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Morphological, Biochemical, and Metabolomic Strategies of the Date Palm (*Phoenix dactylifera* L., cv. Deglet Nour) Roots Response to Salt Stress

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Abstract: Numerous Tunisian arid and semi-arid regions are subjected to soil salinity. Thus, they are known for halophytes plants cultivation, including date palms. *Phoenix dactylifera* L., cv. 'Deglet Nour', is a valuable Tunisian cultivar subjected to high salinity levels. In this way, our purpose is to evaluate the response of its roots to long period exposition to increasing salt concentrations. We started by studying the effects of 4 g/L, 8 g/L, 12 g/L, and 16 g/L NaCl on the parameters of germination (Growth rate—GR, Seed Mortality Rate—SLM, Germination Mean Time—GMT, and Germination Speed—GS) of date palm seeds for a 2-month period. We found that 4 g/L NaCl did not affect the seeds germination, and, hereinafter, the parameters of germination and the radicle length decreased with the increase of NaCl concentrations and experiment time. Then, we demonstrated a high antioxidative enzymes CAT and SOD production in case of salt stress augmentation. Lastly, a metabolomic approach was carried out by LC-HRMS, followed by an untargeted and targeted analysis using the XCMS online and MZmine tools, respectively. The roots chemical composition was compared using PCA. We identified 25 secondary metabolites, divided into 3 categories. Metabolites known for their role in salt stress alleviation include δ-tocotrienol, metabolites identified in salt stress for the first time, and other unknown metabolites.

Keywords: salt stress; *Phoenix dactylifera* L., cv. 'Deglet Nour; germination; antioxidant enzymes; LCMS/MS; XCMS online; MZmine; secondary metabolites; δ-tocotrienol

1. Introduction

Soil salinization is becoming a major problem in multiple regions around the world. It is affected by numerous factors, including climate changes and uncontrolled anthropogenic activities by the continuously increasing human population [1,2]. The extent of land salinization is determined by measuring the level of pH, certain ions' (Na^+, Ca^{2+}, SO_4^{2-}, K^+, SO_4^{2-}, Cl^-, and Mg^{2+}) accumulation, and electrical conductivity (> 4 dSm^{-1}) [3,4]. Excess salt concentrations engender a huge disturbance in soil (osmotic and ionic stresses), in agricultural production, and an unstoppable decrease in arable lands annually [5,6]. Harsh arid and semi-arid regions are generally exposed to multiple other stress factors accentuating the salinity stress, such as drought and extreme temperatures [7,8]. Thus, these regions are distinguished by the culture and development of halophytic plants. These plants are known for their ability to cope with high salt concentrations (> 400 mM) due to their mechanisms of salt accumulation and exclusion, limitation of transpiration, ionic adjustment, antioxidant mechanisms activation, and expression of secondary metabolites responsible for salt stress mitigation [9–11].

Date palm plants are one of mankind's oldest cultivated plants [12]. They were domesticated in ~5000–3000 B.C. [13] and first cultivated in hot arid regions of the world particularly in North Africa and the Middle East [14]. They contain about 200 genera and more than 2.500 species. Particularly, the genus *Phoenix* contains 14 species, including *Phoenix dactylifera* L. [15]. Economically and due to the fast-growing demand, the production of dates has been increasing over the years. Indeed, a fully productive date palm tree can yield more than 100 kg of fruit annually (the average productivity depends on the cultivar, environmental conditions and cultivation practices). A date palm plantation has an economic life of about 50 years; subsequently, yield decreases [16]. These plants are known as halophytes, where some cultivars support up to 12.8 dSm^{-1} with no stress symptoms appearance [17,18]. To date, multiple researchers demonstrated the capacities of date palm adaptation to other biotic (*Fusarium oxysporum* f. sp. Albedinis—FOA disease) and abiotic factors (drought, temperature, pH, etc.) [12,19–21].

Tunisia belongs to the top 10 date-producing countries, and it is mostly known for the production of dates from the variety of 'Deglet Nour' (*Phoenix dactylifera* L., cv. Deglet Nour) due to its outstanding agronomic, nutritional, and socio-economic significance [22,23]. Farmers tend to irrigate date palm oases mainly situated in the regions of Kebili, Tozeur, and Gabes, to ameliorate the growth, quality, and productivity of dates. However, the brackish water, warm temperature (high evaporation level), and drought (lack of rainfall) characterizing these regions lead to salt ions accumulation in the soil and/or salt leaching into underground water [24–26]. Thus, excessive salt accumulation beyond the threshold levels has become the primary abiotic stress factor threatening the cultivation and destructing the production of date palm fruits [27]. Some studies used advanced tools, including metabolomics, to study plant responses to abiotic aggressions, including salt stress, by producing valuable secondary metabolites [28,29]. Nonetheless, the mechanism of the secondary metabolites in date palm stress tolerance is least understood and studied today.

Therefore, the main objective of our work is to study the response of the roots of date palm cv. Deglet Nour to increasing concentrations of salt (4 g/L, 8 g/L, 12 g/L, and 16 g/L) and during a period of 2 months. For this to happen, we started with morphological and biochemical tests, and we conducted for the first time an in-depth metabolomic study on date palm roots subjected to salt stress concentrations to investigate the metabolomics changes and to decipher the metabolites enhancing date palm salt tolerance.

2. Materials and Methods

2.1. Plant Material

Healthy seeds from date palm cv. 'Deglet Nour' were harvested from an oasis in the region of Tozeur, Tunisia, and transported to the laboratory for further tests.

2.2. Germination Assay

The harvested seeds from cv. 'Deglet Nour' were washed under running tap water and then surface disinfected three times successively using a laboratory optimized protocol. In the first instance, we dipped the seeds in 100% commercial bleach for 5 min under vigorous agitation, then in 50% (*v*/*v*) commercial bleach for 10 min, and, lastly, in 25% (*v*/*v*) commercial bleach for 10 min. Afterward, seeds were rinsed thoroughly with sterile distilled water (SDW).

Date palm disinfected seeds were then germinated inside sterilized mason jars. Each jar contains 8 date palm seeds, and either 10 mL of SDW for the Control (0 g/L), or a 10 mL solution of 4 g/L, 8 g/L, 12 g/L, or 16 g/L for salt stress tests. Obtained jars were perfectly sealed to avoid any external contamination and incubated at 24 °C in dark for 60 days. Results were assessed every 20 days, and each experiment was conducted 3 times.

2.2.1. Germination Potential (GP)

The percentage of germinated seeds is calculated using the following formula [30]:

$$GP\ (\%) = (N/S) \times 100 \quad (1)$$

with: N = Total number of germinated seeds, and S = Total number of incubated seeds.

2.2.2. Germination Rate (GR)

The germination rate is determined throughout the following formula [30]:

$$GR = n1/d1 + n2/d2 + \ldots. \quad (2)$$

with: n = Number of germinated seeds, and d = Number of germination days.

2.2.3. Germination Speed (GS)

Germination speed is calculated using the following formula [30]:

$$GS = N/D \quad (3)$$

with: N = Total number of germinated seeds, and D = Total number of germination days.

2.2.4. Germination Mean Time (GMT)

Germination mean time is calculated by the following formula [30]:

$$GMT = (n1 \times d1 + n2 \times d2\ldots)/D \quad (4)$$

with: *n* = Number of germinated seeds, and D = Total number of germination days

2.2.5. Seeds Mortality Rate (SMR)

The seeds mortality rate was estimated using the following formula [30]:

$$SMR = M/D \quad (5)$$

with: M = Number of non-germinated seeds, and D = Total number of germination days

2.3. Evaluation of Biochemical Parameters

The roots of germinated seeds of each experiment (0 g/L, 4 g/L, 8 g/L, 12 g/L, and 16 g/L) were biochemically assayed.

2.3.1. Total Soluble Proteins Extraction

Total proteins test was measured using the colorimetric protocol described by Reference [31]. First, 0.5 g of fresh date palm roots of each treatment (0 g/L, 4 g/L, 8 g/L, 12 g/L, and 16 g/L) were grinded in 1.5 mL phosphate buffer (0.1 M, pH 7), 1 mM EDTA, 1% (w/v) of polyvinylepyrolidone (PVP), and 200 µL Bradford reagent (Sigma, Buchs, Switzerland). Then, the obtained extract was centrifuged at 4 °C for 15 min at 13,000 g, and the supernatant was kept at –20 °C for further utilization in enzymatic assays. The concentration of total proteins was measured using a spectrophotometer at OD = 595 nm. The calibration curve was determined using bovine serum albumin (Sigma, Buchs, Switzerland).

2.3.2. Catalase (CAT) Activity Assay

CAT activity was determined following the protocol of Reference [32]. Briefly, the reaction mixture contains 0.05 g of enzymatic extract, 1.5 mL potassium phosphate buffer (0.1 M, pH 7), 0.1 mM EDTA, and 0.5 mL H_2O_2. The CAT activity was determined by following the H_2O_2 disappearance at OD = 240 nm ($E = 39.4\ mM^{-1}\ cm^{-1}$).

2.3.3. Superoxide Dismutase (SOD) Activity Assay

SOD assay was determined by evaluating the rate of inhibition of the photochemical reduction of nitro blue tetrazolium chloride (NBT) at OD = 560 nm [33]. The mixture solution involved 25 µL enzyme extract, phosphate buffer (50 mM, pH 7.5), methionine (10 mM), riboflavin (2 µM), EDTA (0.1 mM), and NBT (70 µM). One unit of SOD is equal to the amount of enzyme required to cause 50% inhibition of the NBT photo-reduction rate.

2.4. Liquid Chromatography-Mass Spectrometry (LCMS) Analysis

Sample Extraction

Approximately 10 mg of the date palm roots were extracted by 10 mL of methanol. The methanolic extract was evaporated under a vacuum to 1 mL. Ultrahigh-pressure liquid chromatography (UHPLC) and high-resolution mass spectrometry (HRMS) were used for further analysis. The UHPLC-HRMS experiments were carried out according to Reference [12].

2.5. XCMS Online for Untargeted Metabolomics

The XCMS was used for untargeted metabolomics to identify differences between the samples and the control, which presents the metabolite profiles [34].

2.6. Targeted Metabolomics

2.6.1. Data Processing

The data obtained from the LCMS/MS (Liquid Chromatography with tandem mass spectrometry) analysis was processed by MZmine 2.22. The MZmine was used to target the metabolites detected after 4 min to extract features from the raw data. The data processing consists of the following steps: peak detection, mass detection, and chromatographic builder, deconvolution, deisotoping, filtering, alignment, and gap filling. MZmine parameters were used for the data processing: noise level at 10^4; lorentzian function for the peak shape; minimum peak height at 5×10^4; and m/z tolerance at 0.005 m/z or 5.0 ppm. Data were then exported as a CSV file. The spreadsheet was categorized by peak area, the retention time for each sample extract. The data were then subject to multivariate analysis. All potential data information was highlighted and rechecked by comparing the samples to the control sample and the Control as a step for the cleanup

process, adducts, and peaks present in both samples, and their controls (control sample and Control) were removed.

2.6.2. Dereplication Process of the Extracts for the New Hit

Identification of fragments, adducts, and peak complexes and formula prediction steps were carried out to predict possible molecular formulas for each feature and to minimize misassignment of features by eliminating adducts and complexes. Metabolites were tentatively identified by dereplication of their molecular formulae and fragmentation pattern with reported compounds in the Dictionary of Natural Products (V. 23.1 on DVD).

2.7. Statistical Methods and Data Analysis

All experiment data presented are mean values based on 3 replicates. Multiple group analysis was used to compare the control and the samples. The principal component analysis was used to identify the metabolic profiling at different time frames and different concentration and compare it to the control sample. The statistical significance was identified by ANOVA, and results were considered significant when *p*-values were $p \leq 0.05$. The groups were compared using a post-hoc Tukey's HSD (Honestly Significant Difference). The statistical program used was IBM SPSS Statistics v. 22 [30].

3. Results

3.1. Effects of Salt Stress on Date Palm Seeds Germination

Figure 1A demonstrates the date palm germination kinetic curves of 4 treatments with increasing concentrations of NaCl (4 g/L, 8 g/L, 12 g/L, and 16 g/L) compared to the Control (seeds germinated with SDW). There is no significant difference between the germination kinetics of the Control curve (86%) and the treatment curve with 4 g/L (90%) during the 2-month treatment period. However, the percentage of seeds germination dropped at 8 g/L, 12 g/L, and 16 g/L concentrations in the 20th and 40th day of germination compared to the Control and raised again from the 40th day, reaching up to 80%, 77%, and 55% of germination for the 8 g/L, 12 g/L, and 16 g/L NaCl concentrations, respectively, on the 60th day of germination.

Similarly, in the other germination tests, the results of the Control and the treatment with 4 g/L NaCl of GR (0.53) and SLM (0.01) were very close to each other. The TMG (13.55) and GS (0.12) of the 4 g/L test slightly exceeded the TMG (13.11) and GS (0.11) of the Control (Figure 1B).

The results of the treatments with 8 g/L and 12 g/L decreased significantly compared to the Control and were similar to each other in all GR, GMT, SMR, and GS tests. The treatment with the 16 g/L test showed a significant drop compared to the Control and the other treatment concentrations with 0.2, 7.88, 0.05, and 0.07 in the GR, TMG, SLM, and GS tests, respectively (Figure 1B).

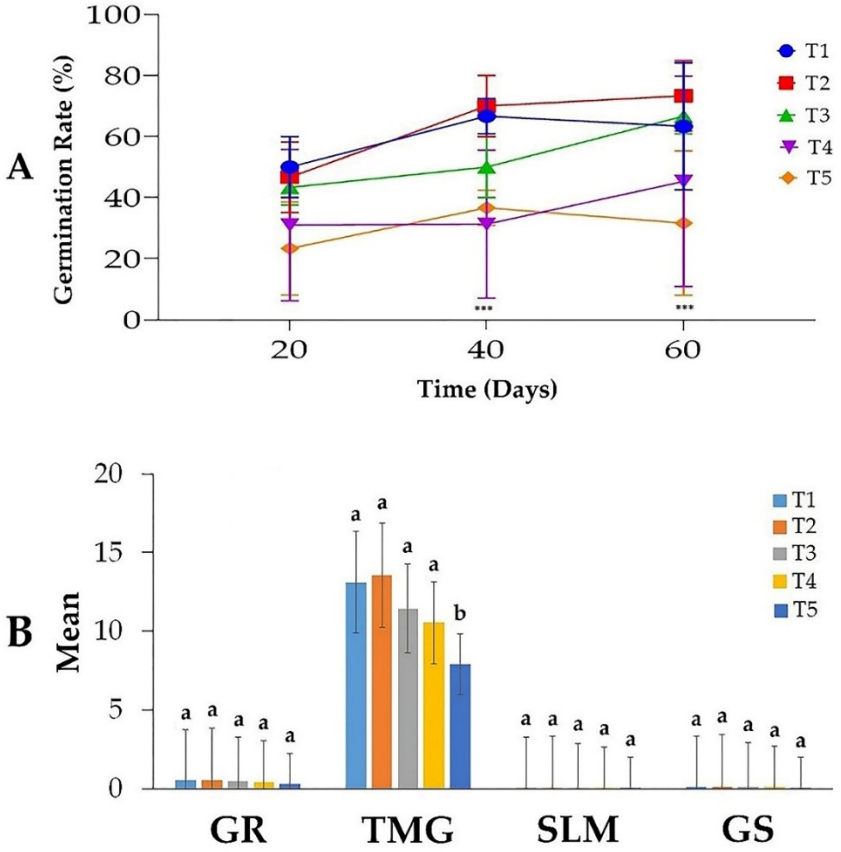

Figure 1. (**A**) Date palm germination rate at different time under salt stress at different NaCl concentration (T1 = 0 g/L (Control), T2 = 4 g/L, T3 = 8 g/L, T4 = 12 g/L, and T5 = 16 g/L) (**B**) Germination rate (GR), Germination Mean Time (GMT), Seed Mortality Rate (SMR), and Germination Speed (GS) under salt stress at different NaCl concentration (T1 = 0 g/L (Control), T2 = 4 g/L, T3 = 8 g/L, T4 = 12 g/L, and T5 = 16 g/L). The value represents the mean ± standard deviation (SD) of six replicates. Two-way ANOVA followed by Turkeys multiple comparison test $p < 0.05$. Starts and letters indication for the statistical differences from the control sample.

3.2. Effects of Salt Stress on Date Palm Radicle Length

The results of radical length in Figures 2 and 3 show that the increase of salt concentration from 0 g/l up to 16 g/l (~0.7 cm) on the 20th day of germination was not significant ($p \geq 0.05$). After 40 days of germination, the ANOVA analysis demonstrated three significantly ($p \leq 0.05$) different groups. The first group involved the root length of the Control (3.6 cm) and the first treatment with 4 g/L (3.2 cm). In the second group, both the 8 g/L (2.4 cm) and 12 g/L (1.9 cm) salt stress treatments were not significantly ($p \geq 0.05$) different. Lastly, the treatment with 16 g/L was the most affected by salinity, where the radicle length was 1.1 cm compared to the Control, which reached 3.6 cm. On the 60th day of germination, the radicle length of the Control (4.25 cm) and the 4 g/L (4.1 cm) treatment were very similar to each other. In contrast, we observed a significant ($p \leq 0.05$) drop in the 8 g/L (3.07 cm), 12 g/L (2.2 cm), and 16 g/L (1.1 cm) treatments compared to the Control (Figures 2 and 3).

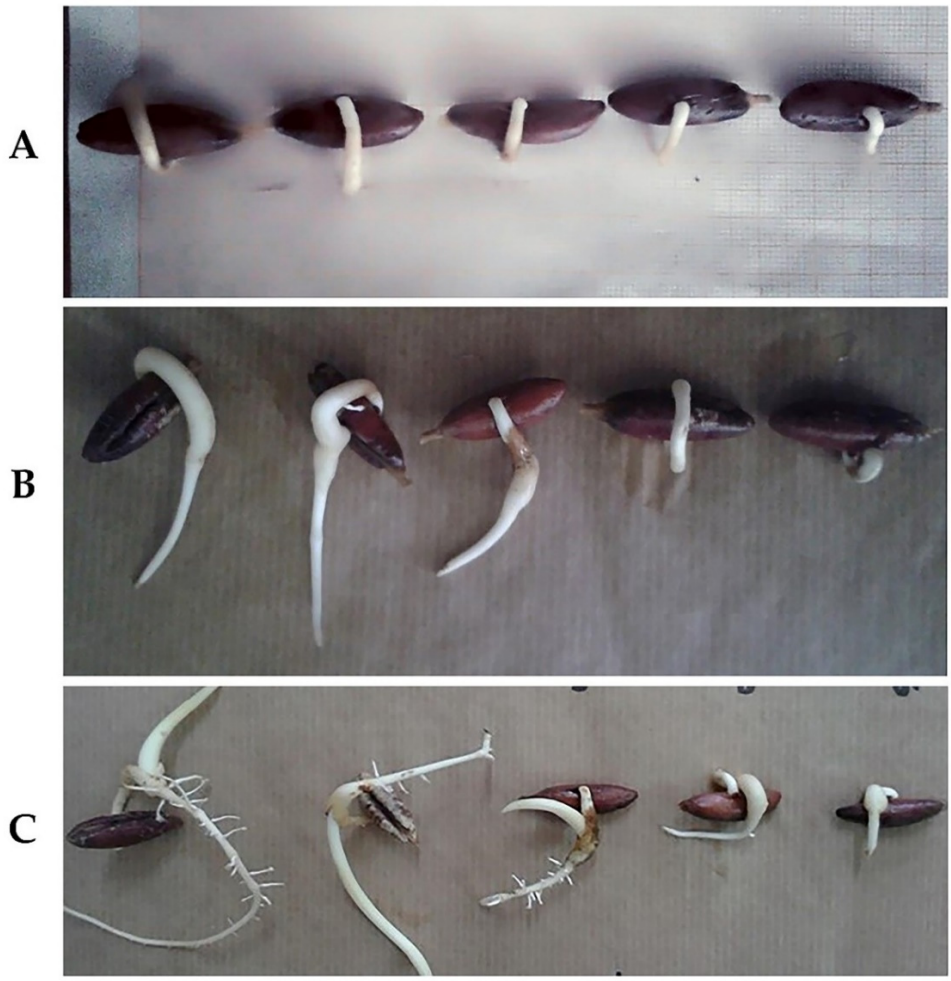

Figure 2. (**A**–**C**) Date palm seeds radical germination after 20, 40, and 60 days, respectively, under 4 g/L, 8 g/L, 12 g/L, and 16 g/L NaCl concentrations.

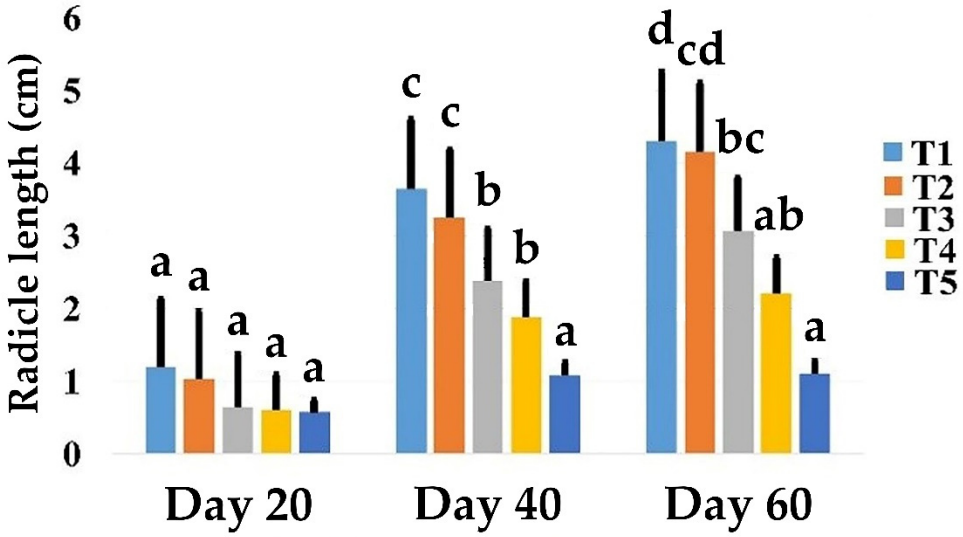

Figure 3. Date palm seeds radical length (cm) results after 20, 40, and 60 days and under 4 g/L, 8 g/L, 12 g/L, and 16 g/L NaCl concentrations. The value represents the mean ± Standard deviation (SD) of three replicates. The value represents the mean ± standard deviation (SD) of six replicates. Two-way ANOVA followed by Tukey's multiple comparisons test. ($p < 0.05$). Treatments with different letters in each column are statistically different from the control sample.

3.3. Effects of Date Palm Salt Stress on Total Proteins Content

The germinated roots of date palm cv. 'Deglet Nour' seedlings produced soluble proteins by an average of 6 mg/mL/g MF compared to the Control (8.02 mg/mL/g MF) after 20 days of germination. Total soluble proteins content raised remarkably after 40 days of germination, and the results were not significantly ($p \geq 0.05$) different between the applied salt concentrations (4 g/L, 8 g/L, 12 g/L, and 16 g/L). Lastly, we did not notice a remarkable rise in the total protein content after 60 days of treatment (~ 12.1 mg/mL/g MF).

3.4. Effects of Date Palm Salt Stress on CAT and SOD Enzymes

As shown in Figure 4A, the results of CAT activity were significantly ($p \leq 0.05$) different after 20, 40, and 60 days of exposure to increasing (0 g/L, 4 g/L, 8 g/L, 12 g/L, and 16 g/L) NaCl concentrations. on the 20th and 40th days, the salt stress did not affect the CAT activity. However, after 2 months of roots salt exposure, we observed that the increase in salt concentrations was inversely proportional to the CAT activity. It dropped from 2.35 μmol H_2O_2/mg protein in the Control to 1.8 μmol H_2O_2/mg protein in the test with 16 g/L NaCl.

The general histograms aspects of the SOD content are very analogous to each other after 20, 40, and 60 days of treatments with salt. Indeed, the Control (0 g/L), 4 g/L, and 8 g/L NaCl tests displayed a low SOD content compared to the 12 g/L and 16 g/L NaCl concentrations, where date palm roots exhibited a high SOD content, reaching up to 27 unit/mg protein and 29.8 unit/mg protein in the treatments with 12 g/L and 16 g/L NaCl, respectively, compared to the Control with 8.5 unit/mg protein [35].

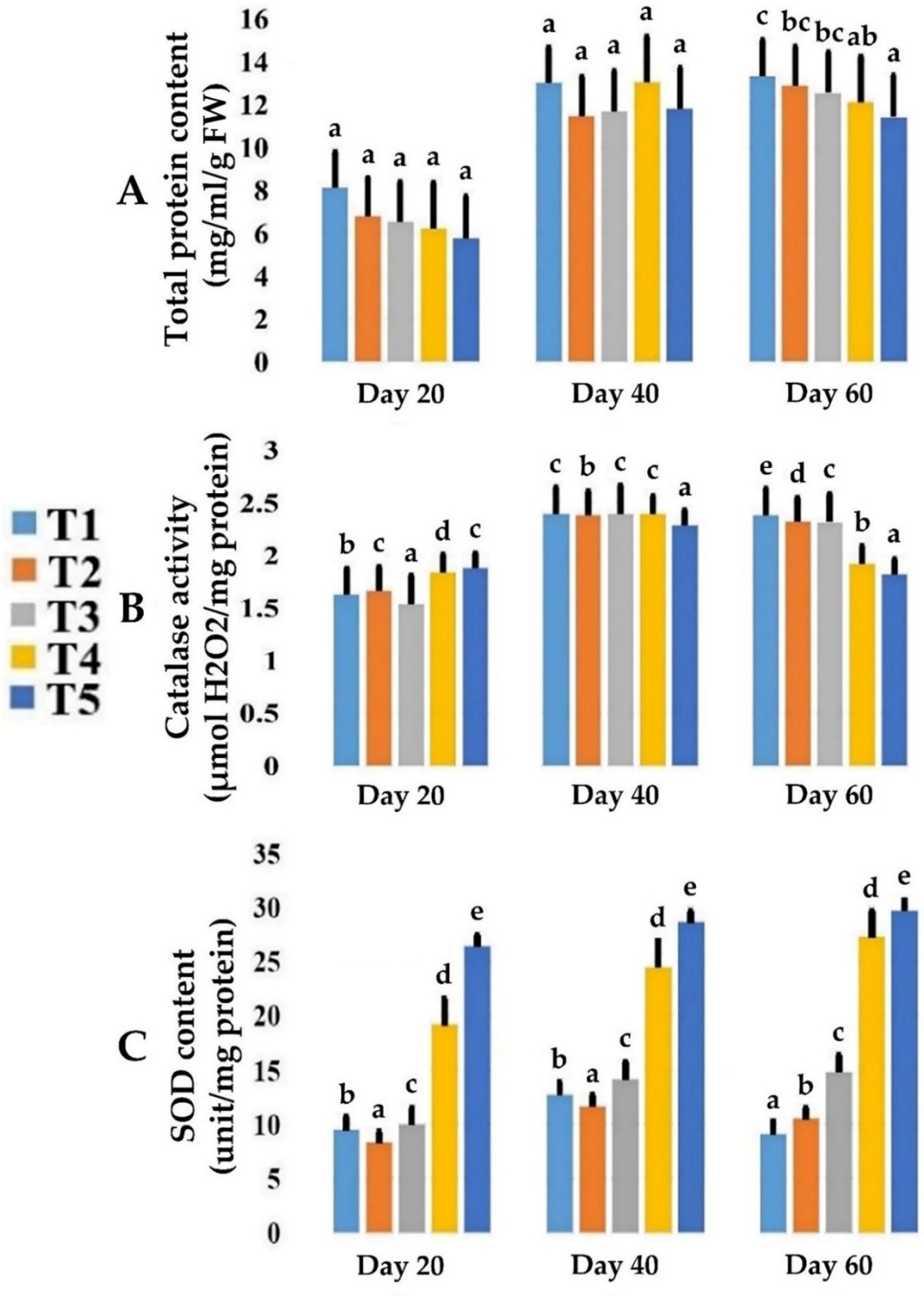

Figure 4. Results of (**A**) total protein content (mg/mL/g FW (Fresh Weight)), (**B**) catalase activity (µmol H_2O_2/mg protein), and (**C**) SOD content (unit/mg protein) after 20, 40, and 60 days and under 4 g/L, 8 g/L, 12 g/L, and 16 g/L NaCl concentrations. The value represents the mean ± Standard deviation (SD) of six replicates. Two-way ANOVA followed by Tukey's multiple comparisons test. ($p < 0.05$). Treatments with different letters in each column are statistically different from the control sample.

3.5. Metabolomics Profiling

Figure 5a shows the cloud plot for the significant metabolites generated by XCMS online. The number of detected and predicted significant metabolites features was 319 at different salt concentrations (0 g/L, 4 g/L, 8 g/L, 12 g/L, and 16 g/L) and time frames. It was possible to predict and hypothesize the chemical features of the date palm L., cv. 'Deglet Nour', in rationale with increasing salt stress concentrations and 3 different growth times (after 20, 40, and 60 days of germination). We noticed variations in the metabolic profiles perceived through the occurrence of metabolites changing with the variation of time and concentration of the samples.

Figure 5b shows the interactive multigroup cloud plot with customized metabolomic data visualization. All features of metabolites are represented by a colored bubble. As an example, m/z 739.4266 was detected in both the control sample and the treated sample, and this feature was increased with the increasing concentrations of salt and growth time (Figure 5b). The m/z 739.4266 feature reached a maximum (peak) after 60 days of growth on the soil contaminated with 8 mg/mL NaCl, and it decreased at higher concentrations (Figure 5b). Figure 6a shows that m/z 739.4266 feature was still available after 60 days of growth on the soil contaminated with 8 mg/mL NaCl. Structure of the compound was tentatively identified by using natural product databases, including AntiBase and Dictionary of natural product (Figure 6b).

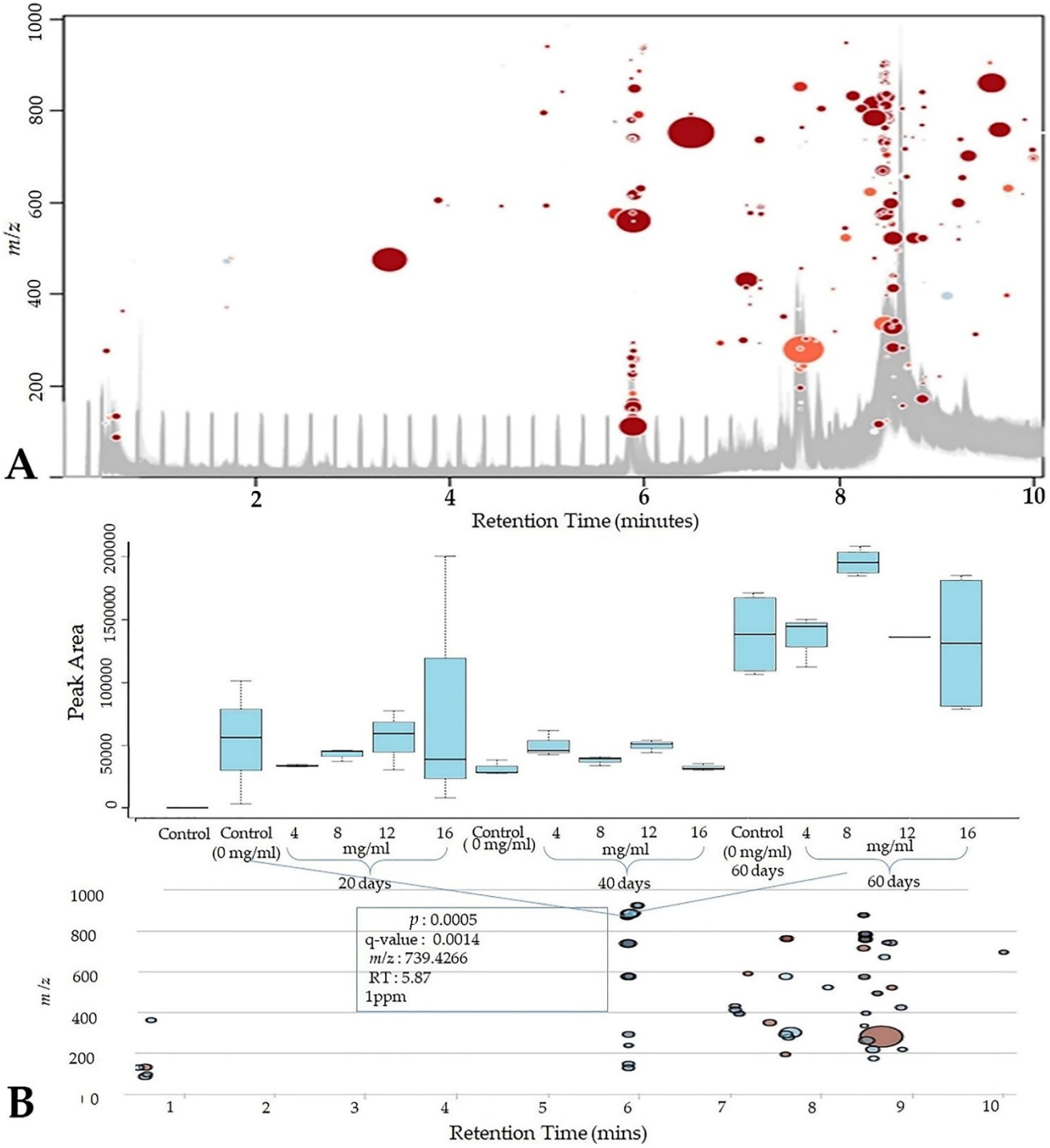

Figure 5. (**A**) Cloud plot for the significant metabolites generated by XCMS online. (**B**) Interactive multi-group cloud plot of the metabolite features and customized metabolomics data visualization and standard deviation.

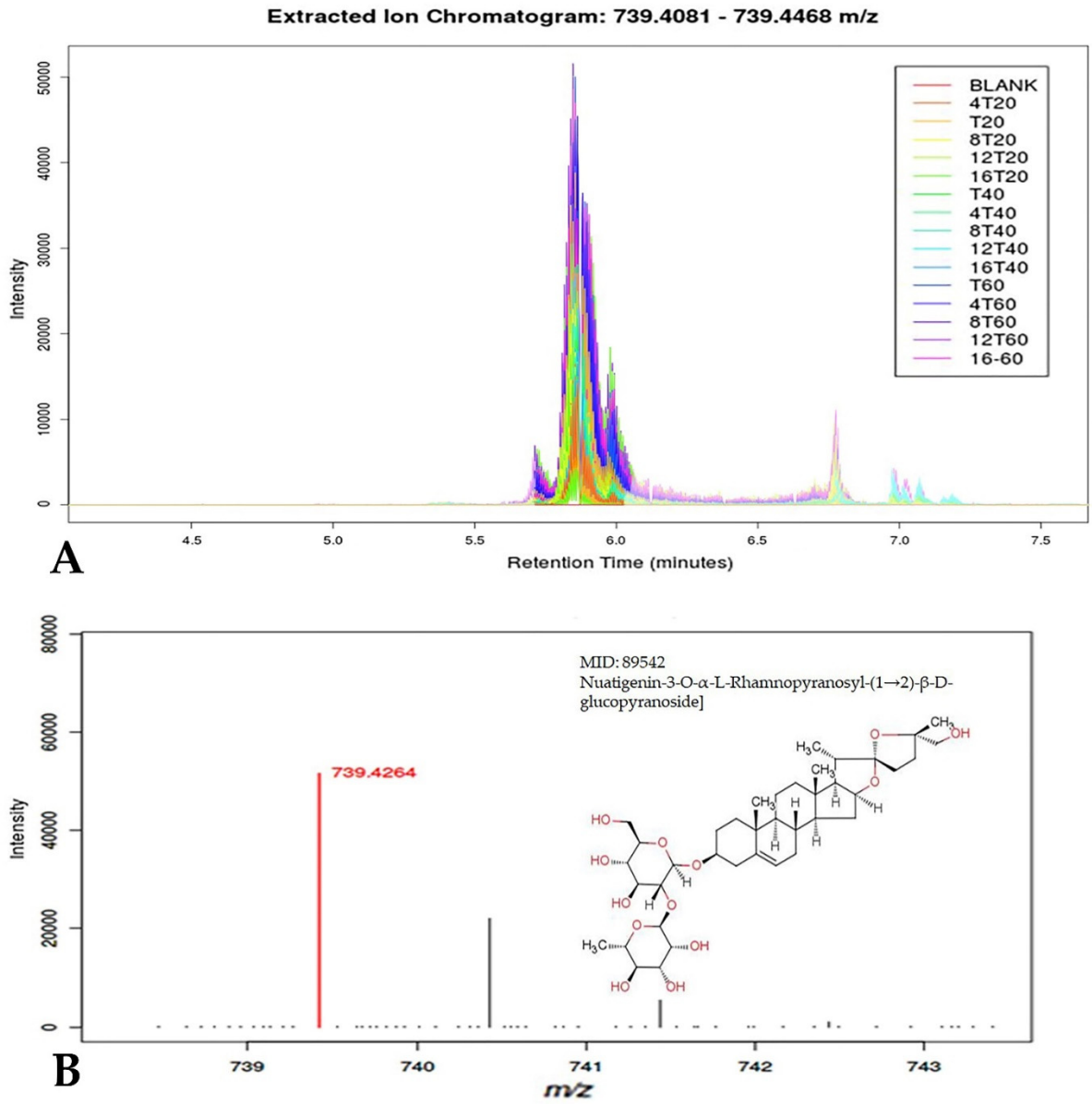

Figure 6. (**A**) Extracted Ion chromatogram of m/z 739.4266 feature. (**B**) The spectrum of m/z 739.4266 and tentatively identified compound structure by LCMS after 60 days of growth on the soil contaminated with 8 mg/mL NaCl.

3.6. Principal Component Analysis (PCA)

The multivariate non-targeted metabolomics statistical analysis method PCA allowed a rapid inspection of metabolic patterns within the salt-stressed date palm roots. The similarity and diversity of metabolites in the samples at different times were determined by principal component analysis (PCA). Results in Figure 7 showed that the metabolites were clustered together in response to salt concentration and time progression. The samples were compared to the control. There was a significant difference in the metabolites produced over the period of progression time. Metabolites produced during the first 20 days were completely different from the ones produced after 40 days and 60 days of growth on the soil treated with salt. Additionally, in the first 20 and 40 days of growth more metabolites were produced in comparison to 60 days of growth. However, the concentration of these metabolites was less compared to those produced in the 60 days.

The salt concentration affected the metabolites produced in each period. The metabolites patterns changed with changing the salt concentration. The metabolite produced at 0 mg/mL were completely different from the one produced at a higher concentration. Furthermore, these metabolites were statistically different $p \leq 0.05$ from the metabolites produced at 0 mg/mL concentration (control) and from the control (solvent). The circles in the plot clusters are the data observations; the closer the circles, the more identical the data (Figure 7).

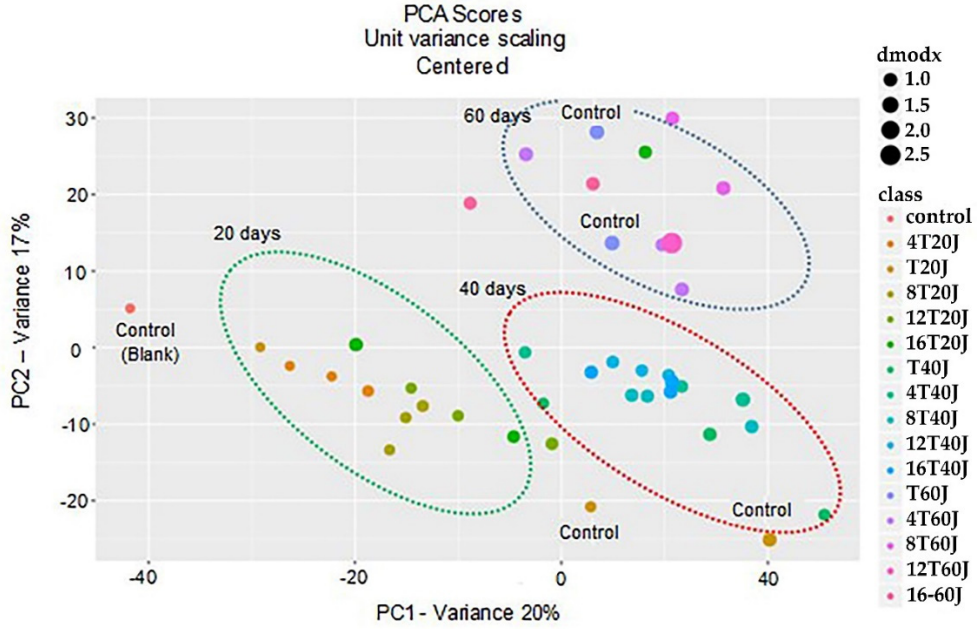

Figure 7. Multivariate analysis using principal component analysis (PCA) for salt–stressed date palm roots at different time frames.

3.7. Customized Metabolomics Data

Figure 8a illustrates an interactive heat map with customized metabolomic data. Each column represents a sample in different salt concentrations (0 g/L, 4 g/L, 8 g/L, 12 g/L, and 16 g/L) and growth times (20, 40, and 60 days) used in the experiment. Each row represents a metabolite feature. The significant metabolites features ($p \leq 0.01$) are identified by the white color. The heat map in Figure 8b showed the multidimensional untargeted metabolites. The customized heat map for feature 552.40 indicated that it was not available in the Control sample. The concentration of this feature increased with increasing time and salt concentration. The optimum concentration was achieved in 16 g/L at 60 days of growth (Figure 8b).

3.8. Secondary Metabolites Prediction

To confirm our results, another investigation was carried out by using the software Mz mine 2.22. Figure 7 represents all metabolites exhibited after 20, 40, and 60 days of treatment under 4 g/L, 8 g/L, 12 g/L, and 16 g/L NaCl concentrations. Greater emphasis was given to the metabolites detected after RT = 5 min. As result, significant ion peaks were found in the treated samples. They correspond to 25 predicted and identified metabolites, including $[M + H/Na]^+$ ion peaks at m/z; see Table 1 and Supplementary Figures S1 and S2.

Table 1. Tentative identification of secondary metabolites produced by date palm roots under 16 g/L salt concentration.

*Rt	ǂHRESIMS	[M + H/Na]⁺	Tentative Identification
5.85	739.4262	$C_{39}H_{63}O_{13}$	Nuatigenin-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranoside] (terpenoid)
5.87	885.4833	$C_{45}H_{73}O_{17}$	Nuatigenin − 3-O-[α-L-Rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside] (terpenoid)
7.58	415.3215	$C_{27}H_{43}O_3$	Diosgenin (terpenoid)
7.60	397.3109	$C_{27}H_{41}O_2$	δ-tocotrienol (terpenoid)
8.40	280.2648	$C_{18}H_{34}NO$	Linoleamide (fatty acid derivative)
8.48	552.4000		Unknown
8.46	715.5225	$C_{45}H_{72}O_5Na$	Belamcandaquinone L (Phenolic derivative)
8.47	352.3221	$C_{22}H_{42}NO_2$	Unknown terpenoid derivative
8.47	279.2084	$C_{16}H_{27}N_2O_2$	Lycocernuine (alkaloid)
8.48	758.5683	$C_{42}H_{81}NO_8P$	L-α-phosphatidylcholine (Glycerophospholipid)
8.48	782.5677	$C_{44}H_{81}NO_8P$	Dilinoleoylphosphatydylcholine (Glycerophospholipid)
8.50	784.5819	$C_{44}H_{83}NO_8P$	α-Oleyl-β-linoleoylphosphatydylcholine (Glycerophospholipid)
8.52	614.4843	$C_{35}H_{65}N_3O_4Na$	Unknown
8.54	570.4582	$C_{35}H_{60}N_3O_3$	Unknown terpenoid derivative
8.55	526.4322	$C_{31}H_{57}N_3O_2Na$	Unknown
8.56	219.1759	$C_{15}H_{23}O$	Germacrone (terpenoid)
8.61	429.3736	$C_{29}H_{49}O_2$	Cholesteryl acetate (terpenoid)
8.61	256.2649	$C_{16}H_{34}NO$	Palmitamide (fatty acid derivative)
8.64	282.2804	$C_{18}H_{36}NO$	Oleamide (fatty acid derivative)
8.65	336.3273	$C_{22}H_{42}NO$	Pipericine (fatty acid derivative)
8.67	742.5819	$C_{42}H_{80}NO_9$	Asteriacerebroside G (cerebrosides)
8.96	284.2939	$C_{18}H_{38}NO$	Stearamide (fatty acid derivative)
9.23	708.5113	$C_{37}H_{71}N_3O_8Na$	Unknown
9.26	663.4533	$C_{40}H_{64}O_6Na$	Scapaundulin B (terpenoid)
9.31	338.3425	$C_{22}H_{44}NO$	Cis-docosenamide (fatty acid derivative)

* (Rt) Retention time. ǂ HRESIMS High Resolution of Mass Spectrometry.

The results showed that the compounds structures belong to known and unknown terpenoids and their derivatives, fatty acids derivatives, phenolic derivative, alkaloid, glycerophospholipid, cerebroside, and other unknown compounds (Table 1). The secondary metabolites production of date palm roots was enhanced with the increase of NaCl concentrations and experiment time (Figure 8B).

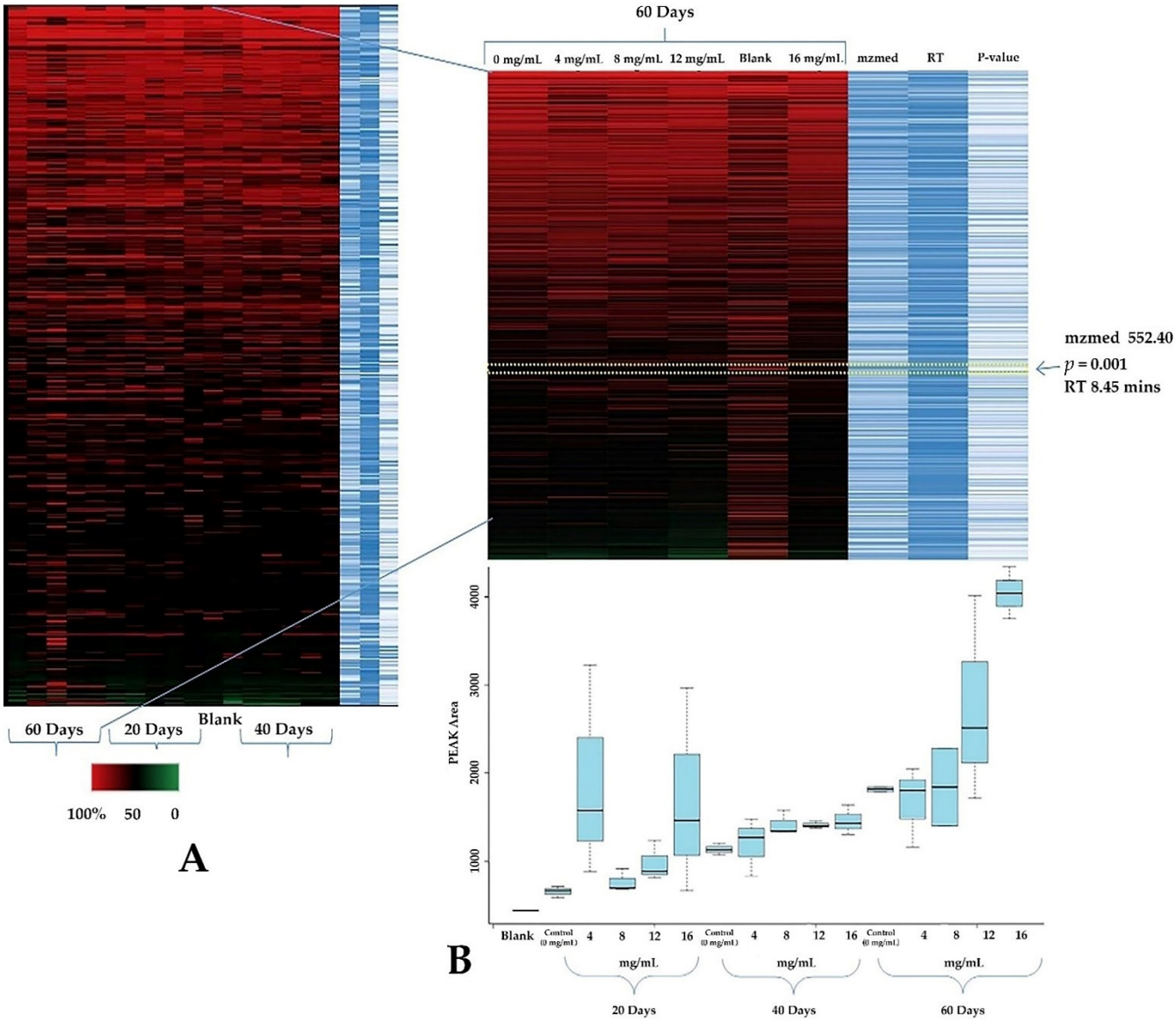

Figure 8. (**A**) Heat-map of all metabolites exhibited after 20, 40, and 60 days of treatment under 4 g/L, 8 g/L, 12 g/L, and 16 g/L NaCl concentrations. (**B**) Heat-map of the produced metabolites after 60 days of treatments and customized metabolomics data visualization under 4 g/L, 8 g/L, 12 g/L, and 16 g/L NaCl concentrations.

4. Discussion

Numerous date palm growing fields are progressively affected by NaCl contamination [36]. The level of date palm salt resistance differs from one variety to another, yet, they are all known as outstanding halophytes surviving saline desert and seashore environments [36–41]. Nonetheless, high salt levels in soil or irrigation water affect fruit quality and productivity [42]. The date palm cultivar Deglet Nour is known as a valuable Tunisian cultivar known by its translucent aspect. In our work, we started by testing the response of the date palm seeds germination to increasing concentrations of NaCl (0 g/L, 4 g/L, 8 g/L, 12 g/L, and 16 g/L). We found that the root germination of control and the treatment with 4 g/L NaCl were very close to each other with slight germination progress in the treatment with 4 g/L. This result could be explained by the date palm tendency to germinate in a salt containing medium [43,44]. The remaining 8 g/L, 12 g/L, and 16 g/L percentages of germination progressed slowly till the 40th day of germination and then rapidly, attaining ~75% on day 60, except for the 16 g/L treatment (54% t). Multiple ancient researchers evaluated the seed germination of other date palm cultivars, including 'Zahedi', 'Khalas', 'Lulu', 'Barhee', and 'Boman', treated with salt solutions between 1 g/L

and 25 g/L, and they found that salt concentration below 8 g/L was not affecting the germination of seeds and that they continued to germinate up to 20 g/L NaCl concentration [45,46]. Similarly, Alhammadi and Kurup [7] mentioned that there is a negative correlation between the elevation of salt concentration and the level of seed germination when the Ca^{2+}, Na^{+}, Mg^{2+}, and K^{+} ions exceed an electrical conductivity of 4.3 dSm^{-1}, and they also mentioned that date palm seedlings stop growing when the soil salt concentration rich 12.8 dSm^{-1} [7]. Additionally, we conducted other germination tests, and we demonstrated that the TMG and GS of the date palm cv. 'Deglet Nour' seeds treated with 4 g/L NaCl were slightly above those of the Control. Concerning the other salt concentrations, we observed a significant drop in all GR, GMT, SMR, and GS tests. These results are in line with Furr and Ream (1968), who studied the salt tolerance on the leaves of date palm cv. 'Deglet Nour' and found that the GR of leaves decreased as the NaCl treatment concentrations increased [47]. In the same context, Reference [27] mentioned that roots are the first plant organs to face and to defend salinity stress.

The effects of salt stress on date palm radicle length were observed, especially after salt treatment for 40 and 60 days, successively. In fact, we observed a clear drop in radicle length, which was adversely proportional to the increasing salt concentrations. These findings accorded with the scientific work of Hewitt [48], who evaluated the effects of NaCl concentrations from 10 g/L to 30 g/L on 'Deglet Nour' seeds germination. He found that seeds germination was slightly affected at 10 g/L, extremely affected at 20 g/L and totally inhibited at 30 g/L [48]. Otherwise, Reference [49] reported the 'remote germination' which is a specific form of germination in date palms, which causes an embryonic dormancy to protect seeds from harsh abiotic stresses. Other salt-resistant plants were able to induce anatomic differentiation in certain organs, including roots, to avoid excess salt concentrations [18,50–52]. Alrasbi et al. [43] stated that salt tolerance could be attributed to the balance between Na^{+} and Cl^{-} concentrations in date palm organs.

After testing the effects of salt stress on the date palm cv. 'Deglet Nour' seeds germination, we evaluated the level of production of 2 antioxidant enzymes used to scavenge the reactive oxygen species (ROS) produced at high levels in case of plant stress [52–55]. Starting with the CAT activity test, its high production levels after 20 and 40 days, and with 4 g/L and 8 g/L NaCl concentrations on the 60th day, could be explained by the plant production of such antioxidant agent to tolerate the stress induced by salinity [56–59]. Similarly, Mohammadi et al. [60] recorded an increase in CAT activity after *Manilkara zapota* cultivation under saline conditions. A comparative study between a salt resistant (Umsila) and a salt susceptible (Zabad) date palm cultivars showed that the 'Umsila' produced higher CAT activity than the 'Zabad' when subjected to increasing levels of salinity [18].

The SOD results showed that the augmentation of NaCl concentrations from 0 g/L to 16 g/L induced an increase in SOD production. Our results are confirmed by numerous other previous works demonstrating the enhancement of SOD expression in plants stressed with salt [61–65].

We conducted a metabolomics study to predict the metabolites produced by the roots of date palm cv. 'Deglet Nour' under 4 increasing NaCl concentrations (4 g/L, 8 g/L, 12 g/L, and 16 g/L) and in 3 different time frames (20, 40, and 60 days). This is the first metabolomics study on 'Deglet Nour' roots response to salt stress. Some previous metabolomics data on date palms focused on date ripening [66], on seedlings subjected to salt and silicon treatments [67], on pulp and seeds [68], on fruit cultivars [69], on therapeutic applications [70], and the quality determination of different date palm varieties [71]. From the results, it was possible to predict and hypothesize the chemical features of the produced metabolites. We first remarked a rationale increase in the amount of one identified metabolite named Nuatigenin-3-O-[α-L-Rhamnopyranosyl-(1→2)-β-D-glucopyranoside] compared to the control, the increase in experiment time, and introduced salt concentrations. Our result is in accordance with those of Reference [72], who mentioned that the plant metabolite synthesis could be adapted to the external aggressions over a concise evolutionary

duration. Otherwise, a metabolomics study on 2 date palm varieties showed that the halophytic cultivar accumulated a specific collection of metabolites in response to salinity [29].

The PCA method enabled the clustering of metabolites with similar chemical profiles. It demonstrated a change in date palm produced metabolites with the changes of the experiment times. The PCA method proved that the salt aggression forced the date palm to produce unique metabolites that are not available in the control sample. These metabolites could be directly or indirectly related to salt stress tolerance. When we compared our results to the literature, we noticed that other scientific works revealed the possibility of using salinity stress to increase the plants secondary metabolites production [73–76].

A total number of 25 secondary metabolites were identified from the roots of the date palm cv. 'Deglet Nour' grown under salinity stress, based on the RT, m/z, and the protonated molecular ion $[M + H/Na]^+$ corresponding to their formulas (Table 1). Plant metabolites are primarily crucial for plant defense and adaptation, and some of them are also used for other purposes [72]. The studies on date palm secondary metabolites allowed the detection of numerous multifunctional compounds [77]. Ashraf et al. [78] reported that plants biosynthesis of secondary metabolites varies in response to biotic and abiotic conditions. Du et al. [64] mentioned that halophytic plants accumulate stress-protective metabolites under saline conditions. The tentatively predicted metabolites belong mostly to the terpenoids, which are known for their activities against plant biotic and abiotic stresses [28,79,80]. For instance, δ-tocotrienol was produced, and it is a vitamin E compound produced by numerous plants, including wheat plants. This secondary metabolite increased when wheat seeds were exposed to stress conditions [81]. Sattler et al. [82] proved the role of δ-tocotrienol in seedlings germination [82,83], while Yusuf et al. [84] found that tocopherol at high concentrations helps in salt stress mitigation. Awad et al. [85] stated that α-tocopherol was exogenously introduced into date palm seedlings to enhance their growth.

Additionally, we found that glycerophospholipid was produced at a 16 g/L salt concentration. It was documented by Hou et al. [86] that lipids interfere in the case of abiotic stress signals. Sui and Han [87] found that the cultivation of *Thellungiella halophila* in saline conditions provoked an increase in unsaturated fatty acids and lipids concentrations in the plant membrane, thus ensuring a better tolerance to salt stress.

Other predicted metabolites, such as the diosgenin, the linoleamide, and germacrone, were known for their role as powerful medicinal compounds [88–90]. The remaining compounds comprise several metabolites yet to be characterized in salt stress tolerance, however, the role of their corresponding classes was heavily proven in salt mitigation. For instance, the salt treatment of *Cynara cardunculus* enhanced the leaves phenolic content [91]. Similarly, the production of alkaloids increased with the increase of the salt exposure duration of *Catharanthus roseus* [92]. We also found other unknown compounds requiring further investigations to be fully identified. Al Kharusi et al. [29] indicated that several metabolic classes proved to play an interesting role in date palm salinity tolerance. Yang et al. [93] conveyed those plants generate numerous secondary metabolites to alleviate ROS production [94–97]. It is important to mention that date plant-associated microbes could also be involved in the synthesis of powerful secondary metabolites against external aggressions [12,98].

5. Conclusions

Our results confirmed the natural halophytic aspect of date palm, particularly the cultivar Deglet Nour. In fact, even when the roots were exposed to high salt stress concentrations for a long period, the date palm managed to produce antioxidant enzymes to scavenge the ROS levels and maintain the seeds germination. When we conducted an in-depth metabolomics study by using the LCMS/MS chromatography, followed by the analysis with XCMS, MZmine, we managed to characterize date palm roots metabolic profiles.

We identified 25 different secondary metabolites that were produced under high salt concentrations and a long-time frame. In perspective, further investigations are required to understand how the secondary metabolites assist the palm tree to resist soil stress and to identify the unknown metabolites.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/11/12/2389/s1, Figure S1 and S2: Chemical structure for the tentatively identified compounds group 1 and group 2.

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