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Physiological responses of date palm (*Phoenix dactylifera*) seedlings to seawater and flooding

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Summary

- In their natural environment along coast lines, date palms are exposed to seawater inundation and, hence, combined stress by salinity and flooding.
- To elucidate the consequences of this combined stress on foliar gas exchange and metabolite abundances in leaves and roots, date palm seedlings were exposed to flooding with seawater and its major constituents under controlled conditions.
- Seawater flooding significantly reduced CO_2 assimilation, transpiration and stomatal conductance, but did not affect isoprene emission. A similar effect was observed upon NaCl exposure. By contrast, flooding with distilled water or $MgSO_4$ did not affect CO_2/H_2O gas exchange or stomatal conductance significantly, indicating that neither flooding itself, nor seawater sulfate, contributed greatly to stomatal closure. Seawater exposure increased Na and Cl contents in leaves and roots, but did not affect sulfate contents significantly. Metabolite analyses revealed reduced abundances of foliar compatible solutes, such as sugars and sugar alcohols, whereas nitrogen compounds accumulated in roots.
- Reduced transpiration upon seawater exposure may contribute to controlling the movement of toxic ions to leaves and, therefore, can be seen as a mechanism to cope with salinity. The present results indicate that date palm seedlings are tolerant towards seawater exposure to some extent, and highly tolerant to flooding.

Introduction

Different plant species are highly variable with respect to their responses to salt stress. According to their tolerance, plants are divided into glycophytes and halophytes. Glycophytes are highly susceptible to salt stress, whereas halophytes can grow at high concentrations of salt in their environment (Acosta-Motos *et al.*, 2017; White *et al.*, 2017; Van Zelm *et al.*, 2020). The detrimental effects of salinity on plants are dependent on: (1) the potential for Na^+ exclusion from leaf blades, (2) mechanisms compensating for osmotic stress, and (3) metabolic tolerance (Munns & Tester, 2008). Generally, plants tolerant to salt stress have higher concentrations of stress-related metabolites under normal growth conditions and/or accumulate larger amounts of protective metabolites under salt stress. Some halophytes (e.g. many euhalophytes) can accommodate large Na^+ concentrations in their shoots (White *et al.*, 2017). Other salt-tolerant species, that is mangroves, have developed specific apoplastic barriers that can function as filters against salt uptake by their roots, thereby minimising salt movement into the plant (Pannaga *et al.*, 2014). Although molecular pathways underlying salt-adaptation mechanisms have been dissected and partially identified in some plant species, the mechanisms that mediate salt tolerance of date palms are still largely unknown (Yaish & Kumar, 2015; Al Kharusi *et al.*, 2017; Hazzouri *et al.*, 2020).

Date palm (*Phoenix dactylifera* L.) is an economically important perennial plantation crop in several arid and semiarid countries in North Africa, the Middle East and Central America (Chao & Krueger, 2007; Al-Khayri *et al.*, 2015; Yaish & Kumar, 2015; Yaish *et al.*, 2017). In their natural environment, date palms are exposed to a multitude of different stresses, for example drought, heat, air pollution and salinity (Shabani *et al.*, 2012; Yaish & Kumar, 2015; Du *et al.*, 2018; Hazzouri *et al.*, 2020). Although date palms are thought to be a salt-tolerant crop able to

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grow in soils up to 12 dS m^{-1} without showing symptoms of salt stress (Ramoliya & Pandey, 2003; Yaish & Kumar, 2015; Patankar *et al.*, 2019; Al-Harrasi *et al.*, 2020), salinity can lead to significant economic losses in date palms (Tripler *et al.*, 2011; Yaish & Kumar, 2015; Al-Muaini *et al.*, 2019). This is particularly observed in salt susceptible cultivars (Al Kharusi *et al.*, 2017, 2019a; Hazzouri *et al.*, 2020), which showed significantly declined photosynthesis, shoot and root growth and foliar water content under salt stress (Alhammadi & Edward, 2009; Al Kharusi *et al.*, 2017). Salt stress affects their growth and productivity independently of drought and heat (Alhammadi & Kurup, 2012), and constitutes an increasing problem in date palm plantations due to irrigation and fertilisation. This is regularly observed in arid and semiarid regions with high evaporation rates and limited fresh water resources required to leach salt into deeper soil layers (Alhammadi & Kurup, 2012; Darwesh, 2013). For instance, 33.6% of the United Arab Emirates area is salinised, particularly along the coastal regions, where salinity is higher than 200 dS m^{-1} (Abdalla *et al.*, 2015).

In addition to salt stress, both in plantations and in their natural environment, date palms are frequently exposed to water saturation of the soil, either through irrigation or flooding, and hence oxygen deficiency in the rhizosphere (Abul-Soad, 2010; Carr, 2013; Darwesh, 2013; Kreuzwieser & Rennenberg, 2014; Hazzouri *et al.*, 2020). As a consequence, photosynthesis can be impaired either by stomatal or nonstomatal mechanisms, or both (Brugnoli & Björkman, 1992). Reduced stomatal conductance in response to flooding results in impaired gas exchange and, hence, reduced CO_2 availability for photosynthesis as reported in many woody plant species (Kreuzwieser & Rennenberg, 2014; Wang *et al.*, 2017). Nonstomatal responses to flooding include reduced foliar pigment concentrations, reduced ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) abundance and activity, accumulation of carbohydrates in leaves due to reduced export in the phloem, and impaired nutrient uptake by roots (Kreuzwieser & Rennenberg, 2014). Conversely, plants have developed several physiological strategies to mitigate stress by root anoxia, including downregulation of numerous energy consuming anabolic processes to overcome the energy crisis mediated by reduced/impaired respiration, stimulation of fermentation as alternative to respiratory energy production, and accelerating the glycolytic flux at the expense of stored carbohydrate (Ferner *et al.*, 2012; Kreuzwieser & Rennenberg, 2014). The toxic ethanol produced by fermentation is transported out of the roots either by exudation into the rhizosphere and/or by allocation to the leaves where it is emitted into the atmosphere, converted to volatile acetaldehyde and/or acetate, and re-metabolised to sugar by gluconeogenesis (MacDonald & Kimmerer, 1993; Kreuzwieser *et al.*, 1999; Tadege *et al.*, 1999; Copolovici & Niinemets, 2010). However, no information on the response of photosynthesis of date palm leaves to flooding has been reported.

As date palms are not only found at inland sites, but also along the coast line, in their natural environment date palms are irregularly exposed to flooding with seawater. The combined stress of salinity and flooding presented by seawater inundation is of particular significance for developing seedlings, which do not have access to deeper soil layers containing water with reduced salt concentrations (Alhammadi & Kurup, 2012). Numerous studies have started to elucidate the mechanistic basis for abiotic stress tolerance in date palms, as well as the intraspecific variations. However, information on the responses of date palms to salinity and flooding combined stresses is still limited (Srikanth *et al.*, 2016; Al Kharusi *et al.*, 2019a; Hazzouri *et al.*, 2020). Net CO_2 assimilation rates of seawater inundated cabbage palm (*Sabal palmetto*) seedlings dramatically decreased, but continuous root zone inundation appeared to ameliorate the effects of salinity on photosynthesis (Williams, 1996). Ye *et al.* (2010) found increased activities of superoxide dismutase, peroxidase and decreased malondialdehyde content in inundated seedlings of the mangrove *Kandelia candel*. Barnuevo & Asaeda (2018) found significantly lower amounts of reactive oxygen species and antioxidant activities in *Rhizophora stylosa* seedlings in noninundated than in inundated conditions. In this mangrove species, inundation imposed a higher degree of stress on photosynthesis than exposure to salt alone (Barnuevo & Asaeda, 2018). However, information that clearly disentangles the effects of flooding and salt exposure on stomatal conductance has not been reported.

The current study was aimed to elucidate the effects of sea salt exposure on gas exchange and metabolic processes of date palm seedlings and to separate the contributions of salt exposure and flooding. For this purpose, seedlings were inundated with seawater and the exchange of H_2O, CO_2 and isoprene as well as metabolite abundances were determined in a first set of experiments. In a second set of experiments, seedlings were inundated with solutions of different salts to identify the components of seawater responsible for changes in gas exchange. In the third experiment, the second experiment was repeated with detached leaves to distinguish between compounds acting on root and leaf processes. We hypothesised that: (1) flooding with seawater mediates stomatal closure with reduced isoprene emission, (2) stomatal closure upon flooding with seawater is a consequence of increased sulfate uptake taken up from the seawater by the roots, (3) salt effects are counteracted by the accumulation of compatible solutes and flooding, and (4) there are modified shoot–root interactions on compatible solutes and ions.

Materials and Methods

Seed germination and plant material

Seedlings of the commercial date palm (*Phoenix dactylifera* L) cultivar Khodry (Aleid *et al.*, 2015) were germinated from seeds provided by the Research Station of the Faculty of Science of Food and Agriculture, King Saud University, Riyadh, Saudi Arabia. For germination, seeds were cleaned with tap water and air dried. After surface sterilisation with 70% ethanol for 20 min with shaking at 300 rpm, the seeds were washed twice with sterilised distilled water and, subsequently, soaked in sterilised distilled water at room temperature in the dark. After 2 d, seeds were placed in sterile plastic Petri dishes (100 × 100 × 20 mm, L × W × H) (Sarstedt, Nümbrecht, Germany) with two layers of moistened filter paper on the bottom, 12 seeds per dish, and

sealed with Parafilm (Bemis Company, Inc., Chicago, IL, USA). Petri dishes with seeds were incubated in a culture room at 27 ± 2°C, in the dark. After 4 wk, the germinated seeds were carefully transplanted individually into 4.5 l pots filled with 70% gravel (diameter 3–6 mm, Euroquarz GmbH, Dorsten, Germany) and covered with 30% multiplication substrate (Floragard Vertriebs-GmbH, Oldenburg, Germany). Seedlings were cultivated in a glasshouse with a 16 h : 8 h, light : dark cycle (200 ± 50 μmol m^{-2} s^{-1} PFD) at ambient temperature and humidity at the Chair of Tree Physiology, University of Freiburg, Germany (48°01′N, 7°83′E, 236 m asl). Seedlings were watered with tap water once per wk.

Salt exposure and flooding experiments

Three experiments were conducted in the present study: (1) an experiment in which whole plants were inundated with seawater and the exchange of H_2O, CO_2 and isoprene, together with metabolite abundances, were determined, (2) an experiment in which solutions of different salts were applied to whole plants with or without flooding, and (3) an experiment in which different salts were applied to detached leaves.

Whole plant experiments

Six plants with an average cotyledon height of 23 cm, were transferred to a walk-in climate chamber (ThermoTec, Weilburg, Germany) 2 wk before the start of the experiment for acclimation. Growth conditions in the climate chamber were set to day : night cycles of 12 h : 12 h (from 09:00 h to 21:00 h) at 500 μmol m^{-2} s^{-1} PFD during the light period, at a constant temperature of 38°C and a relative humidity of 60%. Plants were watered daily with 50 ml distilled water.

The cotyledon of each individual plant was placed into a custom-designed inert borosilicate glass cuvette (3 cm diameter and 30 cm height, *c.* 0.21 l, round bottomed) and closed with FEP foil (PTFE-Spezialvertrieb, Stuhr, Germany). The cuvettes were supplied with hydrocarbon free air at a constant air flow of 500 ml min^{-1}, 30% relative humidity and 400 ppm CO_2 mixing ratio. The cuvettes were connected to the measuring system with heated (50°C) and isolated perfluoralkoxy-copolymer (PFA) tubing lines (AT125-030, 1/8 inch; Wolf-Technik, Stuttgart, Germany). After 2 wk acclimation, roots of three seedlings were submerged into 3.5% (w/v) seawater made by dissolving sea salt from the Dead Sea (origin: Jordan; Schwarzmann GmbH, Laaber, Germany; for chemical composition refer to Supporting Information Table S1) in distilled water, whilst the other three plants were supplied 50 ml distilled water per day and served as controls. CO_2/H_2O gas exchange and the emission of isoprene by the cotyledons were determined. At the end of the experiment, the leaf area was measured using a scanner (CanoScan LiDE 110, Canon, Krefeld, Germany) and the GSA Image Analyser v.4.09 software (Software Development and Analytics GSA, Rostock, Germany). Subsequently, roots and leaves of the seedlings were harvested, weighed separately, immediately frozen in liquid nitrogen and stored at −80°C for further analysis. The experiment was repeated once using exactly the same procedure with another six plants.

To distinguish the effects of sea salt constituents and flooding on stomatal closure, six date palm seedlings each with two or three leaves (average height 30.9 cm; one plant per pot, 15 × 15 × 20 cm, filled with 70% gravel and covered with 30% multiplication substrate) were subjected to one of six treatments, distilled water with and without flooding as controls, 3.5% NaCl with and without flooding, 3.5% sea salt with flooding and 25 mmol $MgSO_4$ with flooding. For controls and NaCl exposure without flooding, 50 ml distilled water and 3.5% NaCl solution was supplied every day. The electrical conductivities of solutions were measured with a conductivity meter (GLM 020A, Greisinger Electronic GmbH, Regenstauf, Germany) and provided in Table S2. For flooding treatments, plants were placed into 70 × 45 × 30 cm containers. Distilled water or salt solutions were applied up to *c.* 1 cm above the surface of the soil substrate. Assimilation, transpiration and stomatal conductance were recorded 4 d after the beginning of the treatments with the portable GFS-3000 system (Heinz Walz GmbH, Effeltrich, Germany) at 2000 μmol m^{-2} s^{-1} PFD, cuvette temperature of 25°C, relative humidity of 45%, and an air flow rate of 700 ml min^{-1}. To minimise diurnal effects, three plants from each treatment were measured per day from 10:00 h to 14:00 h.

Feeding of detached cotyledons via the petiole

To test the effects of the most abundant anions in seawater, that is sulfate and chloride, on stomatal aperture, the transpiration rate of detached cotyledons of date palm seedlings were measured as described previously by Malcheska *et al.* (2017). After detaching, leaf petioles were placed directly into a solution containing of 0.5 mmol $MgCl_2$ and 0.06 mmol $Mg(NO_3)_2$ adjusted to pH 5.5 for equilibration of transpiration (preincubation). Electrical conductivities of solutions used for this experiment are shown in Table S2. The solution did not contain potassium (K^+) to avoid its influence on stomatal aperture. When detached leaves reached a stable transpiration rate after *c.* 60 min, the solution was replaced by one of the following solutions: 10 mmol $MgSO_4$, 5 mmol $MgCl_2$, 3.5% NaCl or 3.5% sea salt (w/v, g ml^{-1}). The transpiration rates were continuously recorded at 1500 μmol m^{-2} s^{-1} PFD, a cuvette temperature of 30°C, a relative humidity of 30% and an air flow rate of 700 ml min^{-1} by a portable GFS-3000 system (Heinz Walz GmbH) for 1 h in five or six biological replicates. The transpiration rates measured after 60 min of incubation were calculated as the percentage of the transpiration rate determined during preincubation.

Isoprene emission and gas exchange measurements

A 4000 ultra Proton-Transfer-Reaction – Time-of-flight – Mass-Spectrometer (PTR-TOF-MS, Ionicon Analytic, Innsbruck, Austria) was used for real-time isoprene flux measurements (Graus *et al.*, 2010), which was further connected to a differential infrared gas analyser (LI-7000 CO_2/H_2O Analyser; Li-Cor, Lincoln, NE, USA) for determination of CO_2 and H_2O gas

exchange. More information on the mode of operation of this system is found elsewhere (Fasbender *et al.*, 2018; Yáñez-Serrano *et al.*, 2018). The PTR-TOF-MS was operated at 2.7 mbar drift pressure, 600 V drift voltage, at an E/N of 120 townsend (Td), and the drift tube heated to 80°C. The PTR-TOF-MS data processing included: (1) correction for nonextending and extending dead times as well as the correction for Poisson statistics (Titzmann *et al.*, 2010), and iterative residual analysis and cumulative peak fitting (Müller *et al.*, 2013) using the DATA TOF ANALYZER software v.4.48; (2) normalisation of the data to primary ions and water; (3) background subtraction from the signal of the empty cuvette; and (4) application of calibration factors.

In the present study, we focused on *m/z* 69.07, which was identified as isoprene. The calibration factors were obtained from humidity dependent calibrations from a gravimetrically prepared multicomponent gas standard (Ionicon Analytik, Innsbruck, Austria).

The equation used for the calculation of the fluxes was:

$$e = \frac{u_i}{s} \times (c_o - c_i)$$

where e is the volatile emission rate in nmol m^{-2} s^{-1}, u_i is the molar flux in the cuvette inlet in mol s^{-1}, s is the leaf area of measured branch in m^2, c_o is the mixing ratio at the outlet of the cuvette and c_i is the mixing ratio at the cuvette inlet, both in mol mol^{-1}. More detailed information can be found in Yáñez-Serrano *et al.* (2019).

Biochemical analyses

Determination of anions and elements Anions were extracted from 50 mg of leaf or root powder together with 100 mg PVPP in 1 ml double-distilled H_2O for 1 h at 4°C under continuous shaking. The extracts were centrifuged for 10 min at 14 000 ***g*** after boiling for 10 min to precipitate proteins. The anions nitrate (NO_3^-), phosphate (PO_4^{3-}) and sulfate (SO_4^{2-}) were determined by automated anion chromatography with an ion chromatography system (DX 120, Dionex, Idstein, Germany) as described previously (Du *et al.*, 2018). Sodium salts of nitrate, phosphate and sulfate were used as standards for quantification.

Element concentrations of plant materials were determined following acid digestion using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) as described in Du *et al.* (2018). Briefly, freeze dried tissue samples were placed in an oven at 70°C overnight before analysis. About 50 mg dried subsamples were weighed and digested in closed vessels using a microwave digester (MARS Xpress; CEM Microwave Technology, Buckingham, UK). Samples were first digested with 10 ml concentrated nitric acid (HNO_3), before 3 ml of 30% H_2O_2 was added to each vessel to complete digestion. Digested samples were diluted with MilliQ water before element analyses. Total K, Ca, Mg, P, S, Na, Cl, Fe, Mn, Zn, Cu and Ni concentrations were determined on digested material by ICP-MS (ELAN DRCe; PerkinElmer, Waltham, MA, USA). Blank digestions were performed to determine background concentrations of elements and a tomato leaf standard (Reference 1573a; National Institute of Standards and Technology, NIST, Gaithersburg, MD, USA) was used as an analytical control. For the determination element concentrations of Dead Sea salt, 1 g sea salt powder was dissolved in MilliQ H_2O acidified with 4.14% nitric acid, and filled up to 500 ml using a volumetric flask. Subsequently, the solution was filtered using a 0.2 µm Nalgene syringe filter (Thermo Scientific, Erlangen, Germany) to remove insoluble components. The elements in sea salt were determined and quantified following the same procedures as for plant materials.

Extraction and determination of water-soluble metabolites by GC-MS Extraction of water-soluble metabolites was performed as previously described (Du *et al.*, 2016). Briefly, 50 mg of frozen leaf or root powder was extracted with 600 µl cold pure methanol and 60 µl 0.2 mg ml^{-1} of the internal standard ribitol was added. After short vortexing, the solutions were heated to 70°C and shaken at 1200 ***g*** (Eppendorf Thermomixer; Eppendorf AG, Hamburg, Germany) for 10 min. After centrifugation (14 000 ***g***, 5 min), aliquots of 500 µl supernatant were combined with the same volume of MilliQ water and cold chloroform. The mixtures were vortexed and centrifuged for another 5 min. Subsequently, 100 µl aliquots of the methanol phase were transferred to new tubes and dried in a freeze dryer for 48 h (Christ Alpha 2–4; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). For derivatisation, the dried extracts were dissolved in 20 µl of a 20 mg ml^{-1} solution of methoxyamine hydrochloride in anhydrous pyridine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The solutions were incubated at 30°C for 90 min with shaking at 1400 ***g***. Thereafter, 40 µl *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA; Sigma-Aldrich) were added and the mixtures were incubated at 37°C for another 30 min with shaking at 1200 ***g***. Finally, aliquots of 50 µl of these solutions were transferred into glass vials with inserts and sealed ready for GC-MS analysis.

Samples together with a mixture of n-alkane (C8-C20, saturated alkane mixture; Sigma-Aldrich) for retention index calibration were measured using a GC-MS system (Agilent GC 6890N coupled to a 5975C quadrupole MS detector; Agilent Technologies, Palo Alto, CA, USA) equipped with an autosampler (MultiPurpose Sampler MPS2; Gerstel, Mülheim, Germany). The settings of the GC-MS system were as described by Du *et al.* (2016). Peak detection and alignment was performed with the Quantitative Analysis Module of the Agilent MASSHUNTER software (Agilent Technologies). A relative concentration of metabolites was calculated as the peak areas of the chromatograms after normalisation using the peak area of the internal standard, ribitol and the dry weight of samples. Artefact peaks and common contaminants were identified and omitted by analysis of 'blank' samples prepared and analysed in the same manner.

Statistical analyses

Statistical analysis of differences in isoprene emission rates, anion and element concentrations, metabolite abundances between

seawater flooding and controls were performed using Student's *t*-test using the software package SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany) or Statistical Product and Service Solutions (Spss 22.0; IBM, Armonk, NY, USA). Nonparametric tests (Spss 22.0) were performed to examine the differences between seawater flooding and controls of gas exchange parameters, because these data failed to match normal distribution even after denary logarithm transformation. Transpiration rates of detached leaves exposed to different feeding solutions, as well as gas exchange parameters of different salt/water treatments with and without flooding were examined statistically using one-way ANOVA using the software package SigmaPlot 11.0. Raw data were transformed to denary logarithm to match normal distribution if required.

Results

Effects of salt and flooding treatment on CO_2/H_2O gas exchange and isoprene emission

In the first set of experiments, date palm seedlings were exposed to seawater flooding. Assimilation, transpiration, and stomatal conductance declined significantly ($P < 0.01$) 24 h after the onset of flooding (Fig. 1). Compared with controls receiving distilled water, assimilation declined to 37% from 7.6 to 2.7 μmol CO_2 m^{-2} s^{-1}, transpiration to 36% from 1.6 to 0.6 mmol H_2O m^{-2} s^{-1}, and stomatal conductance to 28% from 64.2 to 18.0 mmol H_2O m^{-2} s^{-1}, whereas dark respiration was maintained at −0.5 μmol CO_2 m^{-2} s^{-1}. These impacts of seawater flooding were maintained during the subsequent duration of the experiments (Fig. 1a–c). Despite this effect on stomatal conductance, significant differences in isoprene emissions between controls and seawater flooded seedlings were not observed (Fig. 1d). Isoprene emissions showed a strong diurnal pattern with virtually no emissions during the night and emissions of 20.7 ± 0.78 (SE, $n = 6$) nmol m^{-2} s^{-1} during illumination (Fig. 1d).

In a second set of experiments, both NaCl and seawater exposure reduced assimilation, transpiration and stomatal conductance greatly compared with controls with regular distilled water supply (Fig. 2). Upon NaCl treatment without or with flooding, assimilation, transpiration and stomatal conductance dropped to 28.4% and 6.4%, 31.3% and 12.6%, 23.8% and 10.3% of controls, respectively ($P < 0.001$; Fig. 2). Thus, flooding slightly increased the negative effect of NaCl on stomatal conductance, but no significant differences between NaCl with and without flooding were observed (Fig. 2). Flooding with seawater resulted in an even greater decline of transpiration and stomatal conductance to 4.5% and 4.8% of controls (Fig. 2b,c). By contrast, flooding with distilled water and $MgSO_4$ had no significant effect on CO_2/H_2O gas exchange and stomatal conductance (Fig. 2). No significant effects of salinity and/or flooding on biomass, root : shoot ratio as well as visible symptoms of stress were observed (Table S3; Fig. S1).

To identify the main component of seawater responsible for changes in stomatal closure, feeding experiments were conducted with detached leaves using different ion constituents of seawater (Fig. 3). The transfer of the detached leaves from 0.5 mmol $MgCl_2$ in the pretreatment solution to a 5 mmol $MgCl_2$ solution reduced transpiration to 58% (Fig. 3), but this decline was not statistically significant ($P = 0.186$). Exposure to 10 mmol $MgSO_4$, 3.5% NaCl or 3.5% sea salt solutions caused a greater decline of transpiration to 53%, 16% and 12% of that observed in the pretreatment solution, respectively, and was statistically significant ($P = 0.021$, 0.006 and < 0.001, respectively). However, no significant difference was found between the $MgCl_2$ and $MgSO_4$ treatments ($P = 0.661$; Fig. 3), indicating that sulfate made a negligible contribution to the reduction of transpiration upon seawater exposure of detached leaves.

Effects of seawater flooding on element and ion contents in leaves and roots

To assess the contribution of seawater components to the effect of seawater flooding on date palm seedlings further, element and anion contents were quantified in leaves and roots (Fig. 4; Table S4). Compared with controls, chloride (Cl) and sodium (Na) concentrations were both significantly increased in cotyledons and roots of seawater flooded date palm seedlings (Fig. 4a, b). This increase in concentration was 23.4- and 9.2-fold for Na, and 1.8- and 1.6-fold for Cl, in cotyledons and root, respectively. Both the Na and Cl taken up from seawater was partially retained in the roots. However, although the root Na concentration was greater than the cotyledon Na concentration, both root and cotyledon Cl concentrations were similar. In addition, seawater flooding decreased calcium (Ca) and manganese (Mn) contents in the roots significantly, but did not affect the concentrations of these elements in the cotyledons (Fig. 4c,d). Significant effects of seawater flooding on other elements and anions, including nitrate (NO_3^-), sulfate (SO_4^{2-}) and phosphate (PO_4^{3-}), were not observed (Table S4), supporting the view that sulfate does not contribute significantly to the effect of seawater flooding on stomatal conductance of date palm seedlings.

Effects of seawater flooding on metabolite contents in leaves and roots

Metabolic responses of leaves and roots were observed after 3 d of seawater flooding (Fig. 5). Sugar concentrations were slightly enhanced in roots, but mostly declined in cotyledons. Particularly, monosaccharides such as glucose, galactose ($P < 0.05$) and arabinose ($P < 0.01$), as well as the disaccharide α,α-trehalose ($P < 0.05$) declined significantly in cotyledons in response to seawater flooding. Compared with the controls, concentrations of sugar alcohols, that is galactinol and arabitol, as well as organic acids, that is fumaric acid, 2-methyl-malic acid and shikimic acid decreased significantly in the cotyledons of plants exposed to seawater flooding. Similarly, concentrations of two phenolic compounds, that is catechin and *cis*-ferulic acid, n-heptadecan-1-ol and gluconic acid-1,4-lactone also declined significantly in the cotyledons of plants exposed to seawater flooding (Fig. 5). In roots, seawater flooded seedlings had significantly ($P < 0.05$) higher concentrations of glucuronic acid, fumaric acid and

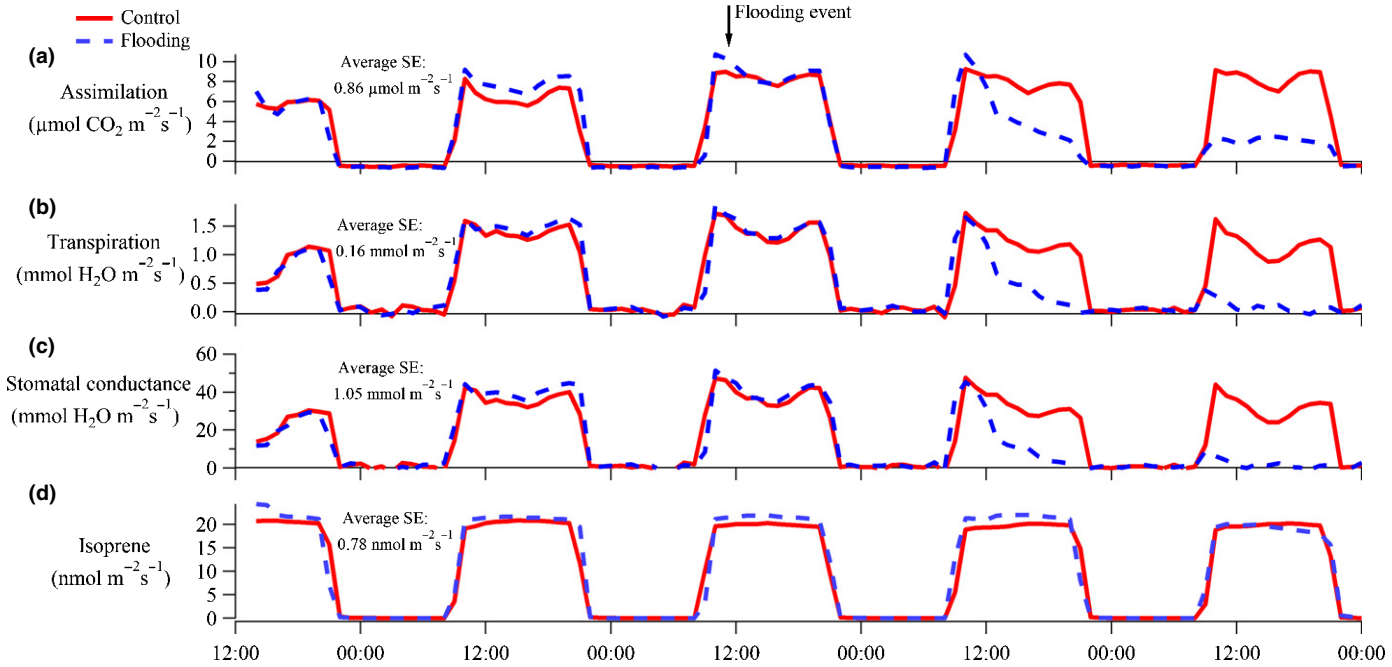

Fig. 1 Assimilation (a), transpiration (b), stomatal conductance (c) and isoprene emission (d) of control (red line) and seawater flooded (blue, dashed line) date palm seedlings. The arrow represents the onset of flooding. Data shown are the mean values of each parameter ($n = 5$ or 6).

sorbitol compared with controls (Fig. 5). Seawater flooding also resulted in increased abundance of many amino acids and other N compounds in cotyledons, except for aspartic acid and tyrosine, which were significantly reduced. The concentrations of phenylalanine and ethanolamine were significantly ($P < 0.001$) greater in roots of plants exposed to seawater flooding, as were concentrations of leucine, isoleucine, γ-aminobutyric acid (GABA) and butyro-1,4-lactam ($P < 0.05$) (Fig. 5).

Discussion

Salinity rather than flooding caused stomatal closure

Stomatal closure is a common response of plants to flooding and salinity, and the extent of this effect depends on the species' tolerance, for example to soil oxygen deficiency, low external water potential and internal nutrient imbalances (Hasegawa *et al.*, 2000; Munns & Tester, 2008; Kreuzwieser & Rennenberg, 2014). Diminished CO_2 assimilation during flooding caused by stomatal limitations and/or nonstomatal constraints has been documented in many trees species, for example citrus (*Carrizo citrange*) (Rodríguez-Gamir *et al.*, 2011), *Quercus robur* and *Fagus sylvatica* (Ferner *et al.*, 2012), *Q. lyrata* and *Q. falcata* (Pezeshki *et al.*, 1996), *Fraxinus excelsior* (Jaeger *et al.*, 2009), *Picea mariana* and *Larix laricina* (Islam *et al.*, 2003), and several mangroves (Krauss *et al.*, 2006). In contrast with the reduced stomatal conductance and/or CO_2 assimilation upon flooding observed in many other species, our results showed that flooding without salinity had no significant effect on stomatal conductance, photosynthesis or transpiration of date palm seedlings (Fig. 2), indicating that date palms are highly tolerant to flooding stress (Kozlowski, 1997; Abul-Soad, 2010; Carr, 2013). Similarly, stomatal conductance was either unaffected or only slightly reduced by flooding in the flooding tolerant ash species *Fraxinus angustifolia* and *F. excelsior* (Jaeger *et al.*, 2009). Naidoo (1985) also found that waterlogging alone had little effects on stomatal conductances of the mangrove species *Rhizophora mucronata* and *Bruguiera gymnorrhiza*.

However, strong depressions in stomatal conductance, as well as CO_2 assimilation and transpiration were observed in date palms seedlings upon seawater flooding (Fig. 1), and salt treatments, that is NaCl with or without flooding (Fig. 2). In line with our results, similar effects of seawater and salinity on stomatal conductance, net photosynthesis and transpiration were also reported in other date palm cultivars (Sperling *et al.*, 2014; Al Kharusi *et al.*, 2017, 2019a; Al-Harrasi *et al.*, 2018; Jana *et al.*, 2019) as well as in the mangroves *R. mucronata* and *B. gymnorrhiza* (Naidoo, 1985; Aziz & Khan, 2001). Osmotically driven stomatal closure was thought to be the main limitation to photosynthesis induced by salinity (Sperling *et al.*, 2014). No significant interaction of flooding and salinity with stomatal conductance or CO_2 assimilation was found in the present study (Fig. 2), which is consistent with previous observations in mangroves (Pezeshki *et al.*, 1990) and *Sagittaria lancifolia* (Pezeshki *et al.*, 1987). The maintenance of low stomatal conductance and transpiration under salt stress further indicated that date palms are to some extent salinity tolerant (Kozlowski, 1997; Aziz & Khan, 2001; Al Kharusi *et al.*, 2019b). Similar responses to seawater exposure are commonly observed in mangroves and are considered to be a feature of salt tolerance (Parida & Jha, 2010) that contributes to controlling the flux of toxic ions within the transpiration stream to the leaves (Acosta-Motos *et al.*, 2017). This result is not surprising, because salinity is not only a common feature of ecosystems in the tidal zone of coastlines, but also

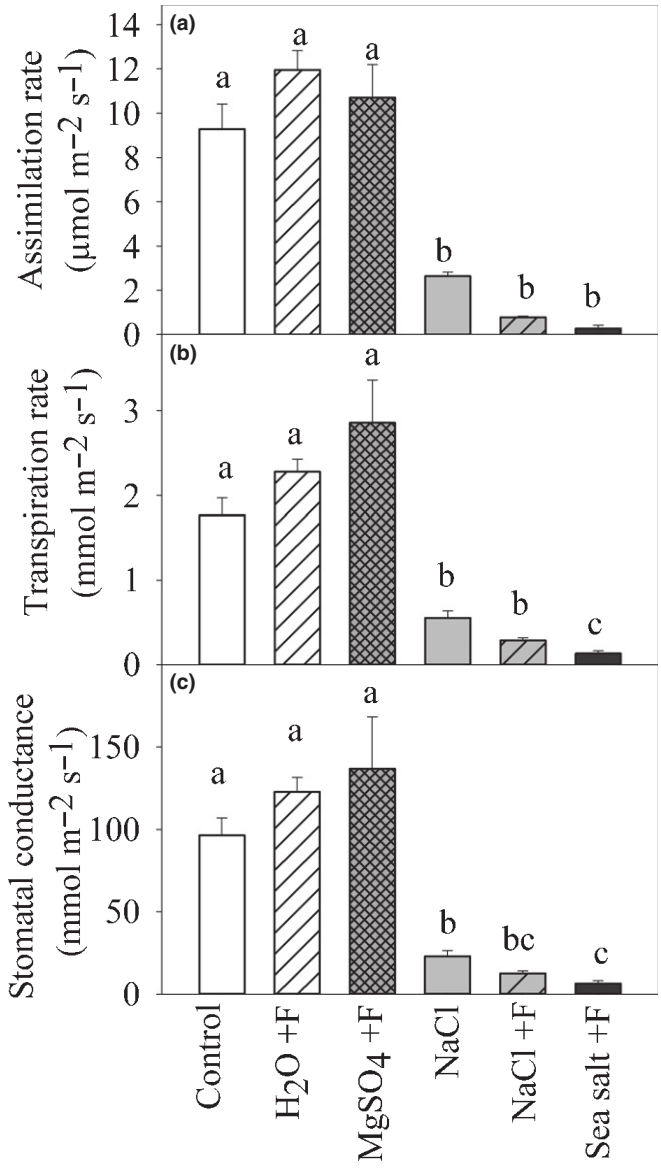

Fig. 2 Assimilation (a), transpiration (b) and stomatal conductance (c) of cotyledons of date palm seedlings received distilled water (control) or exposed to different salt and/or flooding treatments. Data shown are the means ± SE (n = 3). Different lowercase letters indicate significant differences (P < 0.05) between different treatments. Flooding is indicated with +F.

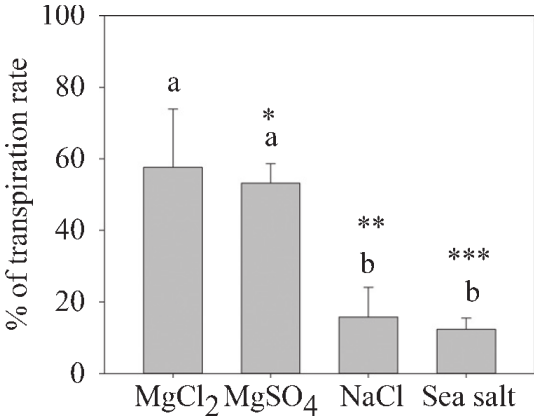

Fig. 3 Transpiration rate of detached date palm leaves fed with 5 mmol $MgCl_2$, 10 mmol $MgSO_4$, 3.5% (w/v, g ml^{-1}) NaCl or 3.5% (w/v) sea salt (from left to right) via the petioles. The detached leaves were first incubated in a preincubation solution containing 0.5 mmol $MgCl_2$ plus 0.06 mmol $Mg(NO_3)_2$ (pH 5.5). The transpiration rates measured during the preincubation period were taken as controls. Data shown are means ± SE (n = 5–6) of relative transpiration rates after 60 min of incubation compared with the transpiration rate of the controls determined during preincubation. Asterisks indicate significant differences compared with the controls (*, P < 0.05; **, P < 0.01; ***, P < 0.001; Student's t-test). Different lowercase letters indicate significant differences (P < 0.05) between the treatments.

in arid and semiarid areas. Apparently, mechanisms to tolerate both low soil water potential caused by salinity as well as drought enable date palms to grow in both types of environments (Munns & Tester, 2008).

Photosynthesis is the main carbon source for isoprene (Schnitzler *et al.*, 2004). There is still only scarce data on the role of isoprene production and emission in response to salinity (Behnke *et al.*, 2013). In particular, the emission of isoprene by date palms has rarely been studied in response to environmental stress, except for heat stress that caused a strongly increased isoprene emission (Arab *et al.*, 2016). Decreased isoprene emission has been observed often in trees exposed to flooding, for example in *Garcinia macrophylla* (Bracho-Nunez *et al.*, 2012), *Populus tremula* and *Q. rubra* (Copolovici & Niinemets, 2010). Therefore, reduced isoprene emissions were expected upon flooding with seawater as a consequence of stomatal closure as described in our first hypothesis. However, seawater flooding had no significant effect on isoprene emission (Fig. 1d) as previously observed upon exposure to drought (Arab *et al.*, 2016). This conserved isoprene emission is likely to be due to increased intercellular isoprene concentrations upon stomatal closure that compensated for decreased stomatal conductance to restore the equilibrium between isoprene synthesis and emission (Fall & Monson, 1992; Behnke *et al.*, 2013). Our results are consistent with observations on salt treated *Eucalyptus globulus* Labill. (Loreto & Delfine, 2000) and *Populus* × *canescens* (Teuber *et al.*, 2008) that both show strongly decreased CO_2 assimilation, but unchanged isoprene emission, upon treatment with saline solutions. Behnke *et al.* (2013) pointed out that isoprene does not offer a considerable advantage for plants coping with salinity. Therefore, an increased isoprene production might not be expected under these conditions. The emission of volatile organic compounds from leaves of flooded plants is closely connected to the altered metabolism of roots under anoxia (Kreuzwieser & Rennenberg, 2013). Therefore, stable isoprene emission in the present study also indicates that seawater flooding does not alter root metabolism, a view supported by their largely conserved sugar and fatty acids composition (Fig. 5).

Sea salt components responsible for stomatal closure

Some salt-tolerant plants maintain low osmotic potential and stomatal conductance despite the accumulation of sodium and

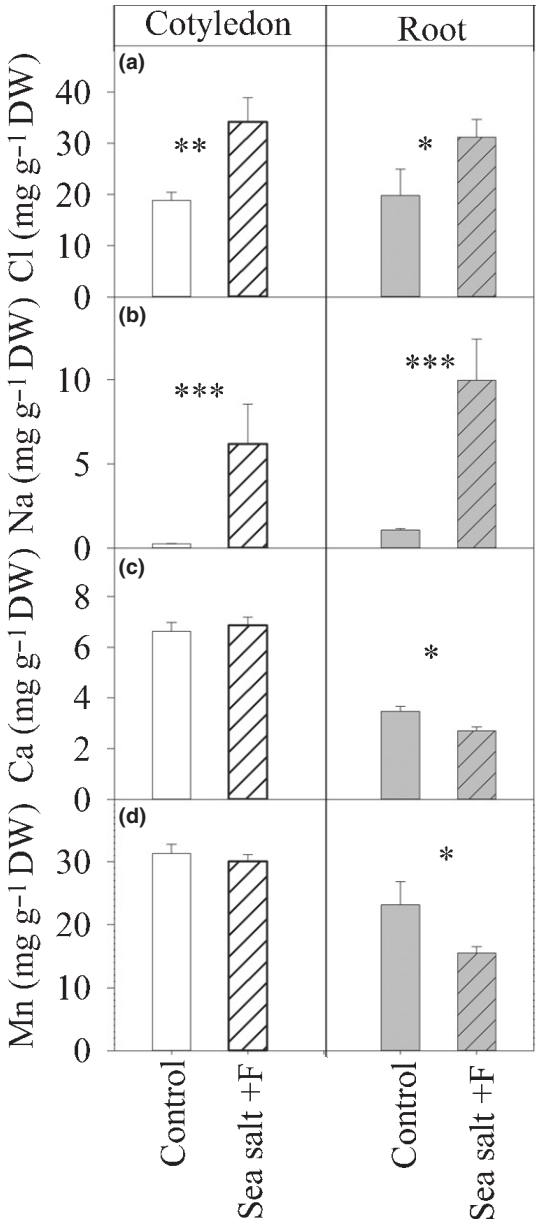

Fig. 4 Chloride (a), sodium (b), calcium (c) and manganese (d) concentrations in cotyledons and roots of control and seawater flooded (hatched bars) date palm seedlings. Data shown are the means ± SE (n = 5–6). Asterisks indicate significant differences at: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; respectively, between seawater flooding and controls examined by Student's t-test (n = 5 or 6). Flooding is indicated with +F.

chloride in their tissues (Munns & Tester, 2008) as frequently observed in mangroves (Naidoo, 1985; Aziz & Khan, 2001). Compared with ionic stress, the responses of stomatal conductance to salinity are immediate, due to disturbed water relations and shortly afterwards due to the local synthesis of abscisic acid (ABA) (Fricke *et al.*, 2004; Munns & Tester, 2008). Many studies have elucidated the effects of sea salt on assimilation and stomatal closure (Naidoo, 1985; Flanagan & Jefferies, 1988; Aziz & Khan, 2001; Koyro, 2006), but only a few studies have sought to disentangle the components of sea salt responsible for stomatal closure. In the present study, both Na and Cl accumulated in roots and cotyledons upon seawater exposure (Fig. 4a,b) concurrent with reduced stomatal conductance (Fig. 1). The simultaneous accumulation of Na and Cl might allow date palms to adapt osmotically and to maintain turgor under high soil salinities as the 'cheapest' form of osmotic adaptation (Munns & Tester, 2008). Under these conditions, Na and Cl are likely to be largely sequestered in the vacuole of the cell to maintain cellular functions (Munns & Tester, 2008; Al-Harrasi *et al.*, 2020).

Besides Cl^-, sulfate (SO_4^{2-}) is the second most abundant anion in seawater with a current concentration of 28 mmol (Canfield & Farquhar, 2009). Recent studies with Arabidopsis (*Arabidopsis thaliana*) and poplar (*Populus* × *canescens*) have demonstrated that, under drought conditions, sulfur is transported from the roots to the shoots in the a transpiration stream, where it is incorporated into cysteine, which then tunes ABA biosynthesis on, to promote stomatal closure as a physiological response to limit water losses (Malcheska *et al.*, 2017; Batool *et al.*, 2018; Patankar *et al.*, 2019). In the present study, in contrast with our second hypothesis, evidence supporting a role of sulfate in stomatal closure upon seawater exposure could not be provided, neither in whole plant experiments nor in experiments with detached leaves. Sulfate did not accumulate in roots or cotyledons of date palm seedlings exposed to seawater and stomatal conductance was not affected by flooding with $MgSO_4$ (Fig. 2; Table S4). Apparently, increased sulfate influx into the roots of date palms exposed to seawater flooding could be avoided. Feeding sulfate via the petiole did not affect stomatal conductance of detached leaves (Fig. 3). Apparently, the responsiveness of stomatal movement to sulfate observed in Arabidopsis and poplar (Malcheska *et al.*, 2017; Batool *et al.*, 2018) does not hold true for date palms and, thus, is not a general mechanism employed by plants to prevent water loss under different stress conditions.

Compatible solutes and root–shoot interactions

Sugars, sugar alcohols and amino acids are metabolites frequently serving an osmolyte function helping to alleviate salinity stress (Hasegawa *et al.*, 2000; da Silva *et al.*, 2008; Munns & Tester, 2008; Acosta-Motos *et al.*, 2017; Al Kharusi *et al.*, 2019a). In the present study, contradicting our third hypothesis, no accumulation of sugars or sugar alcohols was found in cotyledons and the concentrations of these metabolites generally decreased upon exposure to seawater flooding (Fig. 4). Nevertheless, several amino acids and other N containing compounds were slightly, but significantly, accumulated in cotyledons upon seawater flooding (Fig. 5), suggesting that free amino acids were more important as osmolytes than sugars (Hartzendorf & Rolletschek, 2001). However, salt induced accumulations of proline (Djibril *et al.*, 2005; Yaish, 2015; Al Kharusi *et al.*, 2019a) were not observed in the current study, probably due to the intraspecific variations of date palm (Al Kharusi *et al.*, 2019a,b), as well as a counteraction of flooding as reported in several other species, for example cabbage palm (Williams, 1996), *Juncus kraussii* (Naidoo & Kift, 2006). Much stronger accumulation of amino acids were

Classes	Metabolites	Cotyledon	Root
Monosaccharides	Glucose	*	
	Fructose		
	Arabinose	**	
	Galactose	*	
	Altrose		
	Rhamnose		
Disaccharides	Sucrose		
	α, α-Trehalose	*	
	Sophorose		
Trisaccharides	Raffinose		
	6-Kestose		
Sugar acids	Glyceric acid		
	Lyxonic acid		
	Galactonic acid		
	Threonic acid		
	Glucuronic acid		**
Phosphate	Glycerol-3-phosphate		
Sugar alcohols	Threitol		
	Galactinol	*	
	Arabitol	**	
Organic acids	Citric acid		
	Fumaric acid	**	**
	Malic acid		
	2-methyl-Malic acid	**	
	Lactic acid		
	Shikimic acid	**	
Polyols	Sorbitol		*
	myo-Inositol		
	Glycerol		
Antioxidants	Ascorbic acid		
	Dehydroascorbic acid dimer		
Aromatics	4-Methylcatechol		
	Vanillic acid		
	Lumichrome		
	trans-4-caffeoyl-Quinic acid		
	trans-Caffeic acid		
	4-hydroxy-Benzoic acid		
	Benzoic acid	na	
	Catechin	*	
	Guaiacylglycerol		
	Taxifolin		
	cis-Sinapic acid		
	cis-Ferulic acid	*	
	Gallic acid		

Classes	Metabolites	Cotyledon	Root
Amino acids	Glutamic acid		
	Proline		
	Arginine		
	Aspartic acid	*	
	Asparagine		
	Methionine		
	Threonine	*	
	Lysine		
	Valine	*	
	Alanine	*	
	Leucine		*
	Isoleucine		*
	Norleucine	*	
	Phenylalanine		***
	Tyrosine	*	
	Tryptophan		
	Serine		
	Glycine		
Other N compounds	Ornithine-1,5-lactam	*	
	N2-acetyl-Ornithine		
	3-cyano-Alanine		
	γ -Aminobutyric acid (GABA)		*
	dihydro-Uracil	na	
	Uracil		
	Pyroglutamic acid		
	A143018		
	A151021		
	N-Acetylglucosamine		
	Histamine	+	
	Ethanolamine		***
	Dihydroxyphenylalanine		
	Butyro-1,4-lactam		*
	2-amino-Adipic acid		
	1-Pyrroline-3-hydroxy-5-carboxylic acid		
	2-amino-2-deoxy-Gluconic acid		
	Maleamic acid		
Fatty acids fatty alcohol	9-(E)-Octadecenoic acid		
	Octadecanoic acid		
	Hexadecanoic acid		
	hydroxy-Hexanedioic acid		
	n-Heptadecan-1-ol	*	
Others	Phosphoric acid		
	Gluconic acid-1,4-lactone	*	
	IPTG		

Fig. 5 Fold changes (log_2(seawater flooding/control)) of water-soluble metabolites in cotyledons and roots of date palm seedlings. IPTG, isopropyl β-D-1-thiogalactopyranoside; A143018, *N*-methyl *trans*-4-hydroxy-L-proline (2S,4R)-4-hydroxy-1-methyl pyrrolidine-2-carboxylic acid; A151021, *N*-methyl *cis*-4-hydroxymethyl-L-proline. Blocks with 'na' indicate metabolite contents below detect limit; + indicates histamine only abundant in salt flooding treatments. Asterisks indicate significant differences at: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; respectively, between the seawater flooding and control treatments examined using Student's *t*-test ($n = 5$–6).

documented in young leaves of date palms grown at high temperatures (Du *et al.*, 2019) and in needles of drought-treated coastal Douglas fir (*Pseudotsuga menziesii*) provenance (Du *et al.*, 2016). Thus, a significant accumulation of compatible solutes and other metabolites except some N containing compounds in response to seawater flooding is not observed in the present study, and therefore, our third hypothesis has to be rejected.

Obviously, date palms are not able to prevent the uptake of Cl^- and Na^+ ions by the roots and their transport from roots to shoots in the transpiration stream. This is indicated by the accumulation of these ions in both cotyledons and roots (Fig. 4). Apparently, allocation of Cl and Na out of the roots and sequestration in the cotyledons is required to maintain root functions of date palms under seawater exposure. Although the accumulation of Na and Cl is associated with reduced CO_2 assimilation in the cotyledons, sugar concentrations are maintained in the roots (Figs 1,5). Similarly, conserved or increased sugar concentrations in roots of salt stressed rice (*Oryza*) (Nam *et al.*, 2015) and in *Phragmites australis* rhizomes (Hartzendorf & Rolletschek, 2001) have been reported. This result is surprising, since the switch from respiration to fermentation under anoxic conditions requires enhanced consumption of sugar for energy generation (Kreuzwieser *et al.*, 2004; Kreuzwieser & Rennenberg, 2014). The general reduction in coleoptile sugar concentrations upon seawater inundation may therefore be a consequence of both, reduced CO_2 assimilation and enhanced translocation of sugar to the roots. Therefore, our fourth hypothesis is fully supported by the varied shoot–root interactions on compatible solutes and ions.

In conclusion, date palm seedlings responded to seawater flooding with decreased stomatal conductance and reduced CO_2 assimilation. Reduced transpiration upon seawater exposure may contribute to controlling the flux of toxic ions within the transpiration stream to the leaves and, therefore, can be seen as a mechanism to cope with salinity. Salt exposure, but not flooding, contributed to the observed stomatal closure in date palms. Both Na and Cl were accumulated in roots and cotyledons upon sea water inundation. However, although the root Na concentration was greater than the cotyledon Na concentration, suggesting that roots restrict the movement of Na to cotyledons, both root and cotyledon Cl concentrations were similar. The accumulation of sulfate, which is present in seawater in high amounts, is not increased by exposing date palm to seawater flooding and, thus, is not involved in stomatal closure. Experiments with detached leaves further showed that stomatal aperture of date palm cotyledons is not sensitive to sulfate. Our results highlight that date palms are strongly tolerant to flooding and to some extent tolerant to salinity. They further show that sulfate-mediated stomatal closure is not a general phenomenon, but is dependent on the particular stress and the stress exposed species.

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Author contributions

HR, RH, GA and SA conceived and managed the project. HR and BD designed the experiment. HR contributed to writing, reviewing and editing the manuscript. BD cultivated the plants, conducted the feeding experiment and the second salt and/or flooding experiment, performed biochemical measurements and evaluated the data, interpreted the results and wrote the main part of the manuscript. YM performed the first seawater flooding experiment, and determined gas exchange and isoprene emission together with AMYS and LF. YM and AMYS also contributed to writing the manuscript. LA helped in the second salt and/or flooding experiment. PJW measured the elements. CW provided the facilities and instruments. All authors contributed to editing the final draft of the manuscript. BD and YM contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Plant shoot and root images of different treatments.

Table S1 Chemical composition of Dead Sea salt.

Table S2 Electrical conductivities of solutions.

Table S3 Biomass and root : shoot ratio of different treatments.

Table S4 Element and anion contents in cotyledons and roots of control and seawater flooded date palm seedlings.

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