Untargeted metabolomics in halophytes: The role of different metabolites in New Zealand mangroves under multi-factorial abiotic stress conditions

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Abstract

Mangroves are halophytes which live in harsh coastal environments, and can serve as excellent model organisms to understand mechanisms of stress tolerance. Metabolomics is a useful approach to investigate the role different metabolites play during physiological responses of plants to abiotic stress factors. Previous studies indicate that levels of non-structural carbohydrates in plants may be associated with stress tolerance. Here, we manipulated levels of non-structural carbohydrates (NSCs) in the New Zealand mangrove, *Avicennia marina subsp. australasica*, through a light swapping regime. In a subsequent drought × salinity experiment, we then monitored leaf metabolite profiles of two NSC groups (high-NSC and low-NSC). Our results show that fourteen metabolites, belonging to multiple biochemical pathways, were significantly affected by one or more of these factors. The manipulation of non-structural carbohydrates led to increased abundance of amino acids in the low-NSC (L-NSC) plants compared to the high-NSC (H-NSC) plants. Under high drought conditions, the L-NSC plants had higher abundances of leucine and valine in comparison to those with the H-NSC phenotype. The L-NSC plants also had higher abundances of putrescine and aminoadipic acid when exposed to high salinity. Under the combination of drought and salinity, α-ketoglutarate was reduced in plants with the H-NSC phenotype, and soluble sugars accumulated compared to those with the L-NSC phenotype. The increased soluble sugar content in the H-NSC plants can facilitate osmotic adjustment, thereby aiding their survival during low soil water potential conditions. We also detected the presence of stress-protective phenolic compounds (syringic and sinapic acids) not previously reported in mangroves. Relationships between plant growth and the mangrove metabolome were also established, with levels of glucose and phosphoenol pyruvate being primarily

responsible for phenotypic associations. Our findings show that the metabolites detected in this study, and their respective metabolic pathways, play a significant role in salinity and drought stress tolerance of *A. marina subsp. australasica*, providing new information to better understand mangrove mortality.

Introduction

Mangroves are intertidal halophytes that experience periodic inundation and high salinity conditions. In light of increasing threats due to climate change and rising sea levels, mangroves are likely to experience increased mortality (Lovelock et al., 2017). Despite their importance in plant physiology, the functional roles of different primary and secondary metabolites in abiotic stress responses for many plant species is poorly understood (McKiernan et al., 2014; Niinemets, 2016). Characterising the myriad of cellular and biochemical fluctuations in response to different stressors will help to interpret changes in physiological parameters such as leaf water potential, stomatal conductance, and gas exchange (Deshmukh et al., 2014).

Recent research efforts have focused on the role of plant metabolites in ecological and physiological functioning (McKiernan et al., 2014; Niinemets, 2016). Plant metabolomics research has provided advancements in the discovery of novel plant metabolites, metabolic pathways, and their associated functions such as coping with temperature and oxidative stress (Alseekh & Fernie, 2018). Primary metabolites are required for growth and maintenance of cellular functioning. For example, increases in concentrations of simple sugars (glucose, sucrose, fructose) and starch are associated with increased leaf area (Galvez et al., 2013). Secondary metabolites are those involved in plant defence, hormonal regulation, and protein synthesis. For

example, abscisic acid is an important plant hormone, which regulates stomatal closure during drought (Chaves, Maroco & Pereira, 2003; Grant, 2012).

Non-structural carbohydrates (NSCs) such as glucose, sucrose, fructose, and starch are primary metabolites, and their concentrations in plant tissue can reveal important insights regarding their use as osmolytes for drought survival. For example, variations in NSC levels are useful responses for investigating plant/tree mortality under abiotic stress such as drought, low/high temperature, increased precipitation and biotic factors such as pathogen attacks (Dietze et al., 2014; Germino, 2015; Hoch et al., 2002; Wurth et al., 2005). NSCs have been observed to either accumulate (O'Brien et al., 2014; Wurth et al., 2005) or deplete in response to drought (Adams et al., 2013; Ivanov et al., 2019; Tomasella et al., 2017). NSCs represent the bulk of plant carbon, and are well characterised. They also influence the accumulation/depletion of other primary and secondary metabolites, such as amino acids, isoprenoids, and phenolics (Dietze et al., 2014; Niinemets, 2016; Wurth et al., 2005). In order to further advance knowledge regarding the physiological stress responses in plants, broader analyses of other metabolites and their associated biochemical pathways is needed to gain deeper understanding at the metabolic level.

Mangroves exist within extreme environments and employ similar biochemical mechanisms as terrestrial plant species to tolerate water-limited conditions and maintain physiological homeostasis (Aroca, 2012; Ahmad, Azooz & Prasad, 2013). For example, the growth of mangroves in high saline conditions is usually accompanied by accumulation of sugars, polyols (pinitol, mannitol), glycine betaine, proline (Kumari & Parida, 2018; Parida et al., 2002; Parid & Jha, 2010). Similarly, investigations on the effect of abscisic acid on salinity tolerance of *Avicennia marina* (Forssk.) Vierh. revealed that exogenous application of abscisic acid reduced sap flow

and transpiration rates that are typical of plant drought responses (Reef et al., 2012). In addition, in a study on 23 Australian mangrove species, metabolites such as proline and methylated quartenary ammonium compounds were found to be present only in a few species (*Avicennia marina*, *A. eucalyptifolia*, *Acanthus ilicifolius*, *Heritiera littoralis*, and *Hibiscus tiliaceius*), whereas the remaining species accumulated low molecular weight carbohydrates in high quantities, with mannitol and pinitol being the most predominant (Popp, Larher & Weigel, 1985).

Previous studies have examined the effect of salinity and drought in isolation on the plant metabolome (Alla et al., 2012; Gagneul et al., 2007; Hoch et al., 2002; Johnson et al., 2003, Parida et al., 2002). However, the production and roles of different metabolites in plant abiotic stress responses are complex and, when salinity co-occurs with drought, different metabolic adjustments may transpire, with either synergistic (conserved) or antagonistic (divergent) responses occurring (Sanchez et al., 2008b). Synergistic metabolic responses occur when two or more species show accumulation or depletion of similar metabolites. Antagonistic metabolic responses are those where metabolite profiles of different plant taxa show opposing trends (Munns 2002; Sanchez et al., 2008b, 2012). Interpreting plant responses to individual stressors, does not represent natural conditions where different abiotic stresses always co-occur (Jorge & Antonio, 2018).

In this contribution, stress response patterns in primary and secondary metabolites of the New Zealand mangrove, *Avicennia marina subsp. australasica* were investigated under varying levels of drought, salinity, and NSC concentration. We first manipulated NSC levels in mangrove seedlings by exposing them to different light conditions, then subjected them to a combination of salinity and drought treatments to investigate their effects on a comprehensive range of metabolites using an untargeted

GC/MS-based metabolomics approach. Our overall premise is that NSC content plays a fundamental role in stress tolerance via a restructuring of central metabolic pathways, identifiable by changes within the mangrove metabolome. We hypothesize that i) an altered light regimen (light swapping) will cause differences in metabolite accumulation via restructuring of energy and carbohydrate metabolic pathways, and ii) seedlings with higher initial NSC content will be more tolerant to drought and salinity stress through enhanced synthesis of other stress-ameliorating metabolites, leading to better adaptability to different abiotic factors.

Materials and methods

Plant material

New Zealand mangrove propagules were collected from Manghawhai Estuary, northern New Zealand, in December 2014. After initial acclimation and growth, 162 seedlings were placed in 3.7 litre pots with fine sand as the substrate in a shade house (University of Auckland, Tamaki campus). All 162 pots were fertilised every 15 days using commercial garden fertiliser (Thrive all-purpose soluble fertilizer [Yates, New Zealand]; NPK ratio of 25:5:8.8 plus trace elements; 45 gL^{-1} in fresh water; 250 mL provided per pot per application). Environmental conditions (light, temperature and relative humidity) in the shade house were monitored throughout the study period (Hobo Pendant Temp-light Data Logger; Tidbit Mx Temperature Data logger; Hobo Temperature-RH Data Logger [Onset Computer Corporation, USA]). For Jan-Apr 2015, the daily average maximum temperature was 27°C and the average minimum was 18°C. The daily average maximum relative humidity was 84% and the average minimum was 61%. For May-Aug 2015, the daily average maximum temperature was 15°C and the minimum was 9°C. The daily average maximum relative humidity was

64% and the minimum was 55%. The duration of the study from propagule collection, to the final harvest lasted from December 2014 – August 2015.

Manipulation of NSCs

To reduce potential confounding effects from maternal reserves (i.e., to prevent plants from using cotyledonary reserves), cotyledons were removed from all the seedlings immediately prior to NSC manipulation. Before the drought × salinity treatment application, plants with different levels of NSC were obtained by applying a light swapping treatment for 84 days (O'Brien et al., 2014). After 50 days of initial acclimation and growth, seedlings were randomly divided into two groups of 81 plants each (162 plants in total). For the first 42 days, one group of plants was grown under ambient light conditions with a monthly average photosynthetic photon flux density (PPFD) of 307.6 ± 22.8 (μmol m^{-2} s^{-1}), whereas the other group was kept under low light, corresponding to 9–10% of ambient light with a monthly average PPFD of 24.2 ± 4.9 (μmol m^{-2} s^{-1}). The two groups of plants were then swapped to receive the reciprocal light conditions for a further 42 days (Fig. 1a). This resulted in two groups of plants with different NSC levels (Fig. 1b).

Treatment application

After manipulation of NSCs, seedlings were exposed to drought × salinity treatments for 12 weeks. A randomized factorial design was used where three levels of salinity (i.e., Low: 25% seawater, medium: 50% seawater, and high: 75% seawater) were crossed with two levels of drought (low and high), and with two levels of initial endogenous NSC content (low and high). Seedlings within each NSC group were randomly allocated to each of the nine drought × salinity treatments, with three replicate pots per treatment combination and 2–4 plants per pot. In this experiment,

147 the low salinity–low drought treatment combination served as the control. Saline
148 solutions at three concentrations were prepared using artificial sea salt (S9883,
149 Sigma-Aldrich, St. Louis, MO, USA): low salinity (150 mM NaCl; equivalent to 25%
150 seawater), medium salinity (300 mM NaCl; equivalent to 50% seawater), and high
151 salinity (450 mM NaCl; equivalent to 75% seawater). Pots were watered with 400 mL
152 (water holding capacity of pots) of the respective salinity solutions at the start of the
153 experiment (just after light swapping), and then again at weeks 4 and 8. In between
154 salinity treatments, seedlings exposed to low drought conditions received tap water
155 every 3–4 days (200 mL via spray bottle), whereas those exposed to high drought
156 conditions did not receive tap water between salinity treatments. We measured soil
157 moisture content (EC – 10, Decagon Devices, USA) and soil water potential (MPS-6,
158 Decagon Devices, USA) only in the extreme treatment combinations: Low salinity –
159 low drought, high salinity – high drought. Low salinity – low drought treatment plants
160 had a higher soil moisture (36.5% ± 3.2% s.e.; after 8 weeks of treatment, n = 3) and
161 higher soil water potential (-4 MPa ± 1.5 s.e. after 8 weeks of treatment, n = 2).
162 Whereas, the high salinity – high drought treatment plants had a lower soil moisture
163 (15.9% ± 2.4% s.e. after 8 weeks of treatment, n = 3) and lower soil water potential (-
164 7 MPa ± 0.6 s.e. after 8 weeks of treatment, n = 2). The salinity concentrations used
165 in this experiment were within the salinity tolerance range of *A. marina* (Morrisey et
166 al., 2010).

167 **Sample collection**

168 For metabolomic analyses, duplicate leaves from one seedling per pot were collected
169 at the end of the light swapping treatment from both the high NSC (H–NSC; n = 3
170 plants each) and low NSC (L–NSC; n = 3 plants each).

Further sampling of leaves took place after drought and salinity treatments had been in place for 12 weeks. Duplicate leaves from each plant were collected from each treatment combination (*n* = at least 3 each). The leaf samples were snap frozen in liquid nitrogen and stored at -80°C until extraction and further analysis.

Growth and physiological measurements

At the end of week 12, plant growth parameters (leaf area, leaf number and plant height) and physiological variables (stem water potential, maximum hydraulic conductivity and stomatal conductance) were measured around midday (12:00–3:30 pm) immediately prior to destructive harvesting. Plant height was defined as the stem height above soil surface to the first node below the stem apex. Stem water potential was measured using a custom-built Scholander pressure chamber (Scholander et al., 1965) by excising seedling stems (along with leaves) above the soil surface. The excised stem was immediately placed in the pressure chamber for measurement. Xylem embolism was measured from the hypocotyl regions of the stem (cut underwater) using an embolism meter (XYL'EM, INRA, Bronkhorst, France), with degassed KCl (10 mM) as the flushing solution. In order to measure the maximum hydraulic conductivity per sample (each stem segment), the initial flow rate was first recorded then the samples were flushed at 0.1 MPa for about three minutes. This process was repeated until there was no increase in flow rate. Stomatal conductance was measured using a leaf porometer (SC-1, Decagon Devices, Pullman, WA, USA). Measurements were taken from 2–3 leaves per pot and from three pots per treatment, with data representing the mean of the means.

Metabolite Analysis

Leaf metabolite profiling was conducted according to protocols described in detail by Young et al. (2017) and Zarate et al. (2017). Briefly, freeze-dried leaf samples (~10

196 mg) were co-extracted with an internal standard (d4-alanine or D-ribitol) using an
197 established methanol-water method. To increase volatility of the organic acid and
198 carbohydrate constituents, extracted metabolites were derivatized via methyl
199 chloroformate (MCF) alkylation and N-trimethylsilyl-N-methyl trifluoroacetamide
200 (MSTFA) trimethylsilylation (TMS), respectively (Villas-Bôas et al., 2011). Compounds
201 were analysed via gas chromatography mass spectrometry using a Thermo Trace GC
202 Ultra system for the MCF derivatives, and an Agilent GC7890 + MSD5975 for the TMS
203 derivatives. Instrument setups are outlined in Young et al. (2017) and Zarate et al.
204 (2017). Spectral pre-processing was performed using the Automated Mass Spectral
205 Deconvolution and Identification System (AMDISV2.66) software. Chemstation
206 software (Agilent Technologies) and customised R xcms-based scripts (Aggio et al.,
207 2011) were used to identify metabolites by interrogating in-house libraries of MCF-
208 and TMS-derivatised compounds constructed using pure standards. Records were
209 quality assessed and manually checked for presence of contaminants, with aberrant
210 records being removed. Metabolite peak intensities within each dataset were
211 normalised against the relevant internal standard to compensate for potential technical
212 variations (e.g. variable metabolite recoveries), and to sample-specific biomass.

213 **Data analysis**

214 To provide visualisation of the metabolome coverage across plant cellular pathways,
215 pathway mapping was performed on metabolites able to be matched to entries within
216 the KEGG database (reference plant model: *Arabidopsis thaliana*) using Interactive
217 Pathway Explorer v3 (iPATH3; Darzi et al., 2018). Statistical analysis of metabolite
218 data after manipulation of NSCs was conducted using Metaboanalyst 4.0 (Chong et
219 al., 2018), and the R package *nlme* (Pinheiro et al., 2018). For multivariate analyses,

data were log-transformed and range-scaled to reduce heteroscedasticity and provide a Gaussian distribution of metabolite abundances.

After manipulation of NSCs (prior to the drought × salinity treatments), combined heatmap and hierarchical cluster analysis (Euclidian distance; Wards criterion) of the top 50 metabolites ranked by their t-test statistics was conducted to provide an intuitive visualisation of expression patterns and between-sample variability. Quantitative Enrichment Analysis (QEA [Xia & Wishart, 2010]) (Global test statistic) and Network Topology Analysis (NTA [Nikiforova & Willmitzer, 2007]) (Relative betweenness centrality) were used as pathway analysis methods to investigate functional relationships among the annotated metabolites and highlight differential regulation within these collections at the pathway level. Biochemical pathways in the KEGG database (using the *Arabidopsis thaliana* reference library) involving two or more annotated metabolites, QEA p-values < 0.05, and PI scores ≥0.05 were considered as potential target pathways of interest.

Principal components regression (PCR) with Leave One Out Cross Validation (LOOCV) was employed to investigate potential associations between the mangrove metabolome and plant growth (height and leaf area), water potential, hydraulic conductance, and stomatal conductance. Metabolome-response associations between growth and physiological parameters were also investigated via regression-based QEA and NTA.

After drought × salinity treatments, metabolites were modelled using a generalised least squares approach with restricted maximum likelihood (gls, R package *nlme*; Pinheiro et al., 2018) as a function of pre-treatment NSC level, salinity and drought. Variance heterogeneity and normality were assessed using residual

versus fitted plots and quantile-quantile plots respectively for testing violation of model assumptions. For cases where significant interactions were present, these were sliced using a multiple comparison procedure (R package *emmeans*; Lenth, 2018). The resulting p-values from the multiple comparison procedure were corrected for multiple testing using the Benjamini & Hochberg method (Benjamini & Hochberg, 1995). Additionally, because of multiple hypothesis testing involving the reliably identified metabolites (n = 101), Bonferroni correction was used to reduce type I errors, with a marginal significance level (10%, $P \leq 0.0009$). These analyses were performed using R (R 3.4.2, R Development Core Team, 2017).

Results

A total of 149 metabolites including unknowns were detected in *Avicennia marina subsp. australasica* leaves, with 101 being reliably identified. Of these, 69 metabolites, broadly covering a number of modules (collection of manually defined functional units) in the KEGG global metabolic network could be mapped onto the KEGG plant-specific reference network (Fig. 2). These metabolites belong to different metabolic pathways with ~35% involved in energy metabolism, ~38% in amino acid metabolism, ~15% in carbohydrate metabolism, and ~10% involved in biosynthesis of secondary plant metabolites such as phenolic acids.

After the light swapping treatment to manipulate NSC levels, 30 metabolites were identified as being significantly different (t-test; $P < 0.05$) between the high- and low-NSC groups (Supplementary table, S1). A combined heat map and cluster analysis using the top 50 metabolites ranked by their t-test p-values show distinct grouping based on their sample class membership (Fig. 3; Supplementary table, S1). In the high NSC group, the sugars rhamnose, ribose, xylose, myo-inositol, and glyceric

acid were substantially more abundant than in the low NSC group; being primarily responsible for the higher total NSC content. Levels of arabinose, fructose, mannose, galactose, and glucose did not differ between NSC-manipulated groups, and levels of sucrose were unexpectedly higher in the low NSC group.

Topology-based pathway enrichment analysis was conducted to reveal the most relevant pathways associated with the light swapping treatment regime (Fig. 4; Supplementary table, S2). A total of 53 biochemical pathways were recognised from within the KEGG database which contained one or more of the annotated metabolites detected. Pathways involving two or more detected metabolites and with simultaneous QEA p-values < 0.05 and PI score > 0.05, were screened as potential target pathways of interest relating to the treatment effect. According to this selection, 14 biochemical pathways were identified with evidence for differential regulation between NSC treatments (Fig. 4).

Most PCR models were over-fitted (poor LOOCV prediction accuracy) or had low R^2 values, with exception of plant height as the response variable (R^2 = 0.77; Fig. 5). This plant metabolome–growth association was further interrogated via regression-based QEA and NTA.

Five biochemical pathways were found to be associated with mangrove height, consisting of: glycolysis/gluconeogenesis, starch and sucrose metabolism, galactose metabolism, carbon fixation and pyruvate metabolism (Fig. 6; Supplementary table, S3). Pathway modulations were largely driven by differences in levels of glucose and/or phosphoenolpyruvate, with significant positive and negative relationships with plant/mangrove growth, respectively (Fig. 7). Levels of glucose and phosphoenolpyruvate were also largely responsible for metabolic pathway

associations of Starch and sucrose metabolism, and Galactose metabolism with leaf area and with stem water potential (see Supplementary Table S3).

After the drought and salinity treatments, each metabolite was affected by either an interaction effect of NSC, drought and salinity or just the main effect of each factor. We also report metabolites that are prominent in the plant metabolomics literature. The details of this generalised least squares analysis are presented in the Supplementary table, S4.

A total of fourteen compounds were either significantly affected by three-way (NSC × salinity × drought) interactions and main effects (Table 1). Three of them belonged to the energy metabolism, the TCA (Tricarboxylic) cycle. 2-ketoglutamarate abundance was affected by NSC × drought interaction. The interaction was driven by low and high drought conditions in the L-NSC group which had lower abundance. The abundance of α-ketoglutarate was affected by low salinity–high drought conditions. The L-NSC group had higher abundance of α-ketoglutarate than the H-NSC group. Malonic acid, which is also an intermediate in the malonic acid pathway leading to the formation of phenolics, was significantly affected by drought only and not salinity.

Other carbohydrates and amino acids that were significantly affected by interactions or main effects are: Galactose (galactose metabolism) abundance was affected by the NSC × salinity interaction. This interaction was driven by high salinity in the H-NSC group which had higher abundance. Glycerol abundance was significantly affected by drought only. Four amino acids were identified to be affected by interactions or main effects: Leucine and valine which are synthesized via Valine, leucine, isoleucine biosynthetic pathway. Also, putrescine which is synthesized via arginine and proline metabolism and also glutathione metabolism. Leucine and valine

were affected by the NSC × drought interaction. For both compounds, the interaction was driven by high drought conditions. The L-NSC plants had higher abundance of both the compounds. Putrescine and aminoadipic acid (lysine biosynthetic pathway) were affected by NSC × salinity interaction. For putrescine, the interaction was driven by low and medium salinity conditions, whereas for aminoadipic acid the interaction was driven by high salinity conditions. The H-NSC plants had lower abundance of putrescine and, aminoadipic acid in comparison to the L-NSC plants.

Other compound classes, such as phenolic acids, were affected by three-way (NSC × salinity × drought) interactions. Two of them included para-toluic acid, and syringic acid. Under medium salinity – high drought conditions, the abundance of para-toluic acid was high in the H-NSC plants and low in the L-NSC plants. Whereas, the abundance of syringic acid was similar in the H-NSC and L-NSC plants. Additionally, nicotinic acid (nicotinamide metabolism and the tropane, piperidine and pyridine alkaloid biosynthesis) and tridecane (hydrocarbon, intermediate in essential oil synthesis) were identified. The abundance of nicotinic acid was increased in the L-NSC plants under high saline conditions. The abundance of tridecane was affected by medium and high salinity associated with low drought conditions. The L-NSC plants had higher abundance of tridecane in comparison to the H-NSC plants.

Discussion

In our study, light swapping had a major impact on carbohydrate and energy metabolism (supporting our initial hypothesis), and provided seedlings with different NSC contents for the subsequent stress experiment. Following drought and salinity treatments to these seedlings, the abundance of metabolites belonging to different biochemical pathways, and recognised as having roles in ameliorating stress, were significantly affected by interactions of NSC, salinity, and drought (supporting our

second hypothesis). Thus, high initial NSC levels were co-associated with other metabolites which contribute functional roles in the defense mechanisms of mangroves to drought and salinity stress.

To the best of our knowledge, this is the first metabolomics-based study to show metabolite differences following experimental NSC manipulation in mangroves. Our study reveals that a broad range of sugars and metabolites were differently modulated between the light treatment groups; with metabolic impacts extending beyond the more traditionally measured NSC compounds. For example, xylose, arabinose, rhamnose, ribose, glycerol, and myo-inositol were more abundant in the high NSC group compared to the low NSC group, whereas amino acids such as lysine, creatinine, ornithine, isoleucine, valine, glutamine, and phenylalanine were more abundant in the low NSC group.

There was an associated increase in glucose and decrease in phosphoenol pyruvic acid relative to plant height. These compounds play major roles, being differentially regulated in the detected biochemical pathways after treatment application in relation to plant height. We were only able to correlate the metabolic and growth/physiological responses, rather than teasing apart the specific drought or salt effects on metabolite responses in relation to growth or physiology. Further targeted studies would be required to do this. Sugars, such as glucose, have versatile functions in plant growth and development, with key roles as energy sources and signalling molecules. Plants have mechanisms to sense free sugar levels, and can regulate growth and development in response to these levels by controlling expression of growth-related genes (Gibson 2005). Metabolomics-based analyses of *Arabdopsis thaliana* has shown that a specific combination of metabolites are closely linked with plant biomass, and entire metabolite matrices can be used to predict growth (as seen

366 in our study via multivariate regression modelling) (Meyer et al. 2007). Metabolic
367 signatures in *A. thaliana* associated with high plant growth rate primarily include those
368 involved in glycolysis (e.g., glucose and phoshoenol pyruvic acid) and the TCA cycle
369 (Meyer et al. 2007), with glucose being thought to play more of a signalling role rather
370 than as a substrate for synthesis of cellular components (Gibson 2005).

371 Several studies have examined the effect of either drought or salinity in
372 isolation. However, when both treatments are applied together the interpretation of
373 plant physiological responses becomes increasingly difficult to elucidate, involving the
374 synthesis, use and redistribution of many metabolites which belong to multiple
375 biochemical pathways (Flowers & Colmer, 2008; Munns & Tester, 2008; Slama et al.,
376 2015). A recent metabolomics-based study demonstrated that metabolic responses of
377 maize plants to multiple concurrent stresses are distinctly different than when salinity,
378 drought, and heat are applied alone (Sun et al. 2016). The biphasic model of plant salt
379 tolerance suggests that although the initial plant responses to drought and salinity
380 stresses are similar, long-term responses are not (Munns, 1993; Munns & Tester,
381 2008). In the present study, with the combination of drought and salinity stressors, a
382 more generic response was found with metabolites belonging to different pathways,
383 not salt or drought-specific. We have shown how primary metabolic pathways respond
384 to interacting stress factors and overlap with other metabolic pathways leading to the
385 production of different metabolites (Aroca, 2012; Kumari & Parida, 2018; Sanchez et
386 al., 2008a,b). We observed changes in amino acid, sugar and organic acid metabolic
387 pathways consistent with other studies (Alla et al., 2012; Munns & Tester, 2008;
388 Sanchez et al., 2008b).

389 The amino acid proline is an important osmolyte and antioxidant in plants
390 (Slama et al., 2015), and is typically accumulated by many plant species in response

391 to environmental stresses (Kishor et al., 2005; Parida & Jha, 2010). In the present
392 study, the L-NSC plants had higher abundance of proline in the medium salinity–high
393 drought compared to the H-NSC plants. As salinity increased from low to medium
394 saline conditions, proline likely acted as an osmoprotectant. In *Bruguiera parviflora*, it
395 was shown that proline content increased gradually as salinity increased from low (100
396 mM) to medium (200 mM), but decreased at higher salinity (400 mM; Parida et al.,
397 2002). In other halophytes (*Atriplex halimus* and *Lepidium crassifolium*), proline
398 accumulation was observed under high salt conditions and long term drought (Alla et
399 al., 2012; Murakeozy et al., 2003). In *Limonium latifolium*, proline and other
400 metabolites contributed less than 25% to the osmoregulatory activity compared to
401 sugars and hexoses, revealing that soluble carbohydrates are also very important
402 (Gagneul et al., 2007).

403 In most plants, proline accumulation is accompanied by increased levels of
404 carbohydrates during stress. For example, in *Bruguiera parviflora*, proline and total
405 sugar contents increased concomitantly after 45 days of 400 mM salt exposure (Parida
406 et al., 2002). Similarly, in *Atriplex halimus*, exposure to 550 mM salt and 10 days
407 drought simultaneously increased levels increased of proline and sucrose (Alla et al.,
408 2012). Although saline conditions induce a nitrogen deficient environment, halophytes
409 tend to accumulate nitrogenous compounds such as amino acids (Mansour, 2000;
410 Murakeozy et al., 2003). The species in the present study belongs to the physiotype
411 that accumulates both carbohydrates and nitrogenous compounds in higher
412 quantities, as is also seen in the seagrass *Triglochin maritima* (Briens & Larher, 1982).
413 Proline may also provide NAD^+ and $NADP^+$ for photosynthetic and respiratory process
414 (Slama et al., 2015), and plays an important role in stabilising membranes and proteins
415 (Ashraf & Foolad, 2007; Parida & Jha, 2010). In our study of New Zealand mangroves,

the accumulation of proline and sugars in H-NSC plants may have contributed to protein stabilisation of sub-cellular structures and an enhanced capacity for osmotic adjustment.

The organic acids α-ketoglutarate and α-ketoglutamarate, the carbohydrate galactose, and the sugar alcohol glycerol were significantly affected by one or more of the factors (NSCs, drought and salinity). This is consistent with a previous study on *Populus euphratica* leaves, where exposure to heat and high salinity caused decreases in levels of α-ketoglutarate the soluble sugar fructose, and the sugar alcohol mannitol, along with concomitant increases in the sugar alcohol glycerol and its oxidation product glyceric acid (Brosche et al., 2005; reviewed by Sanchez et al., 2008b). These changes were attributed to high levels of sodium in the soil (Brosche et al., 2005; reviewed by Sanchez et al., 2008b). Additionally, in *Lotus japonicas*, physiological responses to high salinity exposure include organic acid reduction and accumulation of amino acids and sugars (Sanchez et al., 2008a, 2012). In the present study, α-ketoglutarate (a key regulator of carbon and nitrogen metabolic interactions) accumulated in mangroves with the L-NSC phenotype compared their H-NSC counterparts under low salinity – high drought conditions.

Levels of α-ketoglutarate decrease under nitrogen starvation, which is usually associated with hypersaline conditions (Stitt & Fernie, 2003). In the present study, the abundance of α-ketoglutarate was affected as drought increased from medium to high, under low saline conditions. We also detected significantly higher levels of free amino acids under increased salinity and drought, which is consistent with limited soil nitrogen availability (Tschoep et al., 2009). In plants grown under mild but continuous nitrogen limitation, total free amino acid levels increased in tissues due to remobilisation of stores, and decreased utilisation of amino acids for protein synthesis

441 and growth (Krapp et al., 2011). Amino acid accumulation is a widespread
442 osmoregulatory mechanism of *Avicennia marina* (Parida & Jha, 2010).

443 We also detected increased synthesis of the phenolic compounds sinapic and
444 syringic acids in both the L-NSC and H-NSC plants after drought and salinity
445 treatments. Phenolics function as antioxidants and also absorb UV light, and tend to
446 be highly abundant in certain mangroves species (Asha et al., 2012; Suh et al., 2014).
447 These compounds assist plants in coping with environmental stress through
448 scavenging harmful levels of reactive oxygen species (ROS) and providing UV
449 protection (Goleniowski et al., 2013). Salt and drought stress are well-known to induce
450 overproduction of ROS in plants (De Carvalho, 2008; Miller et al., 2010). ROS are
451 critical secondary messengers and the level of steady-state cellular ROS is used by
452 plants to monitor their intracellular level of stress (Miller, 2002). When ROS rises
453 beyond manageable limits, oxidative damage to DNA, membrane lipids, and proteins
454 occur. Siani et al. (2018) recently demonstrated that salt-tolerant rice cultivars have
455 higher levels of basal ROS than their salt sensitive counterparts, but also express
456 higher activities of ROS regulatory enzymes to keep the system in check. It is thought
457 that hydrogen peroxide in particular can activate diverse signal transduction pathways
458 involved in salt stress amelioration through enhancing several defense-related
459 mechanisms (Sadak, 2016). Sinapic and syringic acids in *Avicennia marina subsp.*
460 *australasica* offers antioxidant potential to manage excess ROS production during
461 abiotic stress events, and protects against harmful UV levels which can be intense
462 within the mangroves' distribution range during summer months (Liley & McKenzie,
463 2006).

464 In conclusion, this study is a first attempt to comprehensively profile metabolites
465 in response to interacting abiotic stress factors in mangroves. Our findings indicate

that light swapping differentially modulates synthesis and use of numerous sugars and other metabolites in addition to the usually characterised sugars in plant ecophysiological studies. The abundance of primary metabolites in mangroves influences secondary metabolite accumulation, leading to enhanced salinity tolerance and enabling them to thrive in highly inundated saline conditions. Future studies should investigate seasonal effects in addition to elucidating responses to abiotic factors. Metabolite profiling of mature trees under natural conditions is a way forward to better understand the biochemical mechanisms that halophytes employ to cope with extreme environmental conditions.

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Author Contributions

S.R. and S.L. designed the study. S.R. and T.V.N. performed the experiments. S.R. and T.Y. analyzed the data. S.R., T.Y., C.M-Ng, A.A., M.D. and S.L. wrote the manuscript.

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Supplementary information

Table S1. t-test results for metabolite abundances in mangrove leaves after light swapping treatment.

Table S2. Pathway analysis results of log transformed and range scaled metabolite abundances in mangrove leaves after light swapping treatment.

Table S3. Pathway analysis results of log transformed and range scaled metabolite abundances in mangrove leaves correlated with the measured physiological and growth parameters after 12 weeks or drought and salinity treatment.

Table S4. Generalised least squares analysis to test the effects of NSC, salinity, drought on each metabolite expressed. Likelihood ratio test results using a backwards selection procedure applied to a generalised least squares model, where L = likelihood ratio statistic, and df = degrees of freedom of the L statistic. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure legends

Fig. 1. NSC manipulation. a) Experimental design for NSC manipulation (Light swapping treatment). One group of plants were grown in low light (PPFD = 24.2 ± 4.9 µmol m^{-2} s^{-1}) to high light (PPFD = 307.6 ± 22.8 µmol m^{-2} s^{-1}) conditions; another group of plants were grown in high light to low light conditions (plant growth occurs only in high light and not in low light). b) Starch and soluble sugar levels at the end of the light swapping treatment (data obtained from Ravi et al., 2019, in preparation). The resulting plants with different NSC levels were subjected to a NSC × salinity × drought factorial experiment.

Fig. 2. Projection of annotated metabolites in mangrove (*Avicennia marina subsp. australasica*) leaves overlaid on the KEGG model plant (*Arabidopsis thaliana*) global pathway map. Red filled circles = metabolite matches ($n = 69$), black edges = obtained coverage of KEGG pathway modules with one or more matching compounds.

Fig. 3. Effect of light-swapping regime on mangrove leaf metabolome via heatmap and hierarchical cluster analysis (Euclidean distance; Ward's criterion) of the top 50 metabolites ranked by their t-test *p*-values. Columns represent samples (red = High-NSC group; green = Low_NSC group), and rows represent metabolites. The red/white/blue colour scale represents standardised (log-transformed and range-scaled) metabolite abundances, where dark red = higher values, and dark blue = lower values.

Fig. 4. Topology-based pathway analysis showing metabolic networks in mangrove seedlings significantly altered by the light-swapping technique to manipulate levels of non-structural carbohydrates. The most impacted metabolic pathways are designated by the volume and the colour of the spheres (yellow = least relevant; red = most relevant; small circles = low pathway impact scores, large circles = high pathway impact scores) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA), respectively. Dotted line represents the significance cutoff at $P < 0.05$.

Fig. 5. Principal components regression (Log_{10} Metabolite abundance vs Plant height).

Fig. 6. Topology-based pathway analysis showing metabolic networks in mangrove seedlings significantly altered after the drought and salinity treatments in relation to mangrove growth (plant height). The most impacted metabolic pathways are designated by the volume and the colour of the spheres (yellow = least relevant; red = most relevant; small circles = low pathway impact scores, large circles = high pathway impact scores) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA), respectively. Dotted line represents the significance cutoff at $P < 0.05$.

Fig. 7. Relationships between plant height and the relative abundances of glucose ($P = 0.0024$, $R^2 = 0.334$) (left) and phosphoenol pyruvic acid ($P = 0.0018$, $R^2 = 0.35$) (right). Black line indicates the line of fit. Grey bars indicate the 95% confidence intervals.

Table 1. Metabolites that were significantly affected (Bonferroni corrected; $P<0.0009$) after drought and salinity treatments (tested interactions = NSC × salinity × drought, NSC × salinity, NSC × drought, salinity × drought; tested main effects = NSC, salinity, drought).

Metabolite	Biochemical pathways	Interactions and main effects
Malonic acid	Tricarboxylic acid cycle (TCA) Malonic acid pathway Acetate-malonate pathway	Drought
α-ketoglutarate	TCA cycle Pentose & glucoronate interconversions Ascorbate & aldarate metabolism Alanine, aspartate & glutamate metabolism Lysine biosynthesis Lysine degradation Histidine metabolism Glyoxylate & dicarboxylate metabolism	NSC × salinity × drought
α-ketoglutamarate	Unknown	NSC × drought
Glycerol	Galactose metabolism	Drought
Galactose	Galactose metabolism	NSC × salinity
Leucine	Valine, leucine & isoleucine biosynthesis	NSC × drought
Putrescine	Arginine & proline metabolism Glutathione metabolism	NSC × salinity
p-Toluic acid	Biosynthesis of secondary metabolites	NSC × salinity × drought
Syringic acid	Aminobenzoate degradation	NSC × salinity × drought
Nicotinic acid	Nicotinate & nicotinamide metabolism Tropane, piperidine & pyridine alkaloid biosynthesis	NSC × salinity
Tridecane	Intermediate in essential oil synthesis	NSC × salinity × drought

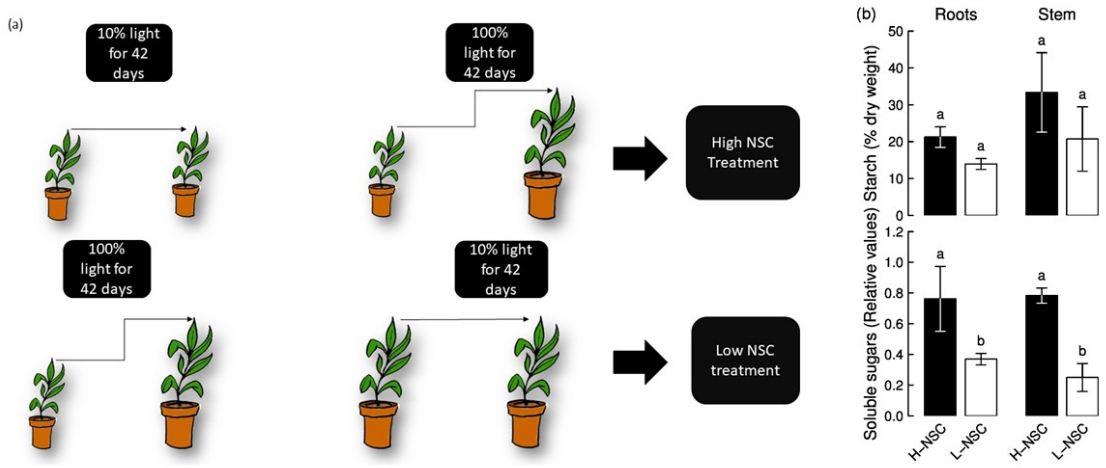

Fig. 1. NSC manipulation. a) Experimental design for NSC manipulation (Light swapping treatment). One group of plants were grown in low light (PPFD = 24.2 ± 4.9 µmol m^{-2} s^{-1}) to high light (PPFD = 307.6 ± 22.8 µmol m^{-2} s^{-1}) conditions; another group of plants were grown in high light to low light conditions (plant growth occurs only in high light and not in low light). b) Starch and soluble sugar levels at the end of the light swapping treatment (data obtained from Ravi et al., 2019, in preparation). The resulting plants with different NSC levels were subjected to a NSC × salinity × drought factorial experiment.

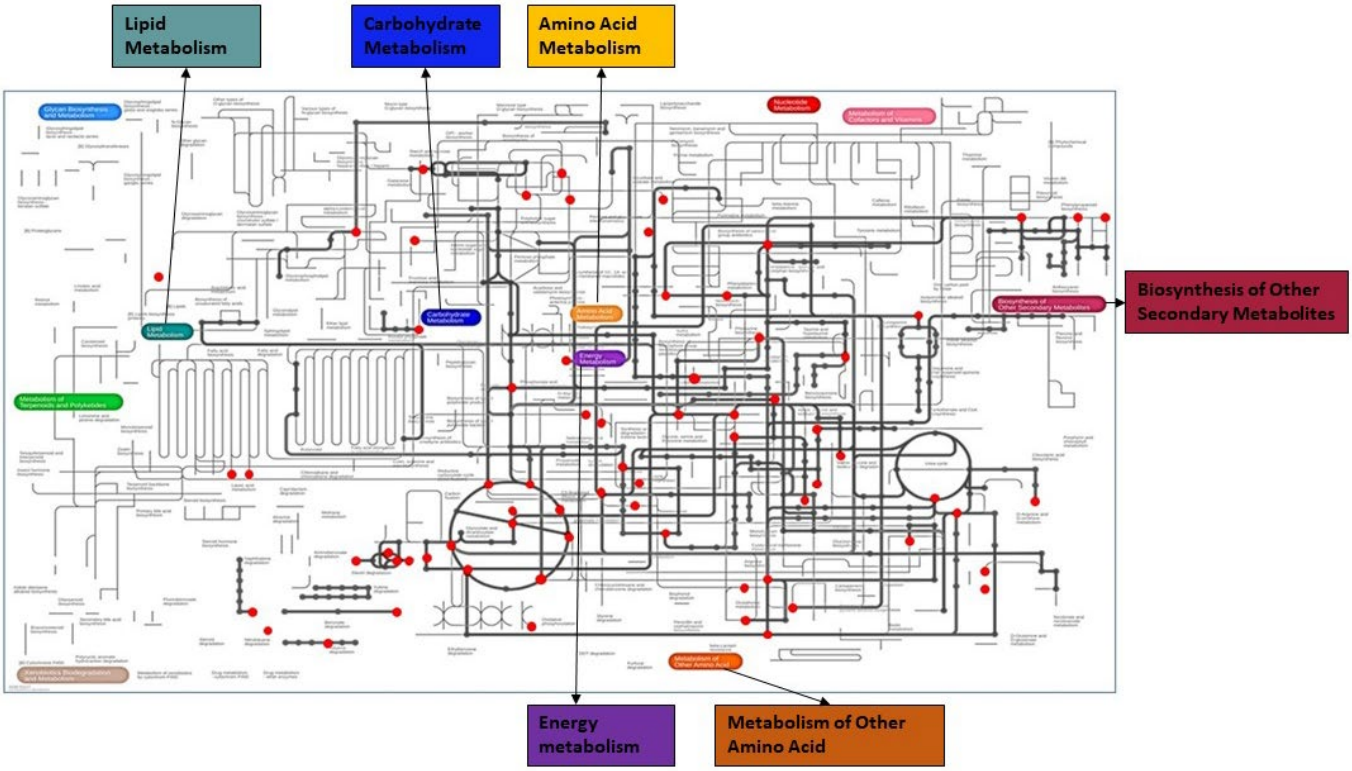

Fig. 2. Projection of annotated metabolites in mangrove (*Avicennia marina subsp. australasica*) leaves overlaid on the KEGG model plant (*Arabidopsis thaliana*) global pathway map. Red filled circles = metabolite matches (*n* = 69), black edges = obtained coverage of KEGG pathway modules with one or more matching compounds.

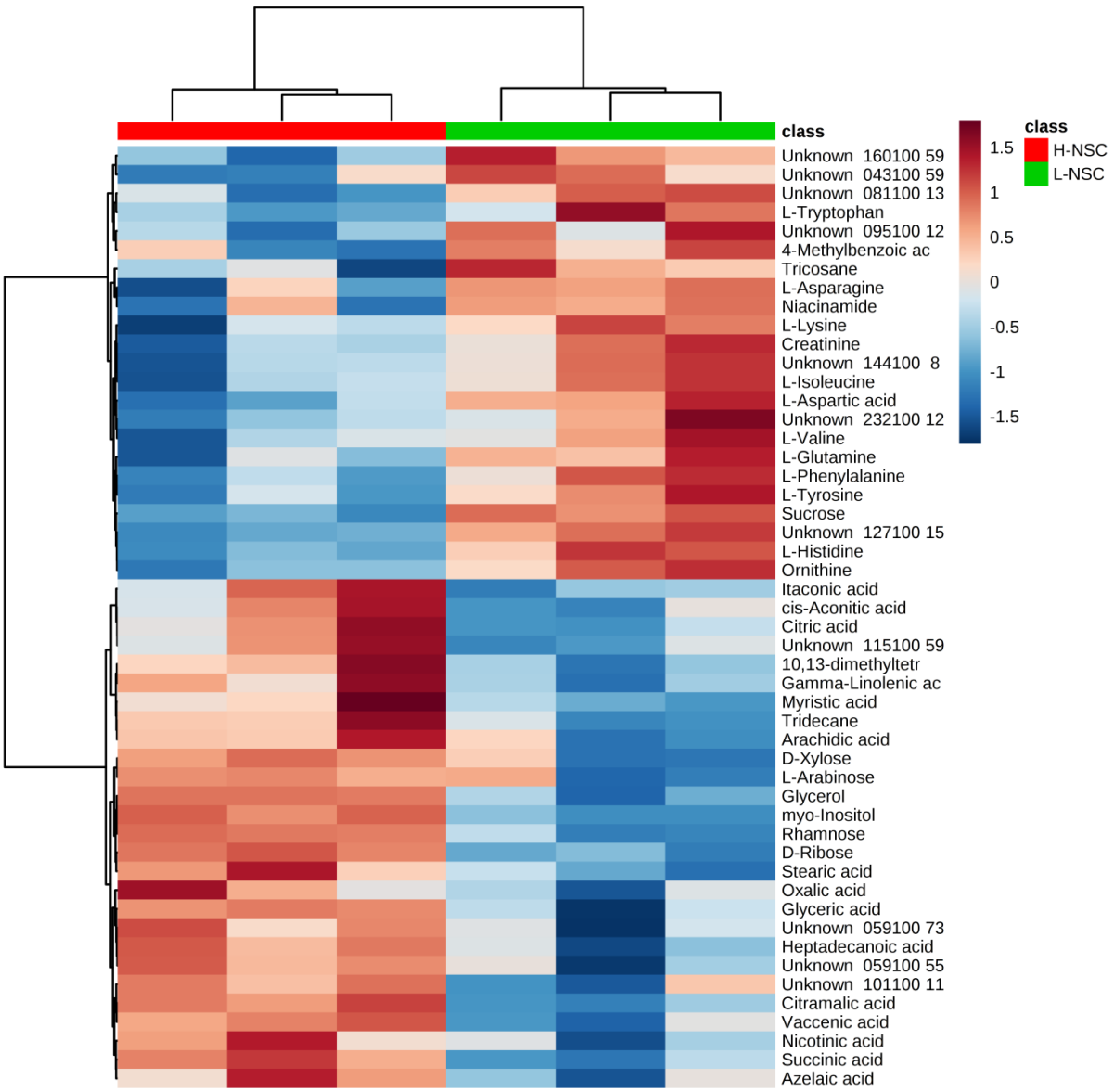

Fig. 3. Effect of light-swapping regime on mangrove leaf metabolome via heatmap and hierarchical cluster analysis (Euclidean distance; Ward's criterion) of the top 50 metabolites ranked by their t-test *P*. Columns represent samples (red = High-NSC group; green = Low-NSC group), and rows represent metabolites. Within the High-NSC and Low-NSC group, each subcolumn represents one biological replicate. The red/white/blue colour scale represents standardised (log-transformed and range-scaled) metabolite abundances, where dark red = higher values, and dark blue = lower values.

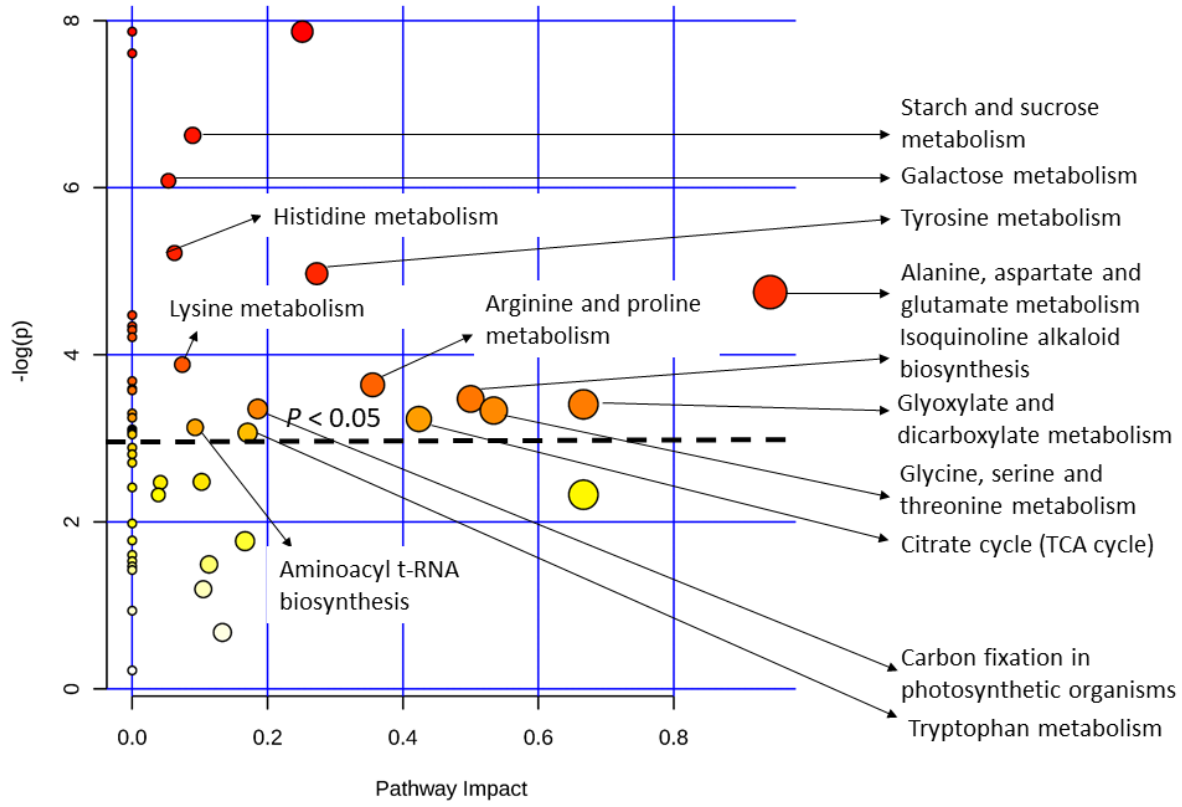

Fig. 4. Topology-based pathway analysis showing metabolic networks in mangrove seedlings significantly altered by the light-swapping technique to manipulate levels of non-structural carbohydrates. The most impacted metabolic pathways are designated by the volume and the colour of the spheres (yellow = least relevant; red = most relevant; small circles = low pathway impact scores, large circles = high pathway impact scores) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA), respectively. Dotted line represents the significance cutoff at $P < 0.05$.

Fit statistics

p-value	<0.001
R^2	0.77
$R^2_{adj.}$	0.67
MSE	0.619
RMSE	0.787
MAPE	16.91

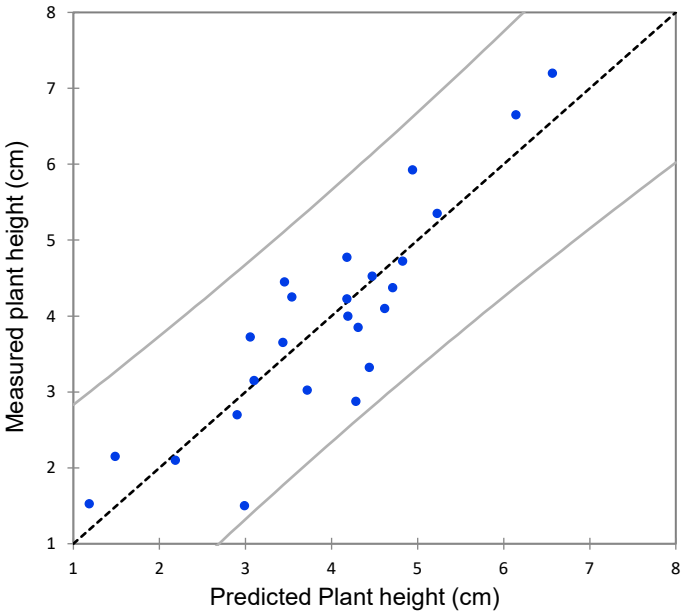

Fig. 5. Principal components regression (Log_{10} Metabolite abundance vs Plant height).

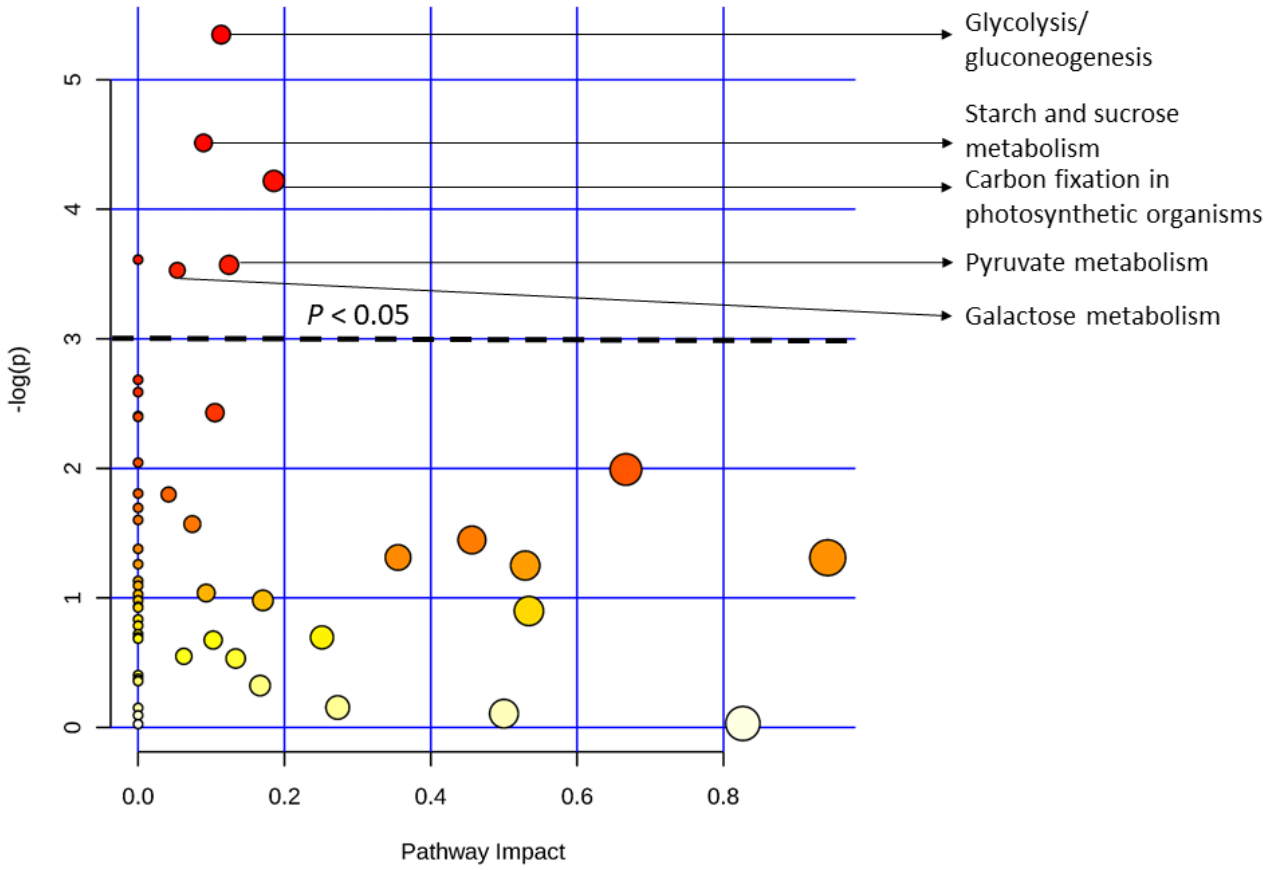

Fig. 6. Topology-based pathway analysis showing metabolic networks in mangrove seedlings significantly altered after the drought and salinity treatments in relation to mangrove growth (plant height). The most impacted metabolic pathways are designated by the volume and the colour of the spheres (yellow = least relevant; red = most relevant; small circles = low pathway impact scores, large circles = high pathway impact scores) according to their statistical relevance and pathway impact (PI) values resulting from Quantitative Enrichment Analysis (QEA) and Network Topology Analysis (NTA), respectively. Dotted line represents the significance cutoff at $P < 0.05$.

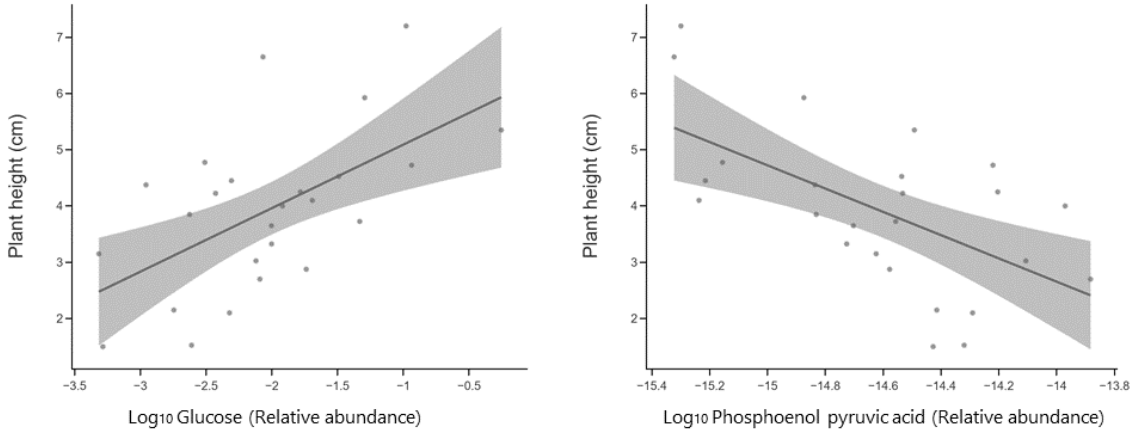

Fig. 7. Relationships between plant height and the relative abundances of glucose (P = 0.0024, R^2 = 0.334) (left) and phosphoenol pyruvic acid (P = 0.0018, R^2 = 0.35) (right). Black line indicates the line of fit. Grey bars indicate the 95% confidence intervals.

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Supplementary table. S1. t-test results for relative abundance metabolite data after light swapping treatment.

Metabolite	t.stat	p.value
Sucrose	-11.82	0.0003
myo-Inositol	11.04	0.0004
D-Ribose	10.32	0.0005
Unknown 127100 15949.8 5948.8	-8.54	0.0010
Citramalic acid	7.21	0.002
Rhamnose	6.14	0.0036
Glycerol	5.87	0.0042
L-Histidine	-5.47	0.0054
Succinic acid	5.06	0.0071
Ornithine	-4.32	0.0124
Unknown 160100 5943.4 11622.7 split peak 1	-4.23	0.0134
L-Aspartic acid	-4.20	0.0136
Vaccenic acid	3.79	0.0193
Unknown 081100 13651.6 15242.5	-3.69	0.0208
Stearic acid	3.48	0.0253
L-Phenylalanine	-3.47	0.0256
L-Tyrosine	-3.26	0.0311
Heptadecanoic acid	3.21	0.0324
Glyceric acid	3.08	0.0367
10,13-dimethyltetradecanoic acid	3.04	0.0383
Gamma-Linolenic acid	2.88	0.0446
Creatinine	-2.88	0.0447
L-Glutamine	-2.88	0.0448
Itaconic acid	2.88	0.0448
Citric acid	2.86	0.0459
L-Tryptophan	-2.84	0.0466
D-Xylose	2.82	0.0475
Tridecane	2.80	0.0488
Unknown 144100 8851.5 11546.4	-2.77	0.0499
L-Isoleucine	-2.77	0.0499
Unknown 095100 12631.0 3911.2	-2.76	0.0506
L-Asparagine	-2.73	0.0524
Unknown 043100 5994.7 8548.1	-2.72	0.0526
Unknown 059100 5559.0 13143.7	2.69	0.0541
L-Lysine	-2.59	0.0603
Tricosane	-2.56	0.0623
Unknown 115100 5965.5 18950.1	2.51	0.0654

Metabolite	t.stat	p.value
Azelaic acid	2.4627	0.0695
Myristic acid	2.4451	0.0708
Nicotinic acid	2.4408	0.0711
cis-Aconitic acid	2.4363	0.0715
4-Methylbenzoic acid	-2.4127	0.0733
Unknown 232100 12872.0 15651.0	-2.4076	0.0737
Arachidic acid	2.3538	0.0782
Niacinamide	-2.3188	0.0812
L-Valine	-2.2297	0.0896
Unknown 059100 7369.3 7745.8	2.2054	0.0921
L-Arabinose	2.1606	0.0968
Oxalic acid	2.1422	0.0988

Supplementary table S2. Pathway analysis results of log transformed and range scaled metabolite abundance after light swapping treatment.

Pathway name	Hits	p.value (P)	Impact
Starch and sucrose metabolism	3/30	0.0013	0.08
Galactose metabolism	5/26	0.0023	0.05
Histidine metabolism	1/16	0.0054	0.06
Tyrosine metabolism	3/18	0.0069	0.27
Alanine, aspartate and glutamate metabolism	11/22	0.0087	0.94
Lysine biosynthesis	2/10	0.0206	0.07
Arginine and proline metabolism	8/38	0.0263	0.35
Isoquinoline alkaloid biosynthesis	1/6	0.0311	0.5
Glyoxylate and dicarboxylate metabolism	6/17	0.0332	0.66
Carbon fixation in photosynthetic organisms	4/21	0.0350	0.18
Glycine, serine and threonine metabolism	6/30	0.0357	0.53
Citrate cycle (TCA cycle)	8/20	0.0395	0.42
Aminoacyl-tRNA biosynthesis	18/67	0.0437	0.09
Tryptophan metabolism	1/27	0.0466	0.17

Supplementary table S3. Pathway analysis results after correlation of log transformed and range scaled metabolite abundance data with the measured physiological and growth parameters after 12 weeks or drought and salinity treatment.

Pathway name	Hits	Plant height		Leaf area		Stem water potential		Max hydraulic conductivity		Stomatal conductance	
		p.value (*P*)	PI#	p.value (*P*)	PI#	p.value (*P*)	PI#	p.value (*P*)	PI#	p.value (*P*)	PI#
Glycolysis/gluc-oneogenesis	3/25	0.0047*	0.11	0.576	0.11	0.4160	0.11	0.6573	0.11	0.1134	0.11
Starch and sucrose metabolism	3/30	0.0109*	0.08	0.0145*	0.08	0.0484*	0.08	0.3097	0.08	0.8464	0.08
Galactose metabolism	5/26	0.0293*	0.05	0.0448*	0.05	0.0134*	0.05	0.5485	0.05	0.0839	0.05
Carbon fixation	5/21	0.0147*	0.18	0.1503	0.18	0.3175	0.18	0.4556	0.18	0.1524	0.18
Pyruvate metabolism	5/21	0.0281*	0.12	0.2368	0.12	0.2596	0.12	0.4027	0.12	0.0384	0.12

*- p. value < 0.05

#- Pathway impact score (PI)