentration. BL spraying could present an easy application in grapevine productivity in saline soil. However, additional research is required to decide the competence of these materials.

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