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Research Article

Differential effect of water salinity levels on gas exchange, chlorophyll fluorescence and antioxidant compounds in *ex vitro* date palm plants

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Abstract

In this study, the response to salt stress was evaluated in *ex vitro* acclimated date palm plants, regenerated from *in vitro* culture multiplication. The plants, eighteen-month-old, were irrigated with 0 (control), 150, 300 or 450 mM NaCl solutions (high to very high-water salinity). Photosynthesis parameters and antioxidant compounds were determined at the end of the experiment in leaves. At 150 mM NaCl, net CO_2 assimilation rate and internal CO_2 concentration were not impaired; while transpiration and stomatal conductance decreased by 60 and 70%, respectively. By increasing salt concentrations, all gas exchanges parameters were decreased. Measurement of chlorophyll fluorescence and P700 redox state showed that PSII and PSI machineries were significantly enhanced under 150 mM NaCl, conditions. With the 300 mM NaCl, the PSI parameters remained unchanged compared to control, while some of the PSII parameters, such as NPQ and Y (NPQ), were increased. At 450 mM NaCl, photosystems functionality was light intensity (PAR) dependent. Only at low PAR, a significant increase of some PSI and PSII parameters was observed. At the contrary, with high PAR, most of the energy conversion functions were significantly reduced, especially those related to PSI, indicating that PSI was more susceptible for damage by salinity than PSII. To overcome high salinity stress, *ex*

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vitro date palm plants mobilized a cascade of physio-biochemical pathways including the antioxidant activity and proline biosynthesis. Overall, the salinity of irrigation water, and up to 150 mM, improves the physiological performance of *ex vitro* date palm plants, which manage to tolerate very high levels of this stress.

Keywords: *ex vitro* plants; high salinity; *Phoenix dactylifera*; photosynthesis; salt stress adaptation

Abbreviations: ETRI: electron transfer rate of photosystem I ; ETRII: electron transfer rate of photosystem II; Fv/Fm: maximum quantum efficiency of PSII photochemistry in dark; PAR: photosynthetically active radiation; PAM: pulse amplitude modulation; PS: photosystem; qP: coefficient of photochemical quenching; RH: relative humidity; ROS: reactive oxygen species; TPC: total phenolic compounds; TAA: Total antioxidant Activity; YI: quantum yield of photosystem; YII: quantum yield of photosystem II

Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most important fruit crops in arid areas of the world, mainly the Middle East and North Africa for at least 5000 years (Helaly and Hanan El-Hosieny, 2011; Zohary *et al*., 2012). In these regions, they play a very important economic, social and environmental roles due its high capacity to adapt to a harsh edaphoclimatic conditions such as: high temperature and salinity. However, the continuous degradation of the quality of the irrigation water in the oases begins to negatively affect this crop and the entire agricultural ecosystem in which it plays a key role (Elshibli and Korpelainen, 2009; Yaish and Kumar, 2015; Jasim and Shareef, 2016). The threat that salinity represents to the sustainability of date palm cultivation makes it essential to understand the functioning of the mechanisms that control the response of this species to saline stress.

The multiplication of the date palm is classically done with seeds or offshoots. These techniques generate genetically and physiologically heterogeneous plant material. The development of the *in vitro* multiplication technique has allowed obtaining homogeneous and vigorous plant material, which is very interesting for carrying out reliable scientific studies (Al-Khayri and Al-Bahrany, 2004; Fki *et al*., 2011). However, this type of material has been little used in physiological and agronomic studies of the date palm until now (Hammami *et al*., 2022). The little knowledge available about the physiological response of date palm to salinity, such as enzymatic activity, gas exchange and nutrient uptake, was obtained from seedlings, with the inherent doubts and potential interfering factors that such material may generate (Elshibli and Complained, 2009; Sperling *et al*., 2014; Al Kharusi *et al*., 2019). In addition, the behaviour of the two photosystems PSI and PSII have never been studied in any type of date palm material despite their central role in photosynthetic activity and sensitivity to oxidative stress (Goussi *et al*., 2018; Manaa *et al*., 2019).

In order to protect photosystems PSI and PSII from oxidative damage caused by salt stress, plants have evolved several fine-tuning mechanisms such as Na^+ exclusion, especially from the leaf and more precisely from the chloroplasts (Müller *et al*., 2014; Farhat *et al*., 2021). An equally important mechanism is the sequestration of Na^+ in the leaf vacuoles. This indeed offers a cheap osmoticum for cell turgor, compared to the metabolically costly organic solute synthesis (Munns and Tester, 2008). Another protective mechanism against salt-stress is the overproduction of antioxidant metabolites like phenolic and flavonoid compounds in order to scavenge harmful reactive oxygen species (ROS) (Cohen and Kennedy, 2010; Agati *et al*., 2012) and compatible osmolytes such as proline with osmoprotectant activity (Szabados and Savouré, 2010; Dar *et al*., 2015). In the date palm, a new study in *ex vitro* plants has shown new insights on the key role of exclusion mechanisms of Cl^- and Na^+ in the roots and the leaf in the tolerance of this species to salinity (Hammami *et al*., 2022). However, the mechanisms adopted to protect the PSI and PSII in salt stress conditions have not yet been fully elucidated.

To deeper investigate the functioning of the photosynthetic machinery and the plants response to tolerate stressful factors, two sensitive and important parameters were used by several authors, including the chlorophyll fluorescence and the P700 redox state (Kalaji *et al*., 2018; Schlau-Cohen and Berry, 2015). Indeed, chlorophyll fluorescence analysis was proven to be very useful in studying the effect of stresses on photosynthesis. Knowledge about the application of chlorophyll fluorescence was greatly advanced during the last two decades and several other parameters were developed in order to measure PSII photochemical efficiency and the detection of the ways light energy was channelled through alternative dissipative mechanisms (Guidi and Landi, 2019).

The purpose of the present work was to evaluate the effect of different levels of water salinity on the photosynthetic gas exchange, chlorophyll fluorescence parameters and antioxidant compounds in *ex vitro* date palm plants.

Materials and Methods

Plant material and stress treatments

The study used eighteen-month-old *ex vitro* date palm (cv. 'Barhee') plants. The plants were grown in black 5 L pots with a peat: soil: sand (3:1:7, v/v/v). During the all the period of the study the pots were placed in a greenhouse with daily average temperature of 25±2 °C, relative humidity (RH) of 65±2%, and photosynthetically active radiation (PAR) of 555±15.4 µmol m^{-2s-1}. Saline treatments consisted of two irrigations per week with 150, 300 and 450 mM NaCl, for four months, and control plants were irrigated with 0 mM NaCl water. At each irrigation, a quantity of water equal to the retention capacity of the substrate was added to avoid any water deficit (added water quantity was determined by weighing pots before each irrigation).

The experimental design was a complete randomized block with four treatments, four blocks, and twelve plants (three replications per treatment and block) per block, giving a total of 48 plants plus border plants.

Leaf chlorophyll index

Leaf chlorophyll index was estimated using a hand-held SPAD-502 meter (Konica-Minolta, Japan) at the heading stage. The average value of thirty SPAD Chl readings was considered as a replication (four replications per treatment) and evaluated in individual leaves of plants measured from the tip to the leaf base, between 10:00 am and 11:00 am.

Photosynthesis-related parameters

Photosynthesis-related parameters such as the net assimilation rate of CO_2 (A), stomatal conductance (gs), transpiration rate (E), and intercellular CO_2 concentration (Ci) were determined using a portable photosynthesis system (LCpro-SD, ADC BioScientific, UK). Measurements were taken between 10:00 am and 12:00 am in the uppermost fully expanded leaf of each plant (leaf closest to the shoot apex). Light intensity was adjusted to above plant light saturation capacity. From these measurements, carboxylation efficiency (A/Ci), instantaneous water use efficiency (A/E) and intrinsic water use efficiency (A/gs) were also calculated.

Chlorophyll fluorescence

Chlorophyll fluorescence measurements were carried out on the date palm leaves at the end of the experiment with a pulse modulated fluorometer (OS1p – Opti-Sciences Inc., USA), using the PAM (Pulse Amplitude Modulation) method which allows analysis of the dependence between the fluorescence yield and the photosynthetic functions (Schreiber and Ulrich, 2004; Schreiber *et al*., 1986). Fluorescence parameters were determined in dark-adapted (30 min) leaves of control and treated plants. Subsequently, those leaves were exposed to modulated light of low intensity for F0 (minimal fluorescence) measurements, then subjected to an

actinic light that initiated electron transport between photosystems (PSII and PSI). The description of the different fluorescence parameters assessed is included as Supplementary Material (Table S1).

Biochemical analysis

Total phenolic concentration (TPC) was determined using the Folin-Ciocalteu reagent, following Singleton's method (Dewanto *et al*., 2002). Root and leaf extracts were obtained by magnetic stirring of 1 g of dry powder with 10 mL of pure methanol for 30 min. The extracts were then kept at 4 °C for 24 h, filtered through Whatman No. 4 filter paper and stored at 4 °C until analysis. Aliquot (125 µL) of a 10-fold diluted sample of extract was added to 0.5 mL of deionized water and 125 µL of the Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min before adding 1.25 mL of 7% Na_2CO_3 solution. The solution was then diluted with deionized water to a final volume of 3 mL and mixed thoroughly. After incubation for 90 min at room temperature, the absorbance was read at 760 nm. TPC was expressed as mg gallic acid equivalents (GAE) per gram of DW.

Total flavonoids concentration was estimated using the colorimetric method of Hatano *et al*. (1988). An aliquot of diluted sample was added to75 µL of sodium nitrite solution ($NaNO_2$, 0.7 M), mixed for 6 min before adding 0.15 mL of aluminium chloride hexahydrate solution ($AlCl_3$ $6H_2O$) (100 g L^{-1}). After 5 min, 0.5 mL of sodium hydroxide (1 M) was added. The final volume was adjusted to 2.5 mL with distilled water and thoroughly mixed. Absorbance was determined at 510 nm against a blank. Total flavonoid content was expressed as mg catechin equivalents (CE) per gram of DW.

Total antioxidant activity (TAA) was assessed following the methodology established by Prieto *et al*. (1999) through the assay of the green phosphate/Mo^{5+} complex. An aliquot (0.1 mL) of samples was combined with 1 mL of reagent solution (0.3 N sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The sample tubes were incubated in a boiling water bath for 90 min., then the absorbance was measured at 695 nm against a blank. Antioxidant capacity was expressed as mg gallic acid equivalents per gram of DW.

Free proline concentration was measured according to the protocol established by Bates *et al*. (1973) (Bates *et al*., 1973). Leaf and root samples (100 mg of fresh weight) were homogenized in 3% (w/v) aqueous sulfosalicylic acid and centrifuged at 14.000 g for 30 min. To the supernatant, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were added, and the mixture was boiled for 1 h. After extraction with toluene, the free proline was quantified at 520 nm from the organic phase. Free proline concentration was expressed as µmol per gram of DW.

Statistical analysis

The experiment was analysed as a randomized complete block design. The analysis of variance and the Tukey's test at $p< 0.05$ were used to assess the differences between treatments. All statistical analyses were performed using Statistix 8.0.

Results

Effects of salt on total chlorophyll content

Leaf chlorophyll content was estimated in plants at the end of the experiment (after 4 months) using the SPAD-502. Plants irrigated with 150 mM NaCl showed similar chlorophyll content to control plants, with value around 65 SPAD units, while those irrigated with 300 or 450 mM NaCl both exhibited a reduction of chlorophyll content of approximately 30% (Figure 1).

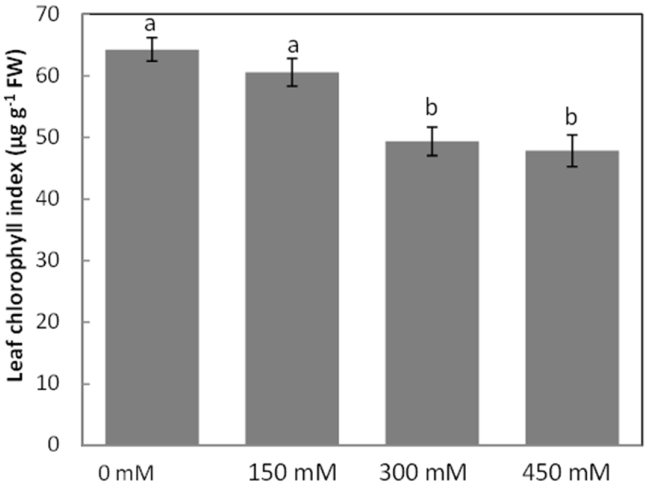

Figure 1. Leaf chlorophyll index in date palm plants under different salt treatments
Treatments were salt-free conditions (0 mM) and plants exposure to 150, 300 and 450 mM NaCl. Values are the means ± standard errors (measurement were performed for each leaf of each plant). Bars with different letters are significantly different at $p < 0.05$ according to Tukey's test.

Effects of salt on leaf gas exchange measurements

The gas exchange of the leaves was very dependent on the level of salinity applied. In fact, we can distinguish two behaviours: the first one is caused by the lower salinity treatment (i.e., 150 mM NaCl) and the second is due to the other two doses of salinity (i.e., 300 and 450 mM NaCl).

The irrigation of date palm plants with 150 mM NaCl did not impair the net CO_2 assimilation rate (A) nor the internal CO_2 concentration (Ci) in the plants. In contrast, a decrease by 60 and 70% for transpiration (E) and stomatal conductance (gs), respectively, was found compared to controls (Table 1). The carboxylation efficiency (A/Ci) was not affected, while the instantaneous (A/E) and intrinsic (A/gs) water use efficiency were increased compared to controls (Table 1).

Table 1. Effects of NaCl concentrations on gas exchange parameters: net assimilation rate of CO_2 (A), internal CO_2 concentration (Ci), transpiration rate (E), stomatal conductance (gs), carboxylation efficiency (A/Ci), instantaneous water use efficiency (A/E) and intrinsic water use efficiency (A/gs) in date palm at the end of the experiment

	A ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	Ci ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	E ($mmol\ H_2O\ m^{-2}\ s^{-1}$)	Gs ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	A/Ci (-)	A/E ($\mu mol\ CO_2/mmol^{-1}\ H_2O$)	A/gs (-)
0 mM NaCl	3.5 ± 0.06a	336.50 ± 3.05a	3.28 ± 0.3a	0.101 ± 0.005a	$10 \times 10^{-3} \pm 1 \times 10^{-3}$ a	1.07 ± 0.004b	34.7 ±0.14c
150 mM NaCl	2.9 ± 0.07a	332.50 ± 1.70a	1.21 ± 0.14b	0.029 ± 0.004b	$9 \times 10^{-3} \pm 1 \times 10^{-3}$ a	2.39 ± 0.001a	100.4 ±0.73a
300 mM NaCl	1.6 ± 0.06b	321.60 ± 4.19b	0.79 ± 0.12c	0.022 ± 0.002b	$5 \times 10^{-3} \pm 1 \times 10^{-3}$ b	2.02 ± 0.008a	72.7 ±0.56b
450 mM NaCl	1.1 ± 0.07b	324.33 ± 2.20b	0.78± 0.13c	0.028 ± 0.003b	$3 \times 10^{-3} \pm 1 \times 10^{-3}$ b	1.41 ± 0.016b	39.3 ±0.70c

Values are the means ± standard error. Means within a column with the same letter are not significantly different at $p < 0.05$ according to Tukey's test.

With the increase in the salt concentrations, all gas exchange parameters decreased. The 300 and 450 mM NaCl treatments reduce the (A), in comparison to the control, significantly by 54 and 66%, respectively. A greater rate of decrease was observed for the (E) and (gs), under these salt concentrations by around 75% and

82 % compared to the control. Furthermore, for both salt concentrations, the (A/Ci) decreased and (A/E) was unchanged compared to controls (Table 1).

Effect of salt on chlorophyll fluorescence

Chlorophyll fluorescence determinations were sorted by treatment (irrigation salt level) and by photosystem activities (PSI and PSII), and the trends of the PSII and PSI parameters (increased, decreased or unchanged) relative to the control values are summarized in Table 2. Compared to salt-free treatment, most of the PSII and PSI parameters increased and none of the PSI photosystem parameters decreased following the irrigation with 150 mM NaCl (Table 2). For the 300 mM NaCl, the PSI parameters remained unchanged compared to the control, while some of PSII parameters, such as NPQ and Y(NPQ) increased. The effect of the highest salt concentration (450 mM NaCl) seemed to be PAR-dependent. In general, most of the energy conversion functions were significantly reduced, particularly those related to PSI. Only at low PAR (0-200 photons m^{-2} s^{-1}) a significant increase of some PSII and PSI parameters, such as Y(ND) and P700ox were observed (Table 2).

Table 2. Chlorophyll fluorescence parameters changes sorted by PSII and PSI photosystems, in date palm plants under different salt treatments (150, 300 and 450 mM NaCl)

Treatments	Photosystems	Increased	Decreased	Unchanged
150 mM NaCl	PSII	Y(II), ETR II, qP, NPQ, qN, Y(NPQ)	Y(NO), Fv	Fv/Fm, Fo, Fo'
	PSI	Y(I), ETR(I), P700m, P700m' P700ox	-	Y(ND)
300 mM NaCl	PSII	NPQ, Y(NPQ)	Y(NO), qP	Y(II), ETR II, qN, Fv/Fm, Fv, Fo, Fo'
	PSI	P700m, P700m', P700ox	-	Y(I), ERT(I), Y(ND), Y(NA)
450 mM NaCl	PSII	PAR 0-200; Y(NPQ)	PAR ≤200 : qP, Y(II) PAR 400 : ETR(II) Fv/Fm	Fo, Fo', Y(NO), NPQ, qN
	PSI	PAR 0-200 ; Y(ND), P700ox PAR 100-700 ; Y(ND)	PAR 0-600: Y(I), ETR(I) PAR 200-1000: Y(ND), P700ox,	-

The changes relative to control values are indicated as increased, decreased, unchanged) on parameter values were referred to controls. Abbreviations of all the mentioned parameters was listed on supplementary Table 1.

Effect of salt on PSII functionality

Photochemical quenching (qP) showed a gradual decrease with increasing light intensity (Figure 2A). This decrease was less pronounced in plants grown under 150 mM NaCl as compared to other treatments, indicating the higher capability of 150 mM NaCl salt-treated plants to utilize light energy (Figure 2A). Concerning non-photochemical-quenching (NPQ), this parameter was higher in plants treated by 150 and 300 mM NaCl compared to controls, although control plants and 450 mM NaCl stressed plants exhibited similar values of NPQ (Fig. 2B).

The maximum quantum yield of PSII photochemistry (Fv/Fm ratio) did not differ between control and plants treated with 150 and 300 mM NaCl, indicating that there was no photo-inhibitory effect during plant growth, which was not the case for plants exposed to 450 mM NaCl (Table 3). However, the proportion of PSII centers in the open state (with QA oxidized) (qL), as a function of light intensity, a parameter proportional to the amount of PSII centers with a reduced QA primary acceptor, was higher in plants irrigated with 150 mM NaCl than controls. This means that the PSII centers functioned better than in control plants,

and that most of PSII did not show a reduction in the primary acceptor, confirming that at the 150 mM NaCl the PSII centers were not photo inhibited (Figure 2C).

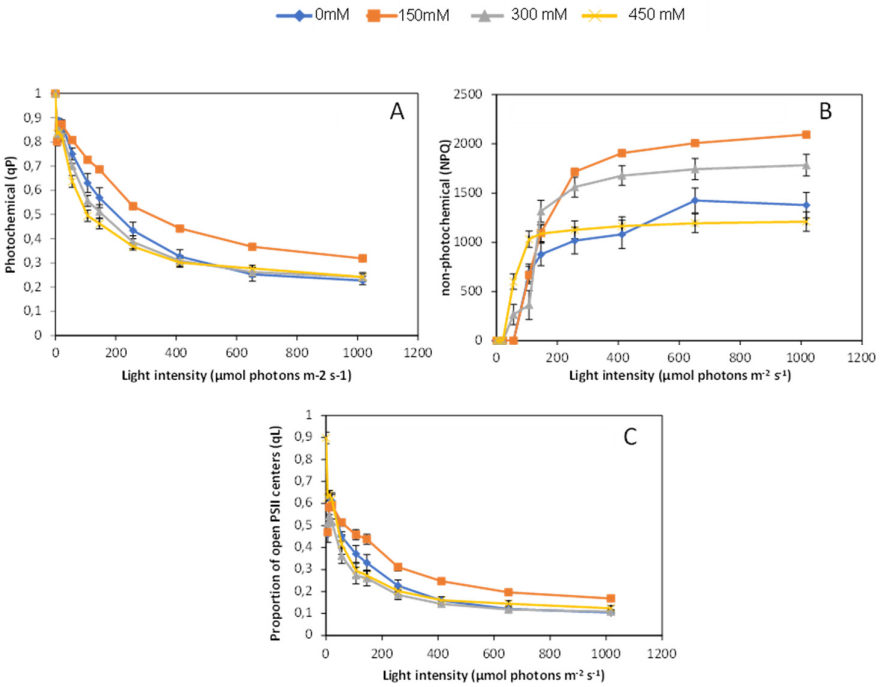

Figure 2. Light-response curves for the photochemical (qP, A) and non-photochemical (NPQ, B) quenching, the proportion of PSII centers in the open state (with QA oxidized) (qL) (C) in leaves of date palm grown under greenhouse conditions for 4 months. Treatments were: salt-free conditions (0 mM), plants exposure to 150, 300 and 450 mM NaCl

Table 3. Effects of salt treatments on the maximal photochemical efficiency of PSII (Fv/Fm) in date palm leaves grown under greenhouse conditions for 4 months

Treatments	Fv/Fm
0 mM NaCl	0.801±0.015a
150 mM NaCl	0.805±0.018a
300 mM NaCl	0.825±0.016a
450 mM NaCl	0.753±0.020b

Different letters indicate significant difference between means (± standard error) according to Tukey's test at p≤ 0.05.

Effect of salt treatments on the electron transport rates around PSII (ETRII) and PSI (ETRI) and the maximum photo-oxidizable P700ox

Relative electron transport rate around PSII and PSI (ETR (II) and ETR (I)) increased with high light intensities (Figure 3). This increase was higher in 150 mM NaCl -treated plants than for the control and the other salt concentrations. The 450 mM NaCl showed the lowest value of ETR (I). Light response curves indicate that ETR (I) was greatly reduced compared to ETR (II) when illuminated at high light intensities (Figure 3A and B). This was confirmed by a decrease in the ETRI/ETRII ratio which indicates a probable decrease of electron transport rate from PSII to PSI and suggests a probable suppression of Cyclic Electron Flow (CEF). The substantial reduction of electron transfer from PSII to PSI served to prevent further photodamage to PSI (Figure 3C) by limiting the risk of a strong supply of electrons from PSII to PSI which could exceed the capacity of PSI electron acceptors. The quantity of efficient PSI complex was evaluated through the maximum photo-oxidizable P700ox as indicated in Figure 3D. The increase in light intensities was

associated with a concomitant increase of P700ox which resulted in a bigger portion of P700 in the oxidized state, preventing the over-reduction of P700. However, plants stressed with 450 mM NaCl showed two trends depending on light intensities: i) higher than control for light intensities ≤300 and ii) lower than control for light intensities ≥300 µmol photons m^{-2} s^{-1}. The P700m, P700m' decreased only in plants exposed to the highest salt concentration, which might indicate the closure of the reaction center (Figure 3E).

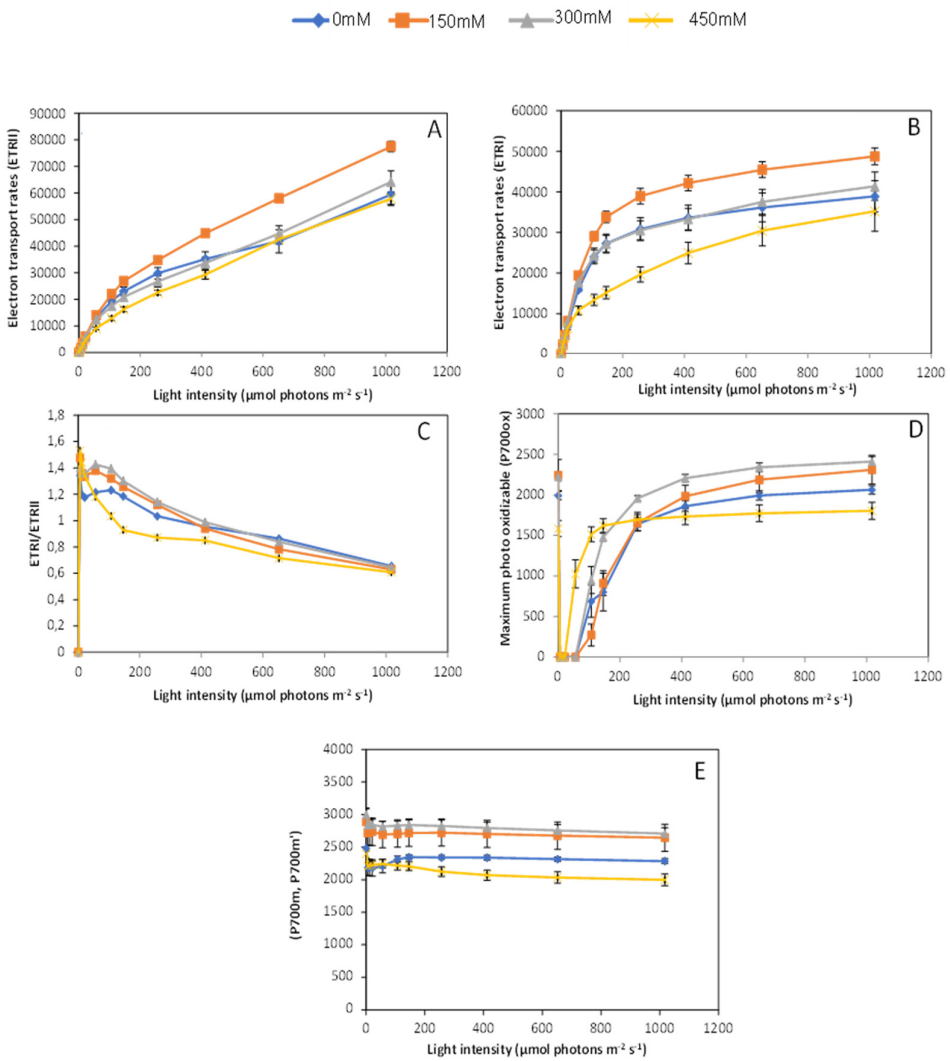

Figure 3. Light-response curves of electron transport rate through PSII (ETRII, A), PSI (ETRI, B), ETRI/ETRII ratio (C), maximum photo-oxidizable P700 (P700ox) (D) and P700m, P700m' (E) in leaves of date palm grown under greenhouse conditions for 4 months Treatments were: salt-free conditions (0 mM), plants exposure to 150, 300 and 450 mM NaCl

Changes in PSI and PSII parameters in date palm under salinity conditions

The present results showed that plants grown under 150 mM NaCl had greater ability to manage the excess in light energy. In fact, plants exposed to this salt concentration presented higher values of Y (II) and lower Y (NO) (Figure 4A, B). Besides, Y (ND) increased by increasing light intensity reaching values similar to that of control plants (Figure 4F). The ability to preserve an efficient electron donation to PSI under stress condition is one of the key traits for a high salinity tolerance. In parallel, the higher values of Y (ND) and the

lower values of Y (NA) indicates that PSI was protected from salinity (Figure 4E, F). The over-reduction of PSI acceptor (NA) was prevented under high light conditions and 150 mM NaCl. No significant difference was noticed for the nonphotochemical quenching parameters of PSI (Y (ND) and Y (NA)) between control and treated plants, possibly indicating an effective electron donation to PSI.

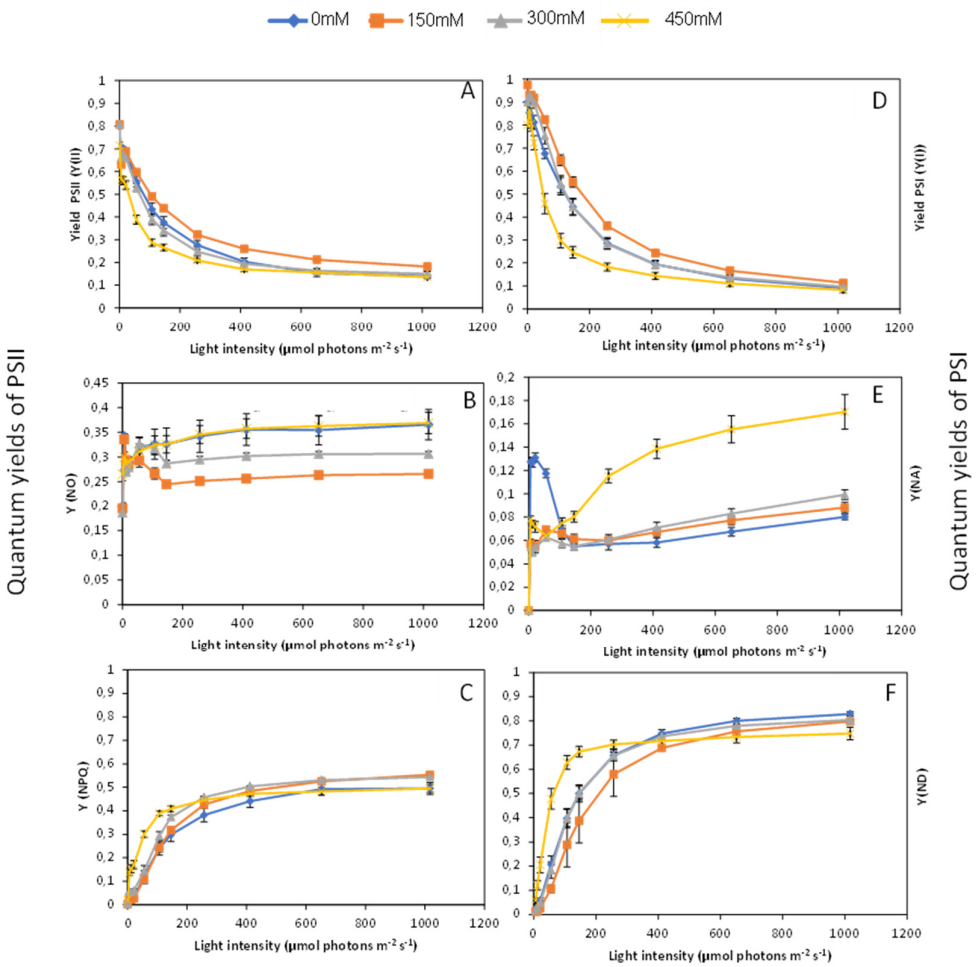

Figure 4. Light-response curves of quantum efficiency of PSII Y(II) (A), fraction of absorbed light lost by either constitutive thermal dissipation and via fluorescence Y(NO) (B), the fraction of absorbed light dissipated thermally via ΔpH and xanthophyll-regulated process Y(NPQ) (C) quantum efficiency of PSI Y(I) (D), the fraction of P700 that cannot be oxidized by a saturating pulse in a given state Y(NA) (E) and the fraction of P700 oxidized in a given state Y(ND) (F), in leaves of date palm grown under greenhouse conditions for 4 months. Treatments were: salt-free conditions (0 mM), plants exposure to 150, 300 and 450 mM NaCl

Plants exposed to 300 mM NaCl exhibited similar values of quantum yields PSII compared to those of control plants, except for the decrease of the Y (NO). Lower Y (NO) together with a high Y (NPQ) is a good indicator that the PSII was protected from the 300 mM NaCl salt concentration. In addition, similar quantum yields of PSI were recorded compared to that of controls which may indicate that PSI was protected from the over-reduction normally caused by the salt stress.

Contrary to plants exposed to the salt concentrations of 150 and 300 mM NaCl, those exposed to 450 mM NaCl, showed an over-reduction of the PSI, indicated by our observance of lower Y(I) and higher Y(NA) compared to controls (Figure 3 D, E). The levels of Y (NO) and Y (NPQ), however, were similar to those of

control plants (Figure 4B, C). In addition, a decrease in the proportion of PSII centers in the open state (with QA oxidized) (qL), a parameter proportional to the amount of PSII centers with a reduced QA primary acceptor (QA) at low PAR, suggests that at 450 mM most PSII centers had a reduced primary acceptor, thus increasing the risk of photoinhibition. Also, the decrease in the maximum quantum yield of PSII photochemistry (Fv/Fm ratio) at the same concentration is indicative of the initiation of the process of photoinhibition in the PSII system.

Radar plot analysis

In order to further analyse the response of date palm plants to salt conditions, the fluorescence parameters derived from the chlorophyll fluorescence curves were presented as radar plots (Fig. 5). All parameters were normalized to the reference value (i.e., the control treatment value of the corresponding parameter), which was assigned a numerical value of 1. The descriptive analysis using radar plots confirmed that date palm plants treated with 150 and 300 mM NaCl and those treated with 450 mM responded differently. Indeed, at 450 mM NaCl and low PAR (12-24 µmol quanta m^{-2} s^{-1}), the plants responded by a 4-fold increase in the functioning of the PSI (Y (ND) and P700ox) and an 8-fold one for PSII (NPQ and Y (NPQ)), compared to controls. However, no changes were observed for the rest of the treatments (Figure 5A, B). When PAR was between 257 to 1017 (µmol quanta m^{-2} s^{-1}), plants treated with 450 mM NaCl enhanced energy dissipation in the form of Y(NA), while, those treated with 150 and 300 mM induced both NPQ and Y(NA) (Figure 5 E-H).

Non-enzymatic antioxidant analysis

For leaves analysed at the end of experiment (4 months), TPC and flavonoids remained unchanged in plants irrigated with 150 mM NaCl compared to non-stressed plants, while an increase in TAA and proline was observed. Plants irrigated with 300 mM of NaCl, showed an increase in all the assessed non-enzymatic antioxidant parameters (Table 4). However, for the highest NaCl concentration (i.e., 450mM) TPC, flavonoids and TAA decreased abruptly (Table 4). For the roots, these parameters decreased at all salt concentrations compared to control plants. However, the decrease was more dramatic in plants stressed with 450 mM NaCl (Table 4). In contrast, the proline content increases with the water salinity.

Table 4. Effects of increasing NaCl concentrations on biochemical parameters Total phenolic compounds (TPC), flavonoids, Total antioxidant activity (TAA) and proline concentrations) in roots and leaves in date palm plants at the end of the experiment

Samples	Treatments	TPC (mg GAE g^{-1} DW)	Flavonoids (mg CE g^{-1} DW)	TAA (mg GAE g^{-1} DW)	Proline (µmol g^{-1} FW)
Leaves	0 mM NaCl	29.20 ± 2.90 b	7.88 ± 1.01 b	43.67 ± 4.30 b	12.49 ± 0.15 c
	150 mM NaCl	30.72 ± 3.00 b	7.96 ± 0.72b	50.05 ± 5.10 a	12.97 ± 0.15 b
	300 mM NaCl	43.03 ± 4.90 a	9.74 ± 0.22 a	52.80 ± 5.09 a	12.80 ± 0.15 b
	450 mM NaCl	4.40± 0.40 c	2.87 ± 0.26 c	31.29 ± 4.01 c	13.19 ± 0.20 a
Roots	0 mM NaCl	40.23 ± 3.01 a	2.72 ± 0.48 a	2.90 ± 0.60 a	3.89 ± 0.16 c
	150 mM NaCl	13.81 ± 0.33 b	1.13 ± 0.18 b	2.77 ± 0.55 a	5.40 ± 0.16 b
	300 mM NaCl	13.28 ± 0.31 b	0.85 ± 0.21 c	2.31 ± 0.40 b	5.99 ± 0.36 b
	450 mM NaCl	7.73± 0.70 c	0.77 ± 0.15 c	2.15 ± 0.38 b	6.92 ± 0.40a

Values are the means ± standard error of four plants per treatment. Means within a column with the same letter are not significantly different at $p< 0.05$ (ANOVA and least significant difference, Tukey's test).

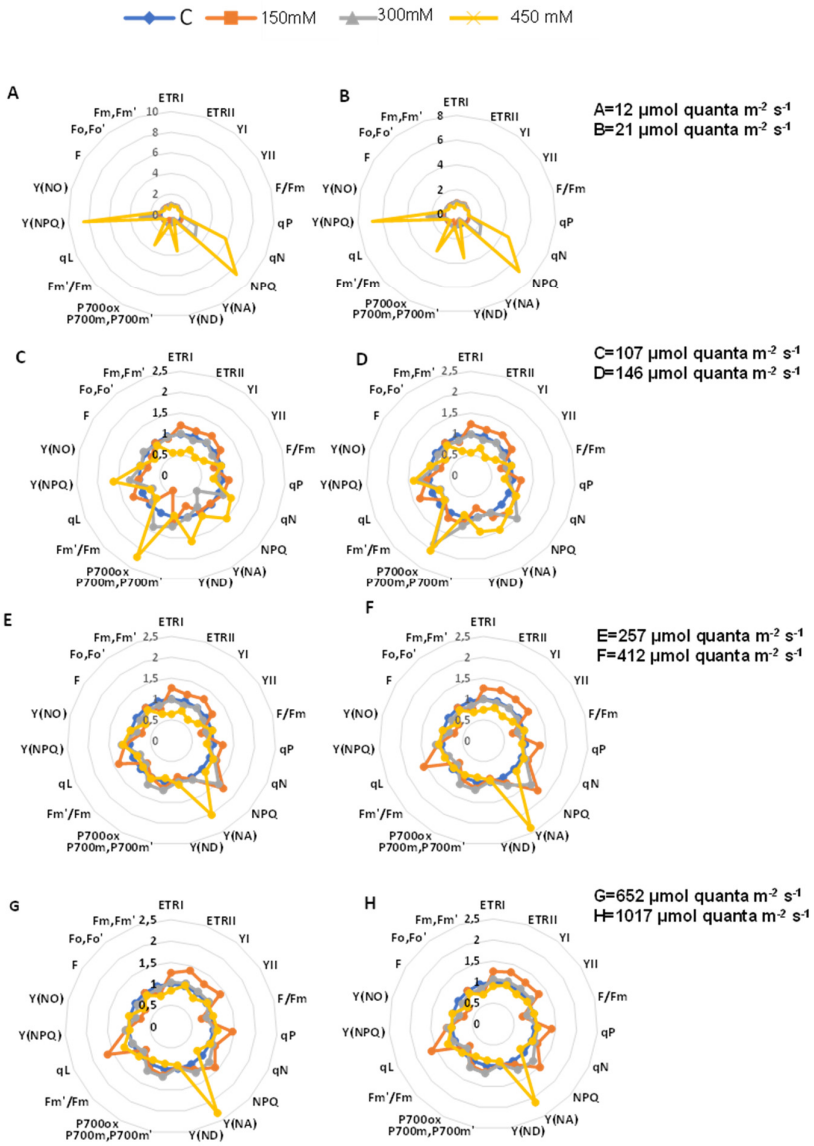

Figure 5. Integrated representation of radar plots in date palm plants exposed to different salt stress
The data refer to the variables related to PSI and PSII according to different light intensity A: 12; B: 21; C: 107; D: 146; E: 257; F: 421; G: 652; H: 1017 µmol quanta m^{-2} s^{-1}. All parameters were normalized to the reference value (i.e. the control treatment value of the corresponding parameter), which was assigned a numerical value of 1.

Discussion

Photosynthetic mechanisms involved in overcoming salt stress of ex vitro date palm plants

The tolerance of plants to salinity stress strongly depends on the plasticity of photosynthesis which, in combination, with other physiological and biochemical mechanisms, strongly affects plant growth and development (Abreu *et al*., 2013). Salinity is known to damage photosynthesis, producing an obviously negative effect on plant growth and development. For example, under salt stress conditions, an imbalance is created between photosynthetic electron transport chain and the Calvin cycle reactions, leading to an over-reduction and excess of the energy absorbed within the thylakoids (Cerqueira *et al*., 2019).

To overcome salt stress, date palm plants exposed to 150 mM NaCl exhibited high net CO_2 assimilation rate (A) and internal CO_2 concentration (Ci), similar to control plants. whilst, they showed a decrease in stomatal conductance (gs) and transpiration rate (E) in order to maintain the water content in plants (Sarwat and Tuteja, 2017; Chalbi *et al*., 2021). The increase in the intrinsic (A/gs) and instantaneous (A/E) water use, observed in our experiment for 150 mM NaCl, suggests that date palm plants maintained higher photosynthesis rate despite stomatal closure.

The parameters of both photosystems PSII and PSI, evaluated by measuring chlorophyll fluorescence transients, provided more insight on the tolerance adaptation of date palms to salinity. Some fluorescence parameters were increased in plants exposed to 150 mM NaCl. The increase in P700ox might suggest that PSI functioning was induced and photoprotection from excess energy was efficient, since it has a fundamental role in the decrease of ROS production and the inhibition of PSI over-reduction (Wada *et al*., 2019). Actually, it is considered as a key regulator process for the protection of PSI from photoinhibition (Miyake *et al*., 2005; Farhat *et al*., 2021).

With 300 mM NaCl, growth and leaf gas exchange were slightly reduced; however, the majority of the photosynthesis-related variables did not show significant difference compared to control plants, and some of them (NPQ and Y (NPQ) and P700ox) even increased. Considering the results obtained, we suggest that palm tree plants could survive and grow at 300 mM NaCl since PSII was not damaged, as reflected by increase in the efficiency of energy removal by the non-photochemical pathway (NPQ and Y (NPQ)), and PSI was protected by increasing P700ox (Wada *et al*., 2019). Our findings were corroborated with what was reported previously by Al-Mulla *et al*. (2013), where the degree of tolerance of one-year-old 'Barhee' seedlings to saline irrigation water went up to 20dS/m (around 275 mM NaCl). Despite the origin of our plants (tissue culture), they shared a similar, or slightly higher, degree of tolerance to that of seedlings.

Plants exposed to 450 mM NaCl, showed a reduction in all gas exchange parameters. The reduction in CO_2 assimilation could probably be due to stomatal closure and lead to a decrease in the photosynthesis rate (Flexas *et al*., 2007; Chaves *et al*., 2009). Similar results were reported by other researchers for date palm plants grown under high saline conditions (Sperling *et al*., 2014; El Rabey *et al*., 2016). The decrease in A/Ci ratio together with a decrease in the intercellular CO_2 concentration indicates that salt stress affected photosynthesis by metabolic limitations and might be linked to a decrease in Rubisco carboxylase activity (Christophe *et al*., 2018; Silva *et al*., 2011) or to damage in the photosynthetic apparatus (Al Kharusi *et al*., 2019b; Hura *et al*., 2007; Sperling *et al*., 2014). This was confirmed in our case by the observed damages in PSII and PSI. The reduction of Fv/Fm obtained in our experiment suggests that PSII photosystem was damaged (Tikkanen *et al*., 2014). Furthermore, the decrease in P700m, P700m' might indicate an increase in a non-photochemical quenching process at or close to the reaction center (Gururani *et al*., 2015). The decrease in Y (II) indicates a compromised capacity of photochemical conversion accompanied by a reduction in the flow of electrons from PSII to PSI, as confirmed by a decrease in ETR (II) in our data. However, this was not efficient since those plants failed to dissipate the excess energy through the xanthophyll cycle pathway and there was no change in NPQ and qN. Additionally, the non-changeable Y (NO) curve means that date palm plants might have channelled the excess energy to photorespiration or other alternative electron sinks like the Mehler reaction. Indeed, several studies have proposed photorespiration as a wasteful process (Peterhanse and Maurino, 2011). Nevertheless, it was proven to be an electron sink under stressful conditions (Wingler *et al*., 2000), making it an effective pathway for excess energy elimination. With respect to the results here explained, we can suggest that PSII was damaged by high salt stress, since its potential activity and primary light energy conversion efficiency were obviously weakened. Thus, the process of photoinhibition was undoubtedly initiated. Besides, the PSI photosystem seems to also have been photo-inhibited, and the damage was obvious from the decrease in all the previously mentioned parameters. This might be related to the fact that stomatal closure at high salt concentration probably caused an enhancement of the oxygenase activity of Rubisco and an increase in

photorespiration that consumes a lot of ATP. Hence, the NADPH/ATP ratio could have increased and then causes PSI photoinhibition (Huang *et al*., 2018).

Our data showed that the photosynthetic response of date palm grown at the highest saline level (450 mM NaCl) was PAR- dependent, which is quite unusual since photosynthesis is always inhibited at high PAR (Wu *et al*., 2019). It seems that date palm tolerates better high salinity level at low light compared to high light, which may be related to the C3 nature of the plant (Li *et al*., 2021; Ye *et al*., 2013). This observation should be considered for further investigation since the adverse effects of salt on photosynthesis can be partially alleviated by changing light spectra in strawberry plant (Esmaeilizadeh *et al*., 2021). In cynobacterium *Spirulina platensis* cells, the response of PSII to salt stress consisted of two distinct phases. The first phase which was independent of light, was characterized by a rapid decrease in PSII activity followed by a subsequent recovery. In the second phase, a progressive decrease in PSII activity was observed only in the light, and the higher incubation light intensity, the greater the decrease in PSII activity (Lu and Zhang, 2000). In our case, it is possible that the variation in PSII and PSI activity may not result from salt stress itself but from the interaction of high salt stress and light.

Antioxidant molecules and proline involvement in date palm responses to salt stress

As a result of PSII and PSI damage caused by the highest saline concentration, the assessment of the antioxidant activity is considered a method to determine the plant defense response against harsh environmental conditions (Gill and Tuteja, 2010). Phenolics and flavonoids are non-enzymatic antioxidants with high scavenging potential against free radicals produced by the oxidative stress which results from plant growth under salt and drought conditions (Baskar *et al*., 2018; Naikoo *et al*., 2019). In our experiment, the exposure of date palm plants to 150 mM of NaCl, showed no significant effect on phenolics and flavonoids concentrations, and at higher water salinity an increase was observed instead. Salinity stress produces many excited energy, and one way to disperse it is to synthesize polyphenols, particularly in photosynthetic structures (Waśkiewicz *et al*., 2013). Increased biosynthesis of flavonoids is also associated with an increase in glutathione s-transferase enzyme, which is involved in the transfer of flavonoids to the vacuole to inhibit active oxygen species (Czerniawski and Bednarek, 2018; Hosseini *et al*., 2021). The results also showed that all salt treatments increase antioxidant activity in leaves of *ex vitro* date palm. Kaur *et al*. (2014) have shown that antioxidant activity plays an essential function in salinity stress tolerance by trapping and preventing free radicals. All these results suggest a key role of non-enzymatic antioxidants in the tolerance mechanism of date palm to salinity stress.

Unlike the leaves, the saline condition resulted in a decrease in the concentration of total phenolics, flavonoids and antioxidant capacity in roots. This assumes that under high salinity, root phenylpropanoid metabolism is possibly depressed, since phenolic compound was equal or lower than that of non-stressed plants. This could be ascribed to the injury of the cell components caused by the toxic ions at root level, and/or to a priority of energy disposal for the synthesis of phenolic compounds in the leaves, rather than the roots, in order to protect the photosynthetically active leaves (Petridis *et al*., 2012).

The adaptation to salt 150 mM NaCl conditions may also be caused by the evolution of an osmoregulation mechanism, essentially shown by the proline increase (Yaish, 2015; Farhat *et al*., 2021). The increase in proline concentrations in roots and leaves of date palm plants subjected to different saline concentrations and drought stress agreed with findings reported by other researchers for date palm plants grown under similar conditions (Sané *et al*., 2005; Shareef *et al*., 2020). Proline accumulation has been reported after exposure to various abiotic stresses (often interconnected) such as salt, drought, high temperature, low temperature and UV irradiation, among others (Siripornadulsil *et al*., 2002; Hong *et al*., 2000), and this capacity to accumulate proline has been correlated with improved plant performance. Although there is a debate about whether proline accumulation is a stress symptom or a response to reduced growth and metabolism rather than a stress-adaptive response, several potential roles of proline in plant stress tolerance

have been reported (Verbruggen and Hermans, 2008). Hence proline could be involved in reducing photo damage in the thylakoid membranes by scavenging and/or reducing the production of ROS (Kavi Kishor *et al*., 2005).

Conclusions

The obtained results showed, for the first time, the involvement of the photosynthesis machinery in the alleviation of salt stress in date palm *ex vitro* plants. Plants grown under 150 mM NaCl had greater ability to manage excess light energy, at 300 mM NaCl plants managed to protect the PSI and PSII photosystems, while at 450 mM NaCl an over-reduction of both photosystems was observed. It seems that the major mechanisms used by the date palm plants to tolerate salt stress are (in addition to ion homeostasis regulation and biomass allocation found in our previous study), photosynthesis protection and the regulation of oxidative damage by the increase of proline and non-enzymatic antioxidants synthesis. These traits could have accounted for the tolerance of the date palm plants of up to 300 mM NaCl and perhaps even 450 mM, since plant growth and photosynthesis were reduced but plant death did not occur. Our studies using *ex vitro* plants provide significant knowledge regarding the functioning of the photosystem machinery and its potential consequences for plant growth under salinity stress.

Authors' Contributions

BSH and SH: Conceptualization, Software, Validation, Investigation, Data curation, Writing–original draft, Writing – review & editing, Supervision. MS and HBJ: Methodology, Formal analysis. SC, RG and FZ: Formal analysis, Data curation, Methodology. NB, RD, ND: Formal analysis, supervision, and Methodology. HFR, AS, TB, JVJN: Supervision, Writing – review & editing.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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