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Comparative metabolic and ionomic profiling of two cultivars of *Stevia rebaudiana* Bert. (Bertoni) grown under salinity stress

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Highlights

- Fengtian showed increased levels of steviol glycosides, particularly rebaudioside A.
- Salinity stress reduced stevia plant height and biomass, particularly in cultivar Shoutian-2.
- Fengtian maintained higher K^+/Na^+ ratios as compared to Shoutian-2.
- Amino acids and amines were the major osmotica in stevia under salinity stress.
- Fengtian accumulated higher levels of proline and gluconate.

Abstract

This study provides a comprehensive investigation on the impact of increasing NaCl concentrations on hydroponically grown *Stevia rebaudiana* cultivars (Shoutian-2 and Fengtian). Growth parameters including plant height, biomass and physiological responses including osmotic potential were measured. In addition, the levels of steviol glycosides, elements and primary metabolites were measured and statistically evaluated. The cultivar Fengtian grew faster, accumulated less Na^+ and compatible organic solutes, and more K^+ in the leaves, as compared to the cv. Shoutian-2. Metabolite analysis identified 81 differentially accumulated metabolites, indicating an alteration in the metabolite phenotype of both cultivars upon exposure to salinity A general increase in many amino acids, amines, sugars and sugar phosphates with a concurrent decrease in most organic acids; including tricarboxylic acid (TCA) cycle intermediates, was observed. In the more salt tolerant cv. Fengtian, the levels of hexose phosphates and metabolites involved in cellular protection increased in response to salinity. These metabolites remained unchanged in the sensitive cv. Shoutian-2. Interestingly, salt treatment notably increased the rebaudioside A concentration by 53% while at the same time stevioside decreased by 38% in Fengtian which has important implications for controlling the relative amounts of reboudioside A and stevioside. The findings of this study leads to the conclusion that mild salinity stress can increase the yield of sweetener compounds, which is dependent on the cultivar and the level of salinity stress.

Abbreviations: Stevioside: ST; Rebaudioside A: RA; Hydrophilic interaction liquid chromatography: HILIC; Liquid chromatography-electrospray ionization mass spectrometry: LC-ESI-MS; Multiple reaction monitoring: MRM; Steviol glycoside: SG; Gas chromatography-mass spectrometry: GC-MS; tricarboxylic acid: TCA; Leaf osmotic potential: LOP; fresh weight: FW; Principal Component Analysis: PCA; dry weight: DW; sodium: Na^+, potassium: K^+, magnesium: Mg^{2+}; calcium: Ca^{2+}; gamma-aminobutyric acid: GABA; uridine diphosphoglucose: UDPG, uridine triphosphate: UTP, adenosine triphosphate: ATP; electrospray ionization: ESI; Triple quadrupole mass spectrometry: *QQQ*-MS; Hydrophilic interaction liquid chromatography: HILIC; trimethylsilyl group: TMS; Analysis of Variance: ANOVA; Visualization and Analysis of Networks containing Experimental Data: VANTED; Milli Pascal: MPa.

1. Introduction

Stevia rebaudiana Bert. (Bertoni) is a perennial shrub indigenous to Paraguay, South America. It is economically important in Asia and South America and grown for its non-carcinogenic and low-calorie sweeteners present in the leaves (Lemus-Mondaca *et al.*, 2012). Stevia extracts have been used by traditional South American cultures for a range of medicinal applications, and studies have indicated that steviosides are beneficial to human health (Gardana *et al.*, 2010 and references therein). These extracts were associated with antiproliferative effects in different cancer cells (Lopez *et al.*, 2016), and its antidiabetic (Zeng *et al.*, 2013; Ritu and Nandini, 2016), antimicrobial (Atteh *et al.*, 2008), anti-hyperglycemic, and antifungal activities (Lemus-Mondaca *et al.*, 2012) have been investigated. Due to these various beneficial attributes, sweeteners produced from *Stevia* plants are gaining popularity. The US Food and Drug administration (US FDA), European Food Safety Authority (EFSA), Food Standards Australia New Zealand (FSANZ), the Joint FAO/WHO Expert Committee on Food Additives, and recently, the European Union (EU) have also considered the addition of steviol glycosides as a naturally occurring authorised sweetener for the food industry (Kubica *et al.*, 2015).

Steviol glycosides (SGs) are the secondary metabolites responsible for the sweetness of Stevia (Gupta *et al.*, 2014; Urban *et al.*, 2015). Stevioside (ST) and rebaudioside A (RA) are the most abundant diterpenoid glycosides, but more than 30 additional SGs are currently known (Woelwer-Rieck *et al.*, 2010). Recently, two minor diterpene glycosides, rebaudioside R and S were detected in the leaves of *Stevia rebaudiana* (Ibrahim *et al.*, 2016). In dried leaves, ST represents 4-13% of SGs and is 110-270 times sweeter than conventional sugar (sucrose) (Tavarini and Angelini, 2012). In contrast, RA represents 2-4% of SGs but is 180-400 times sweeter than sucrose. As compared to ST, RA has an additional glucose monomer that gives it a higher sweetening potency and therefore is the most preferred component of the stevia leaf extracts (Lemus-Mondaca *et al.*, 2012). The RA also lacks the bitter aftertaste usually found associated with steviosides (de Oliveira *et al.*, 2007). Most importantly, purified RA has no effect on either blood pressure or glucose homeostasis (Carakastos *et al.*, 2008). It is for this reason, the new cultivars of *S. rebaudiana* with a higher content of RA and a reduced content of ST are being developed by the plant breeders to improve the utilization of this source of natural sweeteners (Yadav *et al.*, 2011).

Many countries have shown interest in the commercial cultivation of *Stevia rebaudiana* (Ramesh *et al.,* 2006, Ramesh *et al.,* 2007), but further research is required to better understand the physiological and biochemical responses to a range of abiotic stresses affecting SG production (Ren and Shi, 2012). The accumulation of such commercially important secondary metabolites is affected by abiotic stresses (Arbona *et al.,* 2013), as SGs play important role in the adaptation of plants to stress environments via alleviating stress associated effects (Hill and Roessner, 2015; Ramakrishna and Ravishankar, 2011). Salinity is one of the major environmental stress factors that cause disturbances in plant growth and nutrient balance, reducing crop yields. It leads to alteration in metabolic processes, membrane disorganisation, and oxidative stress, reduction in cell division in addition to inducing water stress and ion toxicity (Blumwald, 2000, Aswathappa and Bachelard, 1986; Popp *et al.,* 1990). The ultimate aim of salinity tolerance research is to increase the ability of plants to maintain growth and productivity in saline soils, thereby reducing the effects on growth and yield by introducing new traits like ion exclusion and tissue tolerance to osmotic stress (Roy *et al.,* 2014). The accumulation of low-molecular compounds, termed as compatible solutes, is one important adaptation mechanism that plants exhibit in response to osmotic stress (Cao *et al.,* 2017). Metabolite profiling has proven to be a powerful tool to gain an overview of biochemical changes occurring in important crops upon exposure to salt stress, and to identify pathways potentially involved in salinity tolerance (Dias *et al.,* 2015; Natera *et al.,* 2016; Shabala *et al.,* 2016; Shelden and Roessner, 2013).

Recently, Zeng *et al.,* (2013) reported that salt stress for four weeks changed growth and physiological responses as well as glycoside contents of *Stevia rebaudiana*. Their study showed a decrease in total dry weight and chlorophyll and an increase in proline concentration in response to with increasing salt concentrations (60, 90, and 120 mM). Both RA and ST concentration also decreased with increasing salt concentrations and the ratio of RA/ST of salt-treated plants changed. Their study indicated that this plant could tolerate salt stress, and there is a possibility of optimising the SG composition by using saline soil for growing *S. rebaudiana*. Another recent study found that stevia copes well with mild (34 and 90 mM), short-term (16 and 25 days) salinity stress, which did not change chlorophyll, RA or ST concentrations, but changed tissue ion concentrations (Cantabella *et al.,* 2017).

In this study, we investigated the growth, physiological and biochemical changes induced by salinity stress in two cultivars of *Stevia rebaudiana* (cv. Shoutian-2 and Fengtian) which

showed contrasting salt responses. As previous studies investigated the effects of short-term and mild salinity stress (Zeng *et al.,* 2013; Cantabella *et al.*, 2017), we focussed instead on investigating long-term exposure to salinity stress (8 weeks treatment) at three levels of salinity, ranging from mild to severe (50 mM, 100 mM, 200 mM). We determined differential changes in plant height, biomass accumulation, osmolarity, chlorophyll, RA, ST, ion and primary metabolite concentrations in both salt stressed and control plants of two cultivars of *Stevia rebaudiana*. The aims of this study were to to investigate the effects of salinity stress on the plant phenotype (growth and physiology), as well as the metabolome and ionome. We identified a cultivar that is tolerant to salinity stress whilst maintaining high yields of SG's, suitable for the food industry.

2. Materials and Methods

2.1. Plant growth conditions and treatments

Seeds of the two cultivars of *Stevia rebaudiana,* cv Shoutian-2 (C1) and Fengtian (C2), were supplied by Mr Andrew Rank (Central Queensland University, Rockhampton, Australia). The seeds were sown in a potting media containing a mixture of washed river sand, commercial potting mix and coconut peat (4:3:3 v/v). These cultivars were selected based their high SG content (Midmore *et al.,* 2012). Fifteen days after germination, seedlings were transplanted into small plastic pots (5 cm × 10 cm) containing perlite and placed in a tray supplied with half strength hydroponic solution (Agromatic Corporation Pty Ltd, Victoria, Australia). The hydroponics solution contained the following macronutrients (mM): nitrate (N) 3.62, potassium (K) 7.18, calcium (Ca) 4.74, sulphur (S) 1.44, magnesium (Mg) 1.17, phosphorus (P) 1.66, and micronutrients (μM): iron (Fe) 37.6, boron (B) 24.98, manganese (Mn) 6.92, copper (Cu) 0.79, zinc (Zn) 1.84, and molybdenum (Mo) 0.1. The half-strength hydroponic solution had a conductivity of 1.062 dS/m. The pH of the solution was monitored and maintained at 5.8 throughout the experiment.

Two weeks after the adaptation period, plants were transferred to 10 L white plastic buckets connected to 200 L tanks containing half-strength hydroponics solution, and were acclimated for one additional week prior to the beginning of the salinity treatment. The salinity treatment consisted of the addition of NaCl to the stock nutrient solution in a step wise manner to obtain concentration of 25 mM every 48 hours, until the concentrations reached 50 mM (T1), 100 mM (T2), 200 mM (T3) and 300 mM (T4). Control plants (T0) were grown in half-

strength hydroponic solution without added NaCl. The plants were severely affected in treatment T4; hence, those plants were not including in the subsequent analyses. The greenhouse conditions were as follows: average day and night temperatures were 20 and 15°C, respectively; and the relative humidity was ~75%. The green house was covered with a translucent polyethylene sheet with 67% of the ambient light at a photoperiod cycle of 16 h light and 8 h dark. Plant height was measured once weekly. The final plant height, leaf number, and shoot fresh weight were recorded at harvest. After 8 weeks of growth, leaf, stem and root tissues of five plants from each treatment were oven dried at 70°C for 2 days and their dry weights recorded.

2.2. Chlorophyll content and osmotic potential

Leaf chlorophyll was measured from the youngest fully expanded leaves once a week using a chlorophyll meter SPAD-502 (Konica, Minolta, Japan). After 7 weeks of treatment, leaf osmotic potential was measured in the youngest fully expanded leaf which was harvested and frozen. The frozen samples were thawed and squeezed to release the sap. The squeezed sap was placed on the vapour pressure osmometer (5500 WESCOR). The osmometer readings (mmol kg^{-1}) were then converted to osmolarity (MPa) using the Van't Hoff relation: ψ_s=CiRT, where C is the osmolarity value in mol kg^{-1}, I is an ionising constant assumed equal to unity; R is the ideal gas constant (0.0083143 kg MPa $mol^{-1}K^{-1}$) and T is absolute temperature.

2.3. Elemental analysis

Approximately 100 mg of oven dried, finely ground leaf samples were weighed and digested overnight at room temperature with a mixture of 2 mL of concentrated nitric acid (HNO_3) and one drop of hydrogen peroxide (H_2O_2) (Hansen *et al.*, 2009). Digests were then placed in a water bath maintained at 70ºC for 4 hours, followed by an addition of 3 mL of distilled water. After digestion, additional distilled water was added to the test tubes until the final volume of 10 mL was reached. After centrifugation at 15,000 rpm for 15 min, the concentrations of sodium (Na^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), and potassium (K^+), were measured with an atomic absorption spectrophotometer (Varian Flame AAS Spectra 220) using the method of Munns *et al.*, (2010). Chloride concentration was determined by transferring approximately 500 mg of the oven-dried samples into glass vials containing 2 mL distilled

water and shaking the samples overnight (140 rpm) at 5ºC. Chloride ions present in the supernatant were measured using a chloride meter (Sherwood MKII Chloride Analyzer 926) according to Munns *et al.,* (2010).

2.4. Metabolite analysis on Gas Chromatography-Mass Spectrometry

Leaf tissue (30 mg) was extracted as described by Hill *et al.,* (2013a). Following extraction, 20 µL and 50 µL aliquots of the extract were transferred into glass vial inserts and dried *in vacuo* for GC-MS and analysis. GC-MS analysis was performed as described by Hill *et al.,* (2013b).

2.5. Extraction and analysis of steviol glycoside using LC-ESI-MS

Dried leaf tissue (50 mg) was weighed into a 2 mL cryomill tube packed with ceramic beads (1.3 mm) then 200 µL of deionised water was added and samples were shaken at 3600 rpm for 3× 30 s at 4°C. Following centrifugation at 14,000 rpm for 5 min the supernatant was removed and the pellets were re-extracted twice using the cryomill with 250 µL of water The supernatants were pooled. A 1 mg mL^{-1} combined stock standard was prepared in water. Seven calibration standards of concentrations between 0.1 and 100 µM, containing both ST and RA, were prepared in 80% acetonitrile..

Samples were analysed on an electrospray (ESI) triple quadrupole mass spectrometer operated in negative ionization mode using multiple reaction monitoring (MRM) mode (Agilent 1200LC and 6410B *QQQ*-MS). Stevioside and rebaudioside A were separated on a Phenomonex Kinetix HILIC column (2.1×50 mm, particle size 1.7 µm) with gradient elution. A binary mobile phase gradient was used consisting 10 mM ammonium acetate in water as buffer A and 10 mM ammonium acetate in 95:5 acetonitrile: water as buffer B. The starting mobile phase conditions were 100% B, which linearly decreased to 30% B over 10 min, followed by a 1 min hold. The column was then re-equilibrated at 100% B from 11.1 – 15 min. The mobile phase flow rate was 0.4 mL min^{-1}, column temperature was 30°C and the injection volume was 1 µL.

The MRM fragmentor and collision energies were optimized using authentic standards of RA and ST purchased from Wako Pure Chemical, Japan Pty. Ltd. The ESI conditions were: source gas temperature 300°C, gas flow 10 L min^{-1}, nebulizer pressure 45 psi and capillary

voltage 4000 V. Peak integration, calibration curve plot and qquantitation was carried out using Mass Hunter Quant software (Agilent). Neutral loss scanning mode was used to identify other SGs by monitoring the characteristic neutral loss of glucose (162 units) which occurs under collision-induced dissociation (CID) of the SGs. The $[M-H]^-$ precursor ions for RA and ST were 965.4 *m/z* and 803.4 *m/z*, and the product ions were 803.4 *m/z* and 641.3 *m/z*, respectively. The optimised collision energies were 25 V and 13 V for RA and ST, respectively.

2.6. Data and statistical analysis

The data of growth and ion concentrations were verified for normality, outliers and homogeneity of error variances using a GenStat (16^{th} edition) statistical package (http://www.vsni.co.uk/software/genstat/). Least square differences (LSD) were used to compare the means when the F ratios were significant.

Resulting GC-MS data were evaluated using either the Analyzer Pro Deconvolution Program (Spectralworks, UK) or Agilent Mass Hunter Workstation Software, Quantitative Analysis, Version B.05.00/Build 5.0.291.0 for GC-MS. Mass spectra of eluting compounds were identified using the public domain mass spectra library of Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html) and the *in-house* Metabolomics (University of Melbourne) Australia mass spectral library. All matching mass spectra were additionally verified by determination of the retention time by analysis of authentic standard substances. Relative response ratios (area of analyte divided by area of internal standard, $^{13}C_6$-sorbitol divided by per sample dry weight (mg)) were calculated for each analysed metabolite as described in Hill and Roessner (2013). The data was log transformed prior to statistical analysis. If a specific metabolite had multiple TMS derivatives, the metabolite with the greater detector response and optimal peak shape within the dynamic range of the instrument was selected.

The relative response ratio for each metabolite for each salt stress treatment (T1, T2 and T3) was normalized to the control (T0) of the same cultivar, resulting in fold changes. The Student's *t*-test was also performed using Microsoft Excel 2010. Multivariate analyses, including the Principal Component Analysis (PCA) and heat maps were generated using the open-source software Metaboanalyst 2.0 (Xia *et. al.*, 2012; http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp).

The experimental data were mapped on an author-created metabolite network of the primary metabolism via the built-in graph editor in VANTED (Junker *et al.*, 2006) (https://immersive-analytics.infotech.monash.edu/vanted/). For each *Stevia rebaudiana* cultivar, non-parametric Spearman's rank correlation between the Na^+ concentration and (1) the metabolite response ratios and (2) the other elemental absolute concentrations were performed in VANTED to estimate their statistical dependence, as described in Hill *et al.*, (2013b).

3. Results

3.1. Impact of salinity on plant growth and biomass accumulation

Both cultivars of *Stevia rebaudiana* showed similar height growth response when exposed to salinity for 4 weeks. The plants showed no signs of stress within the first 4 weeks of salinity treatment. After 5 weeks of treatment, leaf chlorosis and a loss of viability (35% and 30% survival) were observed in the 100 mM and 200 mM salt-treated plants respectively. With an increase in salt concentrations, both Fengtian and Shoutian-2 plants showed slow and stunted growth compared to control plants. By 8 weeks, both cultivars showed a significant reduction in height at all NaCl concentrations, with the reduction more pronounced in Shoutian-2 than in Fengtian. Total plant dry weight was assessed after 8 weeks of reaching the salinity treatment. Cultivars differed in total dry weight, and both cultivars showed significant decrease in biomass under different salinity treatments. The most severe reduction occurred at 200 mM NaCl (**Table 1**). The root dry weight decreased significantly ($P<0.05$) in both cultivars in response to salinity stress, but the response did not differ between the two cultivars. Compared to the control (T0), root dry weight of Shoutian-2 and Fengtian was reduced to 97% and 93%, at 100 mM and 200 mM NaCl concentrations.

Cultivar Fengtian showed a higher leaf biomass as compared to Shoutian-2. There was no significant interaction effect between the cultivars and treatment for leaf biomass; the decrease in the leaf biomass was due to the salinity treatments irrespective of the cultivars. Fengtian had greater stem dry weight than Shoutian-2, an effect that was modified by salinity, such that the differences between the cultivars at 100 and 200 mM was less than that shown at the lower NaCl concentration. The same effect was evident for shoot dry weight, but the interaction effect did not carry over to total dry weight.

3.2. The effect of salinity on chlorophyll and osmolarity

Chlorophyll content in the youngest fully-expanded leaf showed a significant interaction between the salt treatments and cultivars (**Table 2**). However, the most interesting result was the decline in SPAD reading with 50 mM NaCl, and then the subsequent rise with increase in salinity. As expected, the leaf osmotic potential declined significantly with an increase in the salinity, but the reduction was much higher in Shoutian-2 than in Fengtian. Notable decrease between the cultivars occurred at 50 mM wherein the osmotic potential decreased from -0.59

MPa to -1.72 MPa in Shoutian-2 whereas in Fengtian, the reduction was much less (-1.65 to -2.22 MPa).

From the morphological and biomass analyses described above, we conclude that Fengtian is more tolerant to salinity than Shoutian-2. Hence, to identify potential salt adaptation and tolerance mechanisms of stevia, a detailed study of ion concentration (Section 3.3), stevioside and rebaudioside A concentrations (Section 3.4), as well as primary metabolites composition (Section 3.5) were subsequently determined for both cultivars of *Stevia rebaudiana*.

3.3. Impact of salinity on the leaf tissue ion concentrations

As expected, the Na^+ and Cl^- ion concentrations in the leaves increased while K^+, Mg^{2+} and Ca^{2+} concentrations decreased (the latter with one exception and ns for Mg^{2+}) in both cultivars under salinity stress when compared to the control (**Table 3**). Ca^{2+} concentrations decreased with an increase in salt concentrations in stressed plants, but not for Mg^{2+}. Increased salinity levels significantly increased Na^+ and Cl^-, and decreased K^+ concentration in the leaves compared to those of the control. There was no interaction between the cultivars and treatments for neither Na^+ nor Mg^{2+}.

For the other ions, Cl^+ increased more at higher NaCl concentration in Shoutian-2, and K^+ declined more in the same cultivar. Shoutian-2 showed strong reductions of K^+ concentration at the slightest NaCl concentration (**Table 3**). The Cl^-/Na^+ ratio did not differ between the cultivars nor treatments, and the K^+/Na^+ ratio was influenced by the cultivar, treatment and their interactions, such that the effect of K^+ rather than the effect of Na^+ came through the interaction, with cv. Shoutian-2 having less K^+ and lower ratio of K^+/Na^+ at higher NaCl concentration.

3.4. Impact of salinity on ST and RA concentrations of Stevia leaves

In comparison to control, there was a substantial reduction in the ST, and an increase in RA accumulation in salt-stressed stevia plants (**Table 4**). Shoutian-2, the cultivar with a low leaf yield **(Table 1),** yet under salinity stress, accumulated higher concentrations of Reb A and SG than those in Fengtian, and showed a more positive response of increase of Reb A following salt stress (**Table 4**).

The ratios of the concentrations RA/ST, RebA/ST and ST/SG were not affected by the cultivar but by the treatments. The ratio RA/ST increased considerably with 50 mM NaCl, compared to the control, with a plateauing thereafter with high NaCl concentration. The ratio RA/ST reached a peak at 100 mM, and declined, the ratio ST/SG also declined with salinity, but did not differ between the three NaCl concentrations.

3.5. Metabolite profiling of salt treated Stevia *plants*

A total of 81 metabolites were detected using a GC-MS untargeted metabolomics approach to profile leaves of stevia. Sixty-one metabolites were unambiguously identified with respect to their chemical nature by comparison to retention times and mass spectra to an *in-house* library. Additionally, 20 unknown metabolites were also detected (**Table 5**).

Shoutian-2 and Fengtian showed differential metabolite responses following exposure to 50 mM, 100 mM and 200 mM NaCl (**Figs 1 and 2**). Principle component analysis (PCA) of the GC-MS metabolomics data of both control and salt-stressed leaves from Fengtian (C2) showed that the control samples shifted more towards the negative axes of both PC1 and PC2 (**Fig 1A**). Salt stress caused a shift in the metabolic profile as indicated in the treatment groups within the PCA score plots, which did not separate due to levels of salt treatment. The cumulative percentage of PC1 and PC2 is 52.5%. To understand the contributory variables to the cluster formation by various groups in PCA scores plot, loadings were plotted. The most contributing metabolites for separation on PC1 for the Fengtian cultivar (C2) (**Fig 2A)** were glycerate, malate, threonate, azelaic acid, proline, glucose and rhamnose. In PC1, the corresponding loading was positive for glycerate, threonine, gluconate, gluconic acid, azeliac acid, rhamnose and glucose, while negative for glucose, galactose, shikimate, glycine, lysine, fumarate, malate and isoleucine.

Based on the PCA scores plot of Shoutian-2 (C1), the metabolite profiles of control and salt-treated samples did not show a clear separation unlike in Fengtian (**Fig 1B**). The cumulative percentage of PC1 and PC2 was 43.3%. There was a progressive grouping from control to increasing concentrations of NaCl. In the Shoutian-2 cultivar, 100 mM and 200 mM NaCl treatment resulted in a similar response compared to the control group. The most contributing metabolites for PC1 for the Shoutian-2 cultivar were xylitol, rhamnose, proline, galactose, and erythritol (**Fig 3B**). These results suggest that all the examined metabolites were strongly altered after salt treatment. In PC1, the corresponding loading was positive for xylitol,

rhamnose, proline, galactose, while negative for melezitol, threonine, glucose, citrate, fumarate, quinate and succinate.

3.5.1 Amino acids and amines responses in Stevia *under salinity stress*

Out of the 19 amino acids and amine derivatives analysed in both cultivars of *Stevia rebaudiana*, the concentrations of 16 amino acids showed significant changes in salt stressed plants in comparison to the respective control plants (**Table 5, Fig 3 and 4**). At 50 mM NaCl, several amino acids and derivatives showed decreased levels in both Shoutian-2 (aspartate, β-alanine, glycine, leucine, phenylalanine, serine and tyrosine) and Fengtian (asparagine, glycine, homoserine, lysine, pyroglutamate, serine, and tyrosine). Several amines increased significantly; notably, proline (+1.9 -fold) and tyramine (+3.8 -fold) in the Shoutian-2, and only proline (+3.6 -fold) in Fengtian at 50 mM NaCl. With an increase in salinity stress (100 mM NaCl), both cultivars showed decreased concentrations of a few metabolites in Shoutian-2 (β-alanine, glycine, homoserine and serine) and Fengtian (glycine, homoserine, leucine, serine and tyrosine). The greatest decrease was recorded for serine (-12.5 -fold) in Shoutian-2 at 100 mM NaCl compared to the control. At 200 mM, significant decreases of metabolites in Shoutian-2 leaves were β-alanine, ethanolamine, glutamate, glycine, homoserine, leucine, phenylalanine, serine and threonine. These reductions were compensated by an increase in proline by +2.5-fold. Similar changes in the metabolite levels were also found in the Fengtian at 200 mM NaCl. GABA, glycine, lysine, pyroglutamate and tyrosine showed lower levels but proline increased by +5.6-fold.

3.5.2 Organic acid responses in Stevia *under salinity stress*

There was a distinct difference in the GC-MS profiles of organic acids in salt-stressed stevia (**Table 5, Fig 3 and 4**). In the Shoutian-2 cultivar, levels of erythronate, fumarate, malate and quinate decreased and gluconate increased (+5.6-fold) following a 50 mM NaCl treatment. In Fengtian, only quinate and shikimate decreased following 50 mM NaCl. Following a 100 mM NaCl treatment, Shoutian-2 showed decreased levels of quinate, succinate and threonate. Fengtian also showed decreased levels of quinate, fumarate and caffeic acid. In this moderate stress condition, there was a significant increase of azelaic acid in the Fengtian by +4.2-fold. At a high stress condition of 200 mM NaCl, a distinct difference was observed among both cultivars. In Shoutian-2, glycerate, shikimate and succinate levels decreased, and gluconate

levels increased (+4.3-fold). In the Fengtian, several organic acid levels decreased (2-keto-L-gluconate, fumarate, quinate, 3-caffeoyl quinate, 4-caffeoyl quinate, 5-caffeoyl quinate, ribonate, shikimate), whereas only gluconate and malonate levels increased (+3.2 and +3.9-fold, respectively).

3.5.3 Response of sugar and sugar phosphates in Stevia *under salinity stress*

There were minimal changes observed with respect to sugar and sugar phosphate levels in both cultivars upon exposure to salinity stress (**Table 5, Fig 3 and 4**). Under low stress (50 mM NaCl), arabinose and galactinol levels decreased, and both digalactosylglycerol (+2-fold) and glycerol (+1.9-fold) increased in Shoutian-2. In Fengtian, erythritol, fructose and threitol increased between 1.6 and 3.7-fold under the same conditions. At 100 mM NaCl, only rhamnose and fructose levels increased in Shoutian-2 (3.4-fold) and fructose (7.4-fold) in Fengtian. Following a 200 mM salt treatment, Shoutian-2 again showed increased levels of fructose of 3.2-fold and rhamnose of 3-fold but also showed decreased levels of arabinose, glucose, glycerol-3-phosphate and melezitose. At 200 mM, the Fengtian showed surprisingly low levels of galactinol, galactose, raffinose and sucrose compared to the control.

3.5.4 Correlation between metabolite and sodium ion concentration in stevia *under salt stress*

A correlation analysis based on non-parametric Spearman's ranking correlation was carried out to determine relationships between sodium ion concentrations with the metabolite and other ion concentrations (**Fig 5 and 6**). Sodium concentrations correlated with a number of metabolites and other ions in both cultivars. In Shoutian-2, sodium correlated positively with gluconate, maleate and azelaic acid, and negatively with several sugars (raffinose, galactinol and sucrose), amino acids (serine, tyrosine, isoleucine, leucine and lysine), phenols and organic acids (caffeic acid, caffeic acid derivatives, quinate, shikimate, citrate and fumarate). In Fengtian, galactose and rhamnose showed strong positive correlations to sodium while a number of amino acids (glycine, serine, tyrosine, homoserine, β-alanine, asparagine and glutamate) as well as two organic acids (threonate and succinate) showed significant negative correlations. In both cultivars, sodium correlated negatively with potassium, and positively with chlorine; however, in Shoutian-2, sodium also correlated negatively with calcium.

4. Discussion

4.1. Exposure to salinity stress leads to a reduction in plant height and biomass

Strategies of plants to cope with saline environments include salt exclusion or sequestration, tissue tolerance to accumulated ions and reduced loss of K^+, osmotic adjustment and control of water homeostasis, biochemical and molecular responses, and changes in growth and development (Tester and Davenport 2003; Shabala and Cuin 2006; Munns and Tester 2008; Sanchez *et al.,* 2008). In this study, growth and biomass production were severely affected by salinity stress in both cultivars of *Stevia rebaudiana*, viz., Shoutian-2 and Fengtian. Comparison of growth inhibition patterns of two cultivars revealed greater differences between the treatments compared to the differences between the cultivars. The reduction in shoot and root dry weight is a result of induced water stress in the tissues by the NaCl stress. Salinity stress also inhibits cell expansion and photosynthesis leading to a failure in the translocation of photo assimilates (Zhang *et al.,* 2016). Dry matter production of stevia was significantly reduced by salt treatments, as compared to the control, and this is consistent with the patterns observed by Shibli *et al.,* (2007) in tomato. The reduced growth under salinity is the result of various salt-induced effects, including reduced carbon fixation due to specific ion toxicity, reduction of photosynthesis due to partial closure of stomata, osmotic adjustment due to plant adaptation to osmotic changes and ion exclusion and growth limitations originating from nutritional imbalances (Munns and Tester, 2008; Aswathappa and Bachelard, 1986). The reductions in growth with increased NaCl concentrations might be due to the use of photosynthates to synthesise chemicals needed for osmotic adjustment (Arndt *et al.,* 2000; Kerepesi and Galiba, 2000; Popp *et al.,* 1990). In the present study, mild salinity (50 mM NaCl) did not affect root development in hydroponically grown stevia which is in agreement with the previous studies of Zeng *et al,* (2013) and Cantabella *et al.,* (2017).

The SPAD readings in plants under stress generally decreased with an increase in salinity, and were more pronounced in Fengtian than in Shoutian-2. SPAD readings represent chlorophyll content of the leaves, thus implying a notable decrease in the chlorophyll content. Reduction in chlorophyll has contributed to reduced plant growth and dry matter accumulation, as a response to salinity stress. Based on the physiological studies, both cultivars tested in this study exhibited reduced growth at higher salinity levels, indicating that stevia is only a moderately tolerant of salinity stress compared to many Australian native species (Ashwath *et al.,* 1986a) and other crop plants (Maas and Hoffman, 1977).

4.2. Differential accumulation of ions during salinity stress

Plants can achieve stress tolerance by physiological and biochemical adaptations, such as the accumulation of inorganic ions and synthesis of organic compounds. Regulation of tissue ion concentrations to prevent excessive accumulation of Na^+ and/or Cl^- appear to be one of the most important mechanisms of salt tolerance in plants (Munns *et al.,* 2010; Aswathappa and Bachelard, 1986). Most plants accumulate both Na^+ and Cl^- in their shoot tissues when grown in saline soils, leading to Cl^- toxicity. This may also be an important cause of growth reduction in plants under salinity stress (Dang *et al.,* 2008). With increasing concentrations of NaCl, more Na and Cl were accumulated in the more salt-sensitive cultivar Shoutian-2 than in Fengtian. This showed relatively higher levels of salt tolerance. Potassium (K^+) contributes to cytoplasmic osmolarity, and hence maintenance of higher K^+ is of great importance during salt stress. A significant reduction in tissue K^+ concentration was observed for both cultivars of stevia under salinity stress. However, the tolerant cv. Fengtian maintained relatively higher levels of K^+ than Shoutian-2. This is due to the improved ability of Fengtian to take up K^+ ions and translocate them to the shoots. Increased Na^+ and Cl^- concentrations followed by reduced growth of plants (reduced leaf expansion) resulted in increased accumulation of salts in the shoots (Tavakkoli *et al.*, 2012). Ca^{2+} and Mg^{2+} concentrations significantly decreased with increasing NaCl concentrations in the root media. The decrease in Ca^{2+} concentration was observed in both cultivars under severe salinity. Regardless of the genotype, Mg^{2+} concentrations in the tissue decreased when the plants were exposed to 100 mM NaCl. The improved tolerance of salt stress by Fengtian might be due to its improved regulation Na^+ and Cl^- within the plant and other physiological processes (Cheeseman, 1988, Aswathappa and Bachelard, 1986; Aswathappa *et al.,* 1986b). The decrease in leaf osmotic potential is likely a sensitive response of the cultivar, as it was unable to resist accumulation of Na^+ and Cl^- ions (Aswathappa and Bachelard, 1986a). The cultivar Fengtian, on the contrary, minimised the accumulation of Na^+ and Cl^- ions and hence it was able to maintain better growth in saline conditions. This was achieved via accumulation of other organic solutes (see the metabolomics section).

4.3. Accumulation of Na and/or Cl leads to changes in the steviol glucoside concentrations in the two cultivars of Stevia rebaudiana

Steviol glycosides, and in particular, stevioside and rebaudioside A, are responsible for the sweetness in stevia. Stevioside concentrations decreased, whereas the RA levels increased in

response to salinity stress. Amongst the two cultivars, Shoutian-2 had higher ST than Fengtian. The ratio of ST/RA shifted towards higher RA at higher salinity levels indicating that the salinity stress could help improve the concentrations of most desired compound, the RA. This increase in RA and decrease in ST concentration is different from that reported by Zeng *et al.,* (2013) and Cantabella *et al.,* (2017). This discrepancy may be due to longer exposure of plants to salinity (>8 weeks) in the current experiment than in previous studies (4 weeks; 16 and 25 days). This may also be due to provision of milder salinity stress of 60, 90, and 120 mM NaCl by Zeng *et al.,* (2013) and 34 and 90 mM NaCl by Cantabella *et al.*, (2017).

4.4. Amino acids and amines are potential biochemical markers for screening of stress tolerance among Stevia rebaudiana cultivars

Plants exposed to NaCl tend to accumulate excessive amounts of Na^+ and Cl^+ in their tissues. In the present study, both cultivars accumulated high levels of Na^+. In response, the plants also accumulated a large number of metabolites. These metabolites assist in coping with salinity stress through osmotic adjustment and/or osmoprotection of intracellular components (Zhu, 2001). These metabolite classes include betaines (Pan *et al.,* 1981), free amino acids (Cano *et al.,* 1996), especially proline (Huang *et al.,* 2009) and soluble carbohydrates (Tavakkoli *et al.*, 2012).

Under salinity stress, amines and amino acids will accumulate in the tissues. Amongst these, proline is considered the most important metabolite (Aspinall and Paleg, 1981) and this could be used as a potential biochemical marker in screening for salinity tolerance in stevia. Proline dominated the list of significantly accumulated metabolites, and many higher plants have also been reported to accumulate free proline in response to salt (Widodo *et al.,* 2009) and drought stresses (Aspinall and Paleg, 1981). In the present study, proline concentration increased in both cultivars but notably to higher levels in Fengtian. Many researchers propose that proline accumulation in plants is a protective counter measure under salt stress, and potentially a salt stress signal (Fan *et al.,* 2012). Stress-induced changes in tyramine levels can be correlated with the regulation of proline accumulation in plants as in tomato (Aziz *et al.,* 1998). In this study, there was an increase in the level of tyramine in both cultivars, and this was more pronounced in Fengtian than in Shoutian-2. Proline accumulation under salt stress also acts as an osmoprotectant, thus safeguarding the organelles and cytosolic enzymes as an osmotic

control factor (Huang *et al.*, 2009), and as a carbon and nitrogen reservoir of energy for post-stress conditions (Huang *et al.*, 2009).

4.5. Sugars assist in osmotic adjustment and in preventing oxidative damage

Sugars (reducing and non-reducing sugars and sugar alcohols) also significantly contribute to stress response. Often the changes in sugar metabolites under salt stress show large variations between the species or the genotypes within the same species (Sanchez *et al.*, 2008). Carbohydrates such as sugars (glucose, fructose, sucrose and fructans) and starch accumulate under salt stress (Parida *et al.*, 2002; Kerepesi and Galiba, 2000; Singh *et al.*, 2000). In this study, both cultivars of *Stevia rebaudiana* displayed marked increase in sugar concentrations upon exposure to NaCl. A higher carbohydrate concentration under salt stress prevents plants from oxidative damage and maintains protein structure (Krasensky and Jonak, 2012). In the present study, in addition to fructose, erythritol, rhamnose and threitol, glycerol also increased in salt-stressed plants. It is likely that the salt stress induces accumulation of total soluble sugars in the leaves of the more salt tolerant cultivar Fengtian. An increase in carbohydrates in salt-stressed leaves may be caused by an inhibition of the distribution of these sugars to storage organs and growing tissues (Krasensky and Jonak, 2012). In this study, increasing total carbohydrate concentrations in the leaves can be associated with a reduced carbon fixation rate through photosynthesis. A reduction in photosynthesis with increasing carbohydrate concentration in the leaves may arise from feedback effects from reduced carbohydrate utilization (Liu *et al.*, 2014) or translocation to storage organs.

4.6. Accumulation of organic acids as an adaptive reaction to salt stress

In both cultivars of *Stevia rebaudiana*, organic acid concentrations changed in response to salt stress. Gluconate is a prominent stress marker and its concentrations increased under salinity stress. Widodo *et al.*, (2009) also noted an increase of 2–3-fold in gluconate levels in salt-treated barley. It is possible that the accumulation of gluconate is an adaptive reaction to salt stress. Increased gluconate concentration might be related to ascorbic acid degradation due to insufficient reducing equivalents or to an impaired pentose phosphate pathway (Pedreschi *et al.*, 2009). There were increased malonate levels in the more tolerant cultivar Fengtian. Researchers have raised the question if malonic acid is a competitive inhibitor of succinate dehydrogenase; therefore diminishing the rate at which oxygen is taken up may lead to the accumulation of succinate (Chen, *et al.*, 2011). However, in the present case, this

mechanism was not observed and succinate did not accumulate in large quantities under salt stress.

5. Conclusions

In conclusion, salinity stress of Stevia resulted in a general increase in the levels of many amino acids, amines, sugars and sugar phosphates, with a concurrent decrease in most organic acids including TCA intermediates. The metabolites involved in salinity response differed between the two cultivars,with results suggesting that the differences in salt response of Shoutian-2 and Fengtian were due to differences in the accumulation of ions and organic solutes. The more salt tolerant cultivar Fengtian showed a smaller reduction in biomass under salinity and increased levels of steviol glycoside, particularly RA. It's better ability to maintain higher K^+/ Na^+ ratios, and accumulate higher levels of proline and gluconate, as compared to the less tolerant cultivar Shoutian-2, appear to confer better salt tolerance in stevia. Of particular significance from these findings is the effect that salinity has on the relative amounts of the two key steviol glycoside. The salinity treatment resulted in 5-6 fold increase in the relative amount of RA, which is up to 400 times sweeter than sucrose and approximately twice the sweetness of ST. These results suggest that there is a potential to maximise the yield of RA in stevia through exposure to mild salinity stress.

6. Contributions

M.D., N.A. and U.R. conceived and designed the experiments. M.D. performed the Stevia growth, salinity and ionomic experiments. D.D. and N.J. performed the metabolite profiling analysis. D.C. performed the steviol glycoside measurements and N.A. and M.D. performed the statistical analysis. M.D., N.A., C.H., D.D., N.J., D.C., D.M. and U.R. analysed and interpreted the data. All authors read and approved the manuscript.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Tables

Table 1:

Effects of salinity on growth parameters of two cultivars of *Stevia rebaudianna*, Shoutian- 2 (C1) and Fengtian (C2), grown in the hydroponic nutrient solution for 8 weeks in a green house. The P values of the two-way ANOVA refer to the variables cultivar (C), salinity treatment (T), and interaction effect of cultivar salinity treatment. Least significant differences of means [LSD at 5%] are provided based on the cultivar values and NaCl stress conditions. Degree of freedom (Df); non significant (NS); P >0.05, significance at *; P ≤ 0.05; **; P ≤ 0.01; ***; P<0.001.

		Treatment with different concentration of NaCl (mM)				**ANOVA**			
PARAMETER	**cultivar**	**0**	**50**	**100**	**200**		**Df**	**Significance**	**LSD (5%)**
Height (cm)	C1	18.27	12.95	8.1	4.79	C	1	***	1.39
	C2	22.1	16.57	13.42	8.47	T	3	***	1.97
	T	0.19^{d}	14.76^{c}	10.76^{b}	6.63^{a}	CxT	3	NS	_
Leaf dry weight(g/plant)	C1	9.24	5.72	2.03	0.23	C	1	*	1.8
	C2	14.58	7.67	2.81	0.5	T	3	***	2.65
	T	11.91^{d}	6.69^{c}	2.42^{b}	0.37^{a}	CxT	3	NS	_

Root dry weight (g/plant)	C1	5.68	3.8	1.1	0.13	C	1	NS	-
	C2	4.1	4.76	1.54	0.23	T	3	***	1.14
	T	4.89[c]	4.28[c]	1.32[b]		CxT	3	NS	-
Shoot dry weight (g/plant)	C1	11.67	6.84	2.93	0.29	C	1	**	2.14
	C2	19.93	10.74	3.65	0.62	T	3	***	3.02
	T	15.8[d]	8.79[c]	3.29[b]	0.46[a]	CxT	3	*	4.27
Stem dry weight (g/plant)	C1	2.43	1.13	0.9	0.06	C	1	***	0.49
	C2	5.35	3.07	0.84	0.12	T	3	***	0.69
	T	3.89[c]	2.1[b]	0.87[a]	0.09[a]	CxT	3	***	0.97

Table 2: The effect on leaf chlorophyll content and osmolarity in cultivars of *Stevia rebaudianna*, Shoutian-2(C1) and Fengtian (C2), grown in hydroponics cultures in a greenhouse and treated with NaCl for 8 weeks. The P values of the two-way ANOVA refer to the variables cultivar(C), salinity treatment (T), and interaction effect of cultivar× salinity treatment. Least significant differences of means [LSD at 5%] are provided based on the cultivar values and stress conditions. Degree of freedom (Df); non significant (NS); $P > 0.05$, significance at *; $P \leq 0.05$; **; $P \leq 0.01$; ***; $P < 0.001$.

Parameter	Cultivar	Treatment with different concentration of NaCl (mM) 0	50	100	200		ANOVA Df	Significance	LSD (5%)
Chlorophyll	C1	50.96	33.66	38.42	47.06	C	1	NS	-
(SPAD reading)	C2	48.68	34.84	40.36	47.06	T	3	***	4.88
	T	49.82d	34.25[a]	39.39[b]	41.16[c]	CxT	3	*	6.90
Osmotic potential	C1	-0.592	-1.722	-2.133	-2.579	C	1	***	0.14
(MPa)	C2	-1.656	-2.222	-2.626	-3.159	T	3	***	0.19
	T	-1.124[d]	-1.972[c]	-2.38[b]	-2.869[a]	CxT	3	*	0.28

Table 3: Effect on salinity on the ionic concentration in leaves of two cultivars of *Stevia rebaudianna*, Shoutian- 2 (C1) and Fengtian (C2), grown in hydroponics cultures in a greenhouse and treated with NaCl for 8 weeks**.** The P values of the two way ANOVA refer to the variables cultivar(C), salinity treatment (T) and interaction effect of cultivar X salinity treatment. Least significant differences of means [LSD at 5%] across the cultivars and stress conditions; degree of freedom (Df); nonsignificant (NS>0.05); Significance at *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. Within a column and under each parameter, the means not followed by common letter differ significantly (P=0.05)

		Treatment with different concentration of NaCl (mM)					ANOVA		
mg/g leaf DW	**Cultivar**	**0**	**50**	**100**	**200**		**Df**	**Significance**	**LSD (5%)**
Na+	C1	0.18	5.74	16.04	11.2	C	1	NS	–
	C2	1.28	2.4	14.5	17.47	T	3	***	5.37
	T	0.73[a]	4.07[b]	15.27[c]	14.34[c]	CxT	3	NS	–
Cl^-	C1	3.4	50.03	85.94	98.67	C	1	**	9.08
	C2	4.33	42.33	47.33	76	T	3	***	12.84
	T	3.87[a]	46.18[b]	66.63[c]	87.33[d]	CxT	3	*	18.16
K^+	C1	5.38	0.51	0.38	0.34	C	1	***	0.14
	C2	5.67	1.18	1.02	0.53	T	3	***	0.20
	T	5.52[c]	0.84[b]	0.7[b]	0.43[a]	CxT	3	*	0.29
Cl^-/Na^+	C1	20.82	8.68	6.83	9.26	C	1	NS	–
	C2	3.41	18.13	3.24	4.41	T	3	NS	–
	T	12.12	13.4	5.03	6.83	CxT	3	*	10.32
$K^+/Na+$	C1	31.14	0.09	0.03	0.03	C	1	***	2.67
	C2	4.55	0.5	0.07	0.03	T	3	***	3.77
	T	17.85[c]	0.3[b]	0.05[a]	0.03[a]	CxT	3	***	5.33
Ca^{2+}	C1	4.43	4.24	3.26	5.41	C	1	NS	–
	C2	4.94	4.03	3.51	3.43	T	3	*	0.72
	T	4.68[b]	4.14[b]	3.38[a]	4.42[b]	CxT	3	*	1.02
Mg^{2+}	C1	2.12	1.94	1.69	1.46	C	1	NS	–
	C2	1.77	1.67	1.54	1.48	T	3	NS	–
	T	1.95	1.81	1.61	1.47	CxT	3	NS	–

852 **Table 4:** Effect on leaf steviol glycoside content in two cultivars of *Stevia rebaudiana*,
853 Shoutian-2 (C1) and Fengtian (C2) when grown in hydroponics cultures in a greenhouse and
854 treated with NaCl for 8 weeks. The P values of the two-way ANOVA refer to the variables
855 cultivar (C), salinity treatment (T), and interaction effect of cultivar × salinity treatment.
856 Least significant differences of means [LSD at 5%] across the cultivars and stress conditions;
857 degree of freedom (Df); non-significant (NS>0.05); Significance at *P ≤ 0.05; **P ≤ 0.01;
858 ***P ≤ 0.001. Within a row and under each parameter, the means not followed by common
859 letter differ significantly (P≤0.05).

Steviol Glycoside (mg g-1 leaf DW)	Cultivar	NaCl treatment (mM) 0	50	100	200	Df	ANOVA	C	T	C*T
ST	C1	23.4	19.5	17.4	16.1	18	Significanc	NS	NS	NS
	C2	19.2	18.8	15.0	11.8	18	LSD(5%)	3.28	5.19	7.34
RebA	C1	2.7	10.5	10.75	6.84	18	Significanc	NS	**	NS
	C2	4.59	8.45	7.2	5.13	18	LSD(5%)	1.21	1.91	2.71
RebA+Stev	C1	26.1	30.0	28.2	23.0	18	Significanc	NS	NS	NS
	C2	23.8	27.2	22.2	17.0	18	LSD(5%)	3.66	5.79	8.19
RebA/Stev	C1	0.1	0.5	0.6	0.4	18	Significanc	NS	NS	NS
	C2	0.2	0.5	0.5	0.4	18	LSD(5%)	0.14	0.22	0.31
RebA/(RebA+Stev)	C1	0.1	0.3	0.4	0.3	18	Significanc	NS	**	NS
	C2	0.2	0.3	0.3	0.3	18	LSD(5%)	0.05	0.09	0.13
Stev/(RebA+Stev)	C1	0.90	0.65	0.62	0.70	18	Significanc	NS	**	NS
	C2	0.81	0.69	0.67	0.70	18	LSD(5%)	0.05	0.09	0.13

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Table 5: Metabolite ratio in Shoutian-2 (C1) and Fengtian (C2) cultivars of *Stevia rebaudiana* in different salinity stress treatments compared to control, which is set to 1. Data obtained from GC-MS analysis of salt treated Shoutian -2 (C1) and Fengtian (C2) leaves were normalized to the mean response calculated to the respective untreated samples to the same stage of growth. Salinity treatments, T0 (control), T1 (50 mM), T2 (100 mM), T3 (200 mM) NaCl values are represented as the ratios ±%SE of five independent determinations. The data was log transformed prior to statistical analysis. Values that are significantly higher at $P< 0.05$ are indicated as blue cells and values that are significant at $P< 0.05$ / (number of metabolites, Bonferroni false discovery correction) are indicated green cells.

	Shoutian(C1)								Fengtian (C2)							
	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem
	C1 Control		C1 Salt 50		C1 Salt 100		C1 Salt 200		C2 Control		C2 Salt 50		C2 Salt 100		C2 Salt 200	
Amino acids & Amines																
Asparagine	1.000	± 0.293	0.867	± 0.292	0.517	± 0.170	0.606	± 0.050	1.000	± 0.205	**0.230**	**± 0.171**	0.383	± 0.251	0.858	± 0.309
Aspartate	1.000	± 0.118	**0.590**	**± 0.090**	0.741	± 0.274	0.741	± 0.248	1.000	± 0.355	0.451	± 0.233	0.534	± 0.488	0.832	± 0.068
beta Alanine	1.000	± 0.127	**0.464**	**± 0.156**	***0.223***	***± 0.018***	***0.157***	***± 0.124***	1.000	± 0.138	0.696	± 0.154	0.574	± 0.475	1.012	± 0.163
Ethanolamine	1.000	± 0.092	1.044	± 0.199	1.215	± 0.281	**0.308**	**± 0.278**	1.000	± 0.257	0.474	± 0.225	0.505	± 0.157	0.617	± 0.297
GABA	1.000	± 0.043	0.834	± 0.309	1.089	± 0.513	0.290	± 0.625	1.000	± 0.278	0.365	± 0.294	0.908	± 0.276	**0.181**	**± 0.157**
Glutamate	1.000	± 0.044	0.677	± 0.210	0.565	± 0.304	**0.449**	**± 0.293**	1.000	± 0.275	0.533	± 0.081	0.654	± 0.369	0.539	± 0.125
Glycine	1.000	± 0.230	**0.428**	**± 0.030**	**0.341**	**± 0.270**	**0.194**	**± 0.166**	1.000	± 0.185	**0.282**	**± 0.117**	**0.307**	**± 0.314**	**0.257**	**± 0.249**
Homoserine	1.000	± 0.016	0.971	± 0.316	**0.374**	**± 0.222**	**0.350**	**± 0.393**	1.000	± 0.146	**0.482**	**± 0.236**	**0.241**	**± 0.379**	0.612	± 0.111
Isoleucine	1.000	± 0.312	0.804	± 0.157	0.885	± 0.280	0.317	± 0.120	1.000	± 0.183	0.848	± 0.157	0.438	± 0.261	0.584	± 0.136
Leucine	1.000	± 0.141	**0.286**	**± 0.334**	0.456	± 0.353	**0.156**	**± 0.388**	1.000	± 0.371	0.437	± 0.216	**0.172**	**± 0.353**	0.232	± 0.230
Lysine	1.000	± 0.663	2.304	± 0.467	1.916	± 0.282	0.943	± 0.618	1.000	± 0.169	**0.242**	**± 0.005**	0.069	± 0.107	**0.092**	**± 0.351**
Phenylalanine	1.000	± 0.212	**0.363**	**± 0.359**	0.660	± 0.314	**0.176**	**± 0.269**	1.000	± 0.434	0.459	± 0.148	0.653	± 0.326	0.299	± 0.039
Proline	1.000	± 0.035	**1.994**	**± 0.202**	1.313	± 0.217	**2.449**	**± 0.159**	1.000	± 0.176	**3.631**	**± 0.084**	2.538	± 0.367	**5.563**	**± 0.287**
Pyroglutamate	1.000	± 0.538	0.562	± 0.154	0.253	± 0.291	0.553	± 0.350	1.000	± 0.170	**0.262**	**± 0.245**	**0.261**	**± 0.352**	**0.332**	**± 0.145**
Serine	1.000	± 0.105	**0.397**	**± 0.078**	**0.084**	**± 0.373**	***0.182***	***± 0.084***	1.000	± 0.170	**0.184**	**± 0.294**	0.192	± 0.692	0.149	± 0.260
Threonine	1.000	± 0.327	0.652	± 0.116	0.545	± 0.279	**0.174**	**± 0.234**	1.000	± 0.178	0.593	± 0.146	0.536	± 0.224	0.663	± 0.058
Tyramine	1.000	± 0.091	**3.813**	**± 0.305**	3.983	± 0.588	1.494	± 0.324	1.000	± 0.195	1.584	± 0.100	1.719	± 0.269	1.386	± 0.202
Tyrosine	1.000	± 0.115	**0.515**	**± 0.158**	0.612	± 0.214	0.309	± 0.429	1.000	± 0.220	**0.408**	**± 0.134**	**0.418**	**± 0.070**	**0.244**	**± 0.129**
Valine	1.000	± 0.147	0.801	± 0.223	0.826	± 0.259	0.444	± 0.368	1.000	± 0.400	0.844	± 0.218	0.659	± 0.256	0.733	± 0.133
Organic acids																
2-Keto-L-gluconic acid	1.000	± 0.137	0.571	± 0.213	0.682	± 0.268	0.593	± 0.148	1.000	± 0.328	0.659	± 0.176	0.729	± 0.245	**0.163**	**± 0.069**
Azelaic acid	1.000	± 0.196	0.911	± 0.446	0.855	± 0.377	1.341	± 0.208	1.000	± 0.372	1.454	± 0.082	**4.182**	**± 0.248**	2.025	± 0.094
Caffeic acid	1.000	± 0.535	0.488	± 0.658	0.421	± 0.351	0.349	± 0.241	1.000	± 0.088	0.837	± 0.109	**0.425**	**± 0.226**	0.513	± 0.317
Citrate	1.000	± 0.200	0.368	± 0.691	0.461	± 0.939	1.189	± 0.398	1.000	± 0.265	0.475	± 0.033	0.487	± 0.209	0.355	± 0.059
Erythronic acid	1.000	± 0.221	**0.422**	**± 0.103**	0.464	± 0.291	0.756	± 0.210	1.000	± 0.302	0.496	± 0.297	0.785	± 0.392	0.384	± 0.055
Fumarate	1.000	± 0.128	**0.134**	**± 0.515**	0.492	± 0.949	0.300	± 0.706	1.000	± 0.110	0.429	± 0.201	**0.197**	**± 0.407**	**0.202**	**± 0.134**
Glucarate	1.000	± 0.086	1.134	± 0.138	0.901	± 0.756	1.842	± 0.214	1.000	± 0.418	0.725	± 0.111	0.765	± 0.587	1.696	± 0.041
Gluconate	1.000	± 0.116	**5.629**	**± 0.191**	0.773	± 0.125	**4.392**	**± 0.330**	1.000	± 0.373	1.406	± 0.154	2.557	± 0.417	**3.221**	**± 0.100**
Gluconic acid,1,4-lactone	1.000	± 0.210	0.645	± 0.266	0.471	± 0.189	0.741	± 0.398	1.000	± 0.080	4.615	± 0.167	4.369	± 0.282	3.411	± 0.119
Glycerate	1.000	± 0.138	0.791	± 0.076	0.589	± 0.363	**0.603**	**± 0.082**	1.000	± 0.130	0.838	± 0.166	0.821	± 0.463	1.235	± 0.049
Malate	1.000	± 0.127	**0.228**	**± 0.389**	0.462	± 0.920	0.570	± 0.251	1.000	± 0.263	0.942	± 0.217	0.358	± 0.454	0.740	± 0.120
Malonic acid	1.000	± 0.215	1.898	± 0.173	0.802	± 0.240	0.968	± 0.217	1.000	± 0.399	1.338	± 0.123	2.351	± 0.303	**3.963**	**± 0.162**
Quinate	1.000	± 0.215	**0.183**	**± 0.440**	**0.250**	**± 0.517**	0.770	± 0.728	1.000	± 0.487	0.225	± 0.058	**0.088**	**± 0.067**	**0.128**	**± 0.072**
Quinic acid,3-caffeoyl	1.000	± 0.159	1.637	± 0.260	1.303	± 0.465	1.182	± 0.285	1.000	± 0.129	1.061	± 0.212	0.576	± 0.411	**0.281**	**± 0.186**
Quinic acid,4-caffeoyl	1.000	± 0.144	0.350	± 0.524	**0.138**	**± 0.704**	0.293	± 0.922	1.000	± 0.294	**0.337**	**± 0.210**	**0.123**	**± 0.402**	***0.061***	***± 0.058***
Quinic acid,5-caffeoyl	1.000	± 0.050	0.889	± 0.343	0.594	± 0.467	0.519	± 0.762	1.000	± 0.444	0.480	± 0.237	**0.080**	**± 0.565**	**0.037**	**± 0.198**
Ribonate	1.000	± 0.233	0.468	± 0.235	0.727	± 0.300	0.632	± 0.078	1.000	± 0.445	0.689	± 0.158	0.722	± 0.220	**0.178**	**± 0.250**
Shikimate	1.000	± 0.093	0.526	± 0.426	0.486	± 0.468	**0.363**	**± 0.188**	1.000	± 0.235	**0.418**	**± 0.067**	0.334	± 0.476	***0.081***	***± 0.025***
Succinate	1.000	± 0.195	0.465	± 0.262	**0.279**	**± 0.368**	**0.352**	**± 0.056**	1.000	± 0.232	0.493	± 0.167	0.411	± 0.591	0.442	± 0.053
Threonic acid	1.000	± 0.004	0.929	± 0.192	**0.542**	**± 0.180**	0.869	± 0.253	1.000	± 0.118	1.540	± 0.103	0.997	± 0.435	**1.883**	**± 0.013**
Sugars & sugar phosphates																
Arabinose	1.000	± 0.018	**0.485**	**± 0.185**	0.929	± 0.242	**0.325**	**± 0.251**	1.000	± 0.207	1.139	± 0.089	1.208	± 0.172	0.636	± 0.053
Cellobiose	1.000	± 0.366	1.642	± 0.292	1.132	± 0.163	0.625	± 0.112	1.000	± 0.089	1.109	± 0.154	1.087	± 0.304	1.202	± 0.271
Digalactosylglycerol	1.000	± 0.028	**2.060**	**± 0.202**	0.861	± 0.358	0.890	± 0.572	1.000	± 0.719	1.754	± 0.126	1.488	± 0.627	1.069	± 0.144
Erythritol	1.000	± 0.217	2.433	± 0.283	1.935	± 0.482	1.495	± 0.430	1.000	± 0.051	**1.673**	**± 0.044**	1.105	± 0.155	1.345	± 0.164
Fructose	1.000	± 0.353	1.538	± 0.434	**3.435**	**± 0.095**	**3.160**	**± 0.101**	1.000	± 0.141	**3.704**	**± 0.261**	***7.350***	***± 0.212***	1.676	± 0.227
Galactinol	1.000	± 0.029	0.598	± 0.228	0.955	± 0.505	0.843	± 0.381	1.000	± 0.450	0.224	± 0.260	**0.018**	**± 0.298**	**0.008**	**± 0.407**
Galactose	1.000	± 0.128	1.179	± 0.093	2.231	± 0.278	1.715	± 0.299	1.000	± 0.221	1.970	± 0.174	1.592	± 0.217	**0.052**	**± 0.400**
Galactosylglycerol	1.000	± 0.298	1.177	± 0.470	1.094	± 0.416	1.106	± 0.168	1.000	± 0.430	1.152	± 0.174	1.667	± 0.115	0.414	± 0.129
Glucose	1.000	± 0.312	1.256	± 0.291	0.894	± 0.591	**0.377**	**± 0.141**	1.000	± 0.254	0.831	± 0.058	1.582	± 0.385	0.615	± 0.038
Glycerol	1.000	± 0.169	**1.870**	**± 0.077**	1.863	± 0.224	1.490	± 0.140	1.000	± 0.254	1.194	± 0.144	1.918	± 0.204	2.563	± 0.442
Glycerol-3-phosphate	1.000	± 0.117	0.792	± 0.127	0.609	± 0.325	**0.289**	**± 0.290**	1.000	± 0.365	0.546	± 0.079	0.589	± 0.328	0.415	± 0.251
Inositol_chiro	1.000	± 0.496	1.028	± 0.141	1.087	± 0.262	0.949	± 0.013	1.000	± 0.426	1.183	± 0.017	1.271	± 0.125	1.392	± 0.187
Inositol_myo	1.000	± 0.380	1.340	± 0.325	1.231	± 0.105	1.240	± 0.065	1.000	± 0.098	1.270	± 0.142	1.511	± 0.146	1.724	± 0.263
Maltose	1.000	± 0.164	1.396	± 0.183	1.115	± 0.347	0.378	± 0.445	1.000	± 0.250	1.304	± 0.180	1.291	± 0.076	0.744	± 0.201
Melezitose	1.000	± 0.223	1.038	± 0.023	0.763	± 0.248	**0.353**	**± 0.235**	1.000	± 0.250	1.106	± 0.059	1.363	± 0.506	0.915	± 0.145
Raffinose	1.000	± 0.757	0.468	± 0.875	0.383	± 0.462	0.146	± 0.977	1.000	± 0.149	0.413	± 0.134	0.060	± 0.476	**0.006**	**± 0.177**
Rhamnose	1.000	± 0.233	1.452	± 0.218	**3.400**	**± 0.097**	**3.018**	**± 0.057**	1.000	± 0.608	1.098	± 0.262	2.416	± 0.338	0.454	± 0.224
Sucrose	1.000	± 0.610	0.837	± 0.314	1.120	± 0.458	0.169	± 0.681	1.000	± 0.370	1.471	± 0.315	0.149	± 0.059	**0.060**	**± 0.249**
Threitol	1.000	± 0.172	0.570	± 0.087	0.615	± 0.088	0.724	± 0.389	1.000	± 0.108	**2.281**	**± 0.250**	1.632	± 0.141	1.260	± 0.064
Trehalose	1.000	± 0.413	0.234	± 0.302	0.164	± 0.578	0.094	± 0.281	1.000	± 0.678	0.751	± 0.258	0.146	± 0.174	0.240	± 0.272
Xylitol	1.000	± 0.145	0.745	± 0.193	0.900	± 0.285	1.442	± 0.212	1.000	± 0.150	0.597	± 0.075	1.339	± 0.350	1.459	± 0.281
Other compounds																
Phosphate	1.000	± 0.255	0.698	± 0.067	0.485	± 0.074	0.622	± 0.127	1.000	± 0.312	1.216	± 0.215	0.749	± 0.178	0.723	± 0.070
Un_12.53_306	1.000	± 0.242	0.353	± 0.578	***0.009***	***± 0.346***	**0.009**	**± 0.515**	1.000	± 0.146	***0.006***	***± 0.194***	0.558	± 0.517	***0.010***	***± 0.072***
Un_16.17_306.2	1.000	± 0.167	0.873	± 0.118	1.164	± 0.428	1.300	± 0.216	1.000	± 0.278	1.297	± 0.067	1.401	± 0.323	2.115	± 0.060
Un_18.69_394.2	1.000	± 0.222	0.970	± 0.189	0.985	± 0.181	1.239	± 0.215	1.000	± 0.192	0.794	± 0.113	1.446	± 0.049	1.465	± 0.097
Un_21.94_361.2	1.000	± 0.066	1.228	± 0.234	0.507	± 0.419	***0.294***	***± 0.046***	1.000	± 0.269	0.823	± 0.083	1.285	± 0.324	1.006	± 0.019
Un_22.12_394.2	1.000	± 0.386	0.982	± 0.094	0.518	± 0.309	1.596	± 0.101	1.000	± 0.476	1.368	± 0.173	1.301	± 0.348	1.852	± 0.086
Un_22.2_361.2	1.000	± 0.116	1.207	± 0.182	0.418	± 0.603	***0.220***	***± 0.088***	1.000	± 0.262	0.866	± 0.094	5.787	± 0.497	1.374	± 0.107
Un_22.7_466.3	1.000	± 0.458	0.656	± 0.306	0.253	± 0.442	0.464	± 0.114	1.000	± 0.294	0.769	± 0.126	1.677	± 0.285	0.607	± 0.244
Un_23.03_333.2	1.000	± 0.181	**2.763**	**± 0.176**	1.653	± 0.463	1.375	± 0.247	1.000	± 0.475	1.116	± 0.136	1.238	± 0.192	2.695	± 0.257
Un_23.3_333.2	1.000	± 0.339	2.653	± 0.165	1.234	± 0.479	0.594	± 0.380	1.000	± 0.422	1.934	± 0.147	**5.156**	**± 0.142**	**4.958**	**± 0.201**
Un_23.43_391.2	1.000	± 0.148	2.115	± 0.220	3.905	± 0.722	**3.303**	**± 0.195**	1.000	± 0.583	1.030	± 0.101	1.080	± 0.460	1.070	± 0.167
Un_23.9_319.2	1.000	± 0.223	0.750	± 0.167	0.650	± 0.149	0.541	± 0.255	1.000	± 0.102	0.618	± 0.261	0.764	± 0.179	0.867	± 0.141
Un_24.09_290.2	1.000	± 0.288	2.471	± 0.299	1.394	± 0.085	1.741	± 0.380	1.000	± 0.606	1.587	± 0.206	2.690	± 0.207	3.479	± 0.161
Un_24.2_319.2	1.000	± 0.246	**0.304**	**± 0.341**	0.317	± 0.376	0.203	± 0.635	1.000	± 0.221	0.728	± 0.266	0.679	± 0.375	**0.177**	**± 0.097**
Un_24.27_319.2	1.000	± 0.248	0.338	± 0.336	0.348	± 0.382	0.204	± 0.625	1.000	± 0.206	0.722	± 0.256	0.597	± 0.355	**0.162**	**± 0.079**
Un_24.81_345.2	1.000	± 0.076	**0.296**	**± 0.416**	**0.093**	**± 0.527**	**0.222**	**± 0.354**	1.000	± 0.346	1.262	± 0.220	0.867	± 0.176	1.243	± 0.147
Un_26.76_549.4	1.000	± 0.888	0.367	± 0.190	0.100	± 0.443	0.582	± 0.486	1.000	± 0.525	**19.803**	**± 0.131**	**6.157**	**± 0.421**	**30.739**	**± 0.229**
Un_26.96_433.3	1.000	± 0.435	0.493	± 0.246	**0.063**	**± 0.507**	0.234	± 0.353	1.000	± 0.395	1.188	± 0.240	1.228	± 0.197	1.641	± 0.458
Un_27.55_351.2	1.000	± 0.351	**13.241**	**± 0.319**	3.006	± 0.388	1.682	± 0.287	1.000	± 0.564	2.617	± 0.161	0.806	± 0.963	0.265	± 0.303
Un_27.87_334.2	1.000	± 0.277	0.499	± 0.385	**0.071**	**± 0.581**	0.183	± 0.534	1.000	± 0.354	0.831	± 0.186	0.595	± 0.482	0.638	± 0.323
Un_28.58_361.2	1.000	± 0.155	1.522	± 0.151	0.937	± 0.052	1.414	± 0.205	1.000	± 0.344	1.031	± 0.141	1.825	± 0.217	1.686	± 0.166

874 **Figure legends**

875 **Figure 1**: Principal component analysis (PCA) score plot with 95% confidence intervals for
876 the two cultivars of *Stevia rebaudiana*, Fengtian (**A**) and Shoutian-2 (**B**). The plants were
877 grown in hydroponic culture and exposed to control, 50 mM, 100 mM and 200 mM NaCl for
878 8 weeks. The distances between the four populations were calculated as described in the
879 "Materials and Methods'' using the log-transformed, normalized data of each of the cultivar
880 from which the means presented in Table 5 are derived. The PCA vectors span a 9-
881 dimensional space to give the best treatment separation with each point representing a linear
882 combination of all the metabolites from individual treatment. Vectors 1 and 2 were chosen
883 for the best visualization of the differences between salinity treatments. Salinity stress
884 profiles differed moderately from the control plants by 33% in Fengtian (C2) in comparison
885 with 28.4% in Shoutian-2 (C1) on the basis of the information derived from metabolic
886 variances.

887 **Figure 2**: Principal component analysis (PCA) showing loading plot of metabolite profile
888 data of two cultivars of *Stevia rebaudiana*, Fengtian (**A**) and Shoutian-2 (**B**). The seedlings
889 were grown in the hydroponics and were exposed to control, 50 mM, 100 mM and 200 mM
890 NaCl stress. The distances between these populations were calculated as described in the
891 'Materials and Methods' using the log-transformed, normalized data of the single
892 measurements from which the means presented in Table 5 are derived. The PCA vectors span
893 a 9-dimensional space to give the best treatment separation with each point representing a
894 linear combination of all the metabolites from an individual sample. For complete annotation
895 of the global responses of different metabolites to salt stress refer to Table 5.

896 **Figure 3**: Mapping of metabolite changes on known pathways for the cultivar Fengtian (C2)
897 of *Stevia rebaudiana*, grown in hydroponics in control and different concentrations of NaCl
898 for 8 weeks. The data from the leaves of each cultivar were normalized to the mean response
899 calculated for the respective unstressed control samples (Tables 5). The control is colored red
900 and the NaCl stressed treatments are colored blue. Maximum intensity in 50 mM and
901 minimum in 200 mM NaCl treatments

902 **Figure 4**: Mapping of metabolite changes on known pathways for *Stevia rebaudiana*, cultivar
903 Shoutian-2 (C1) grown in hydroponics in unstressed control and different concentrations of
904 NaCl stress for 8 weeks. The data from the leaves of each cultivar were normalized to the
905 mean response calculated for the respective control samples (Tables 5). The control is colored

red and the NaCl stressed treatments are colored blue. Maximum intensity in 50 mM and minimum in 200 mM NaCl treatments.

Figure 5: Correlation map based on the non-parametric Speakman's correlation co-efficient showing the combined element and metabolite profile of the leaf tissue of the cultivar Fengtian (C2) of *Stevia rebaudiana* grown in hydroponics in control and different NaCl concentrations for 8 weeks. More over 61 metabolites were identified representing different metabolic cycles and these metabolites were correlated with sodium, calcium, magnesium, potassium and chloride concentrations of the plants exposed to unstressed control, 50 mM, 100 mM and 200 mM NaCl. The experimental data was mapped on a metabolite network of primary metabolism via a built-in graph editor in VANTED.

Figure 6: Correlation map based on the non-parametric Speakman's correlation co-efficient showing the combined element and metabolite profile of the leaf tissue of the cultivar Shoutian-2 (C1) of *Stevia rebaudiana* grown in hydroponics in control and different NaCl concentrations. More than 61 metabolites were identified representing different metabolic cycles and these metabolites were correlated with sodium, calcium, magnesium, potassium and chloride concentrations of the plants exposed to control, 50 mM, 100 mM and 200 mM NaCl. The experimental data was mapped on a metabolite network of primary metabolism via a built-in graph editor in VANTED.

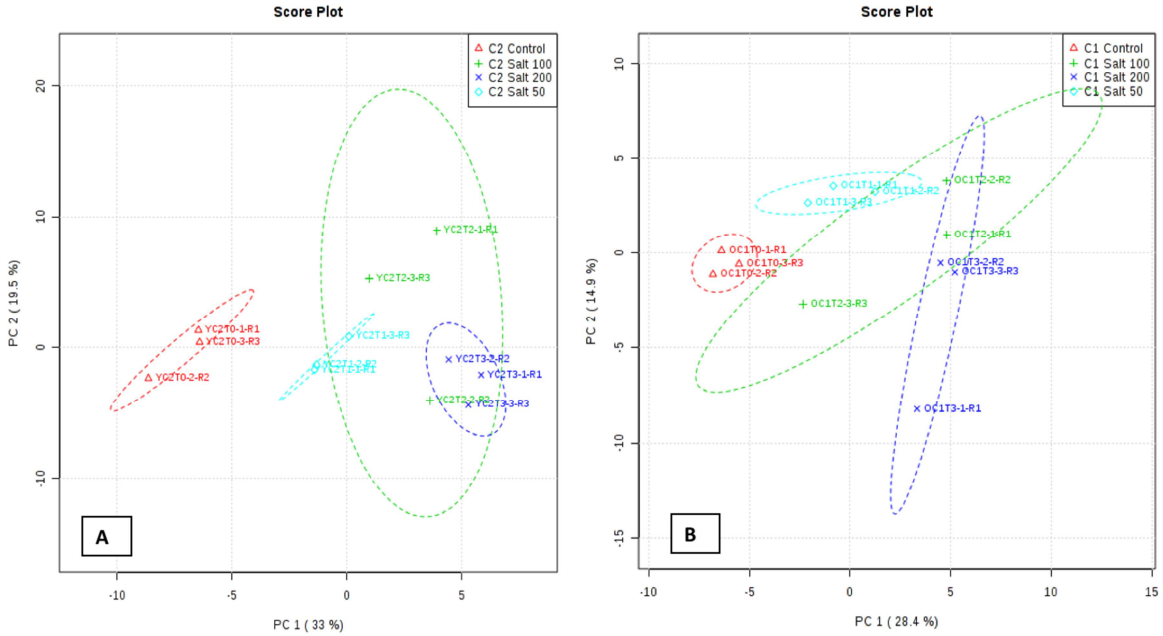
Steviol Glycoside (mg g^{-1} leaf DW)	Cultivar	Treatment with different concentration of NaCl (mM)											
		0	50	100	200	Mean	Df	ANOVA	C		T		C*T	
ST	C1	23.39	19.51	17.41	16.13	17.5	18	Significanc	NS		NS		NS	
	C2	19.16	18.77	14.95	11.84	15.1	18	LSD(5%)	3.28		5.19		7.34	
RebA	C1	2.7	10.5	10.75	6.84	7.46	18	Significanc	NS		**		NS	
	C2	4.59	8.45	7.2	5.13	6.25	18	LSD(5%)	1.21		1.91		2.71	
RebA+Stev	C1	26.09	30.01	28.1	22.98	24.5	18	Significanc	NS		NS		NS	
	C2	23.75	27.23	22.15	1.97	21.35	18	LSD(5%)	3.66		5.79		8.19	
RebA/Stev	C1	0.13	0.54	0.66	0.42	0.48	18	Significanc	NS		NS		NS	
	C2	0.24	0.45	0.52	0.44	0.45	18	LSD(5%)	0.14		0.22		0.31	
RebA/(Reb	C1	0.11	0.35	0.39	0.29	0.3	18	Significanc	NS		**		NS	
	C2	0.19	0.31	0.33	0.3	0.3	18	LSD(5%)	0.05		0.09		0.13	
Stev/(RebA	C1	0.88	0.64	0.6	0.7	0.69	18	Significanc	NS		**		NS	
	C2	0.8	0.69	0.66	0.69	0.7	18	LSD(5%)	0.05		0.09		0.13	

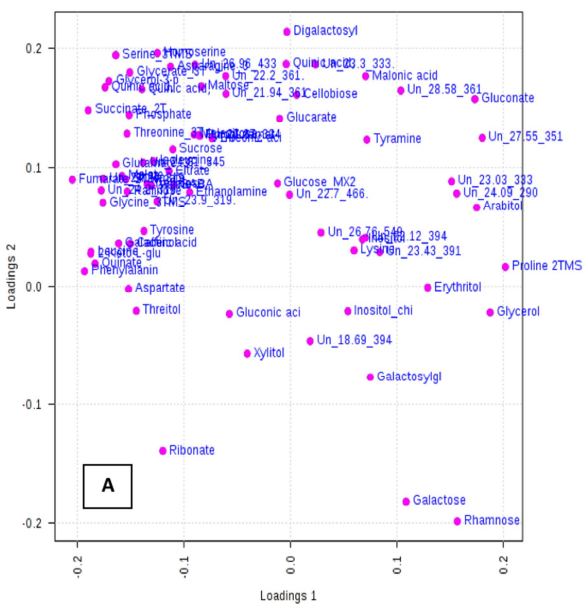
Steviol Glycoside (mg g-1 leaf DW)	Cultivar	NaCl treatment (mM) 0	50	100	200	Df	ANOVA	C	T	C*T	
ST	C1	23.4	19.5	17.4	16.1	18	Significanc	NS	NS	NS	
	C2	19.2	18.8	15.0	11.8	18	LSD(5%)	3.28	5.19		7.34
RebA	C1	2.7	10.5	10.75	6.84	18	Significanc	NS	**	NS	
	C2	4.59	8.45	7.2	5.13	18	LSD(5%)	1.21	1.91		2.71
RebA+Stev	C1	26.1	30.0	28.2	23.0	18	Significanc	NS	NS	NS	
	C2	23.8	27.2	22.2	17.0	18	LSD(5%)	3.66	5.79		8.19
RebA/Stev	C1	0.1	0.5	0.6	0.4	18	Significanc	NS	NS	NS	
	C2	0.2	0.5	0.5	0.4	18	LSD(5%)	0.14	0.22		0.31
RebA/(RebA+Stev)	C1	0.1	0.3	0.4	0.3	18	Significanc	NS	**	NS	
	C2	0.2	0.3	0.3	0.3	18	LSD(5%)	0.05	0.09		0.13
Stev/(RebA+Stev)	C1	0.90	0.65	0.62	0.70	18	Significanc	NS	**	NS	
	C2	0.81	0.69	0.67	0.70	18	LSD(5%)	0.05	0.09		0.13

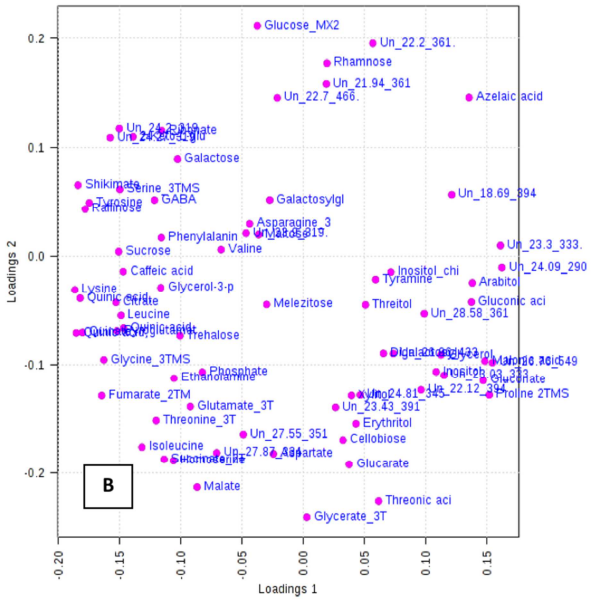
	Shoutian(C1)								Fengtian (C2)							
	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem	x-fold	sem
	C1 Control		C1 Salt 50		C1 Salt 100		C1 Salt 200		C2 Control		C2 Salt 50		C2 Salt 100		C2 Salt 200	
Amino acids & Amines																
Asparagine	1.000	± 0.293	0.867	± 0.292	0.517	± 0.170	0.606	± 0.050	1.000	± 0.205	**0.230**	**± 0.171**	0.383	± 0.251	0.858	± 0.309
Aspartate	1.000	± 0.118	**0.590**	**± 0.090**	0.741	± 0.274	0.741	± 0.248	1.000	± 0.355	0.451	± 0.233	0.534	± 0.488	0.832	± 0.068
beta Alanine	1.000	± 0.127	**0.464**	**± 0.156**	***0.223***	***± 0.018***	***0.157***	***± 0.124***	1.000	± 0.138	0.696	± 0.154	0.574	± 0.475	1.012	± 0.163
Ethanolamine	1.000	± 0.092	1.044	± 0.199	1.215	± 0.281	**0.308**	**± 0.278**	1.000	± 0.257	0.474	± 0.225	0.505	± 0.157	0.617	± 0.297
GABA	1.000	± 0.043	0.834	± 0.309	1.089	± 0.513	0.290	± 0.625	1.000	± 0.278	0.365	± 0.294	0.908	± 0.276	**0.181**	**± 0.157**
Glutamate	1.000	± 0.044	0.677	± 0.210	0.565	± 0.304	**0.449**	**± 0.293**	1.000	± 0.275	0.533	± 0.081	0.654	± 0.369	0.539	± 0.125
Glycine	1.000	± 0.230	**0.428**	**± 0.030**	**0.341**	**± 0.270**	**0.194**	**± 0.166**	1.000	± 0.185	**0.282**	**± 0.117**	**0.307**	**± 0.314**	**0.257**	**± 0.249**
Homoserine	1.000	± 0.016	0.971	± 0.316	**0.374**	**± 0.222**	**0.350**	**± 0.393**	1.000	± 0.146	**0.482**	**± 0.236**	**0.241**	**± 0.379**	0.612	± 0.111
Isoleucine	1.000	± 0.312	0.804	± 0.157	0.885	± 0.280	0.317	± 0.120	1.000	± 0.183	0.848	± 0.157	0.438	± 0.261	0.584	± 0.136
Leucine	1.000	± 0.141	**0.286**	**± 0.334**	0.456	± 0.353	**0.156**	**± 0.388**	1.000	± 0.371	0.437	± 0.216	**0.172**	**± 0.353**	0.232	± 0.230
Lysine	1.000	± 0.663	2.304	± 0.467	1.916	± 0.282	0.943	± 0.618	1.000	± 0.169	**0.242**	**± 0.005**	0.069	± 0.107	**0.092**	**± 0.351**
Phenylalanine	1.000	± 0.212	**0.363**	**± 0.359**	0.660	± 0.314	**0.176**	**± 0.269**	1.000	± 0.434	0.459	± 0.148	0.653	± 0.326	0.299	± 0.039
Proline	1.000	± 0.035	**1.994**	**± 0.202**	1.313	± 0.217	**2.449**	**± 0.159**	1.000	± 0.176	**3.631**	**± 0.084**	2.538	± 0.367	**5.563**	**± 0.287**
Pyroglutamate	1.000	± 0.538	0.562	± 0.154	0.253	± 0.291	0.553	± 0.350	1.000	± 0.170	**0.262**	**± 0.245**	**0.261**	**± 0.352**	**0.332**	**± 0.145**
Serine	1.000	± 0.105	**0.397**	**± 0.078**	**0.084**	**± 0.373**	***0.182***	***± 0.084***	1.000	± 0.170	**0.184**	**± 0.294**	0.192	± 0.692	0.149	± 0.260
Threonine	1.000	± 0.327	0.652	± 0.116	0.545	± 0.279	**0.174**	**± 0.234**	1.000	± 0.178	0.593	± 0.146	0.536	± 0.224	0.663	± 0.058
Tyramine	1.000	± 0.091	**3.813**	**± 0.305**	3.983	± 0.588	1.494	± 0.324	1.000	± 0.195	1.584	± 0.100	1.719	± 0.269	1.386	± 0.202
Tyrosine	1.000	± 0.115	**0.515**	**± 0.158**	0.612	± 0.214	0.309	± 0.429	1.000	± 0.220	**0.408**	**± 0.134**	**0.418**	**± 0.070**	**0.244**	**± 0.129**
Valine	1.000	± 0.147	0.801	± 0.223	0.826	± 0.259	0.444	± 0.368	1.000	± 0.400	0.844	± 0.218	0.659	± 0.256	0.733	± 0.133
Organic acids																
2-Keto-L-gluconic acid	1.000	± 0.137	0.571	± 0.213	0.682	± 0.268	0.593	± 0.148	1.000	± 0.328	0.659	± 0.176	0.729	± 0.245	**0.163**	**± 0.069**
Azelaic acid	1.000	± 0.196	0.911	± 0.446	0.855	± 0.377	1.341	± 0.208	1.000	± 0.372	1.454	± 0.082	**4.182**	**± 0.248**	2.025	± 0.094
Caffeic acid	1.000	± 0.535	0.488	± 0.658	0.421	± 0.351	0.349	± 0.241	1.000	± 0.088	0.837	± 0.109	**0.425**	**± 0.226**	0.513	± 0.317
Citrate	1.000	± 0.200	0.368	± 0.691	0.461	± 0.939	1.189	± 0.398	1.000	± 0.265	0.475	± 0.033	0.487	± 0.209	0.355	± 0.059
Erythronic acid	1.000	± 0.221	**0.422**	**± 0.103**	0.464	± 0.291	0.756	± 0.210	1.000	± 0.302	0.496	± 0.297	0.785	± 0.392	0.384	± 0.055
Fumarate	1.000	± 0.128	**0.134**	**± 0.515**	0.492	± 0.949	0.300	± 0.706	1.000	± 0.110	0.429	± 0.201	**0.197**	**± 0.407**	**0.202**	**± 0.134**
Glucarate	1.000	± 0.086	1.134	± 0.138	0.901	± 0.756	1.842	± 0.214	1.000	± 0.418	0.725	± 0.111	0.765	± 0.587	1.696	± 0.041
Gluconate	1.000	± 0.116	**5.629**	**± 0.191**	0.773	± 0.125	**4.392**	**± 0.330**	1.000	± 0.373	1.406	± 0.154	2.557	± 0.417	**3.221**	**± 0.100**
Gluconic acid,1,4-lactone	1.000	± 0.210	0.645	± 0.266	0.471	± 0.189	0.741	± 0.398	1.000	± 0.080	4.615	± 0.167	4.369	± 0.282	3.411	± 0.119
Glycerate	1.000	± 0.138	0.791	± 0.076	0.589	± 0.363	**0.603**	**± 0.082**	1.000	± 0.130	0.838	± 0.166	0.821	± 0.463	1.235	± 0.049
Malate	1.000	± 0.127	**0.228**	**± 0.389**	0.462	± 0.920	0.570	± 0.251	1.000	± 0.263	0.942	± 0.217	0.358	± 0.454	0.740	± 0.120
Malonic acid	1.000	± 0.215	1.898	± 0.173	0.802	± 0.240	0.968	± 0.217	1.000	± 0.399	1.338	± 0.123	2.351	± 0.303	**3.963**	**± 0.162**

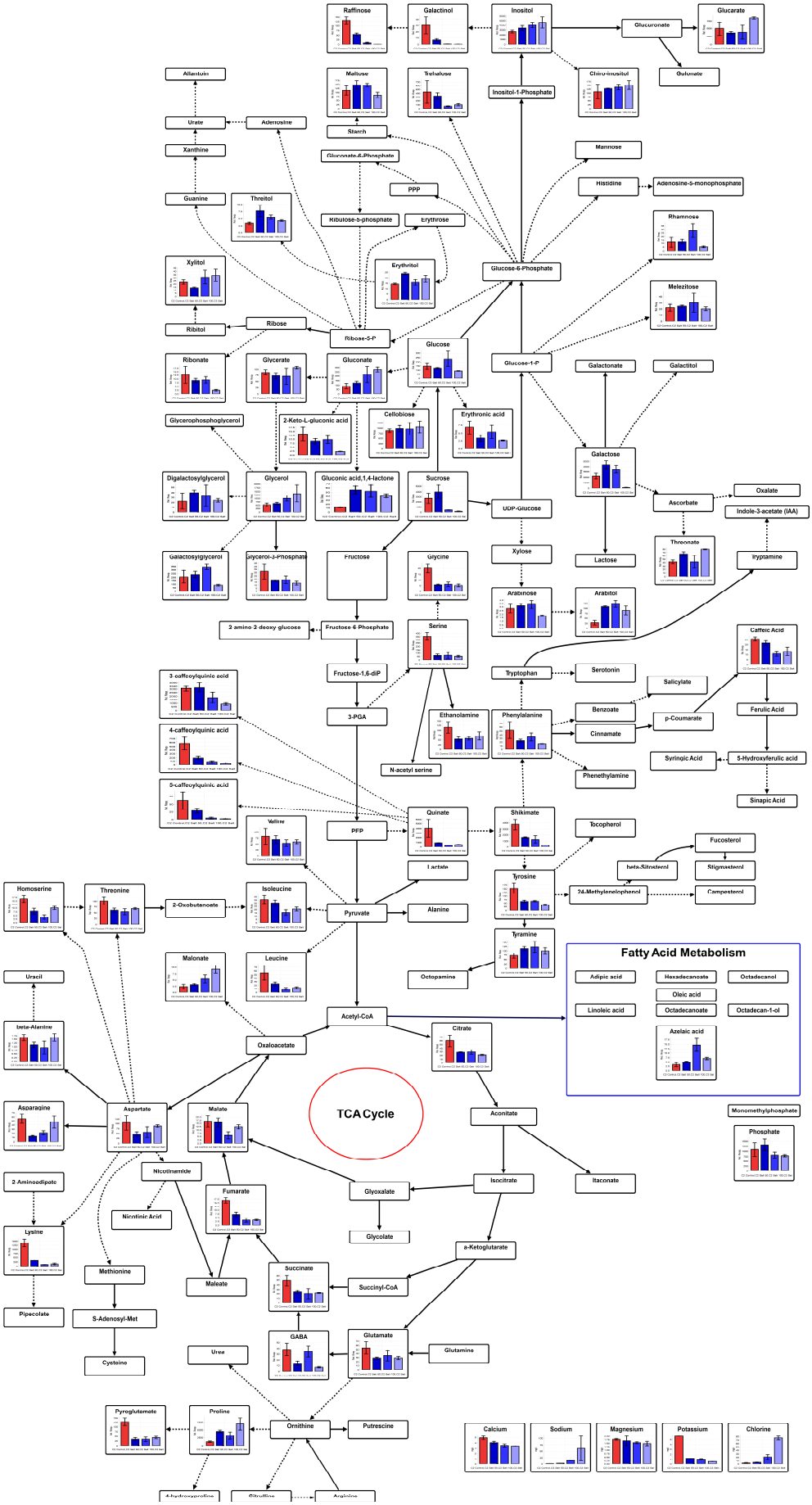
Quinate	1.000 ± 0.215	**0.183 ± 0.440**	**0.250 ± 0.517**	0.770 ± 0.728	1.000 ± 0.487	0.225 ± 0.058	**0.088 ± 0.067**	**0.128 ± 0.072**
Quinic acid,3-caffeoyl	1.000 ± 0.159	1.637 ± 0.260	1.303 ± 0.465	1.182 ± 0.285	1.000 ± 0.129	1.061 ± 0.212	0.576 ± 0.411	**0.281 ± 0.186**
Quinic acid,4-caffeoyl	1.000 ± 0.144	0.350 ± 0.524	**0.138 ± 0.704**	0.293 ± 0.922	1.000 ± 0.294	**0.337 ± 0.210**	**0.123 ± 0.402**	***0.061 ± 0.058***
Quinic acid,5-caffeoyl	1.000 ± 0.050	0.889 ± 0.343	0.594 ± 0.467	0.519 ± 0.762	1.000 ± 0.444	0.480 ± 0.237	**0.080 ± 0.565**	**0.037 ± 0.198**
Ribonate	1.000 ± 0.233	0.468 ± 0.235	0.727 ± 0.300	0.632 ± 0.078	1.000 ± 0.445	0.689 ± 0.158	0.722 ± 0.220	**0.178 ± 0.250**
Shikimate	1.000 ± 0.093	0.526 ± 0.426	0.486 ± 0.468	**0.363 ± 0.188**	1.000 ± 0.235	**0.418 ± 0.067**	0.334 ± 0.476	***0.081 ± 0.025***
Succinate	1.000 ± 0.195	0.465 ± 0.262	**0.279 ± 0.368**	**0.352 ± 0.056**	1.000 ± 0.232	0.493 ± 0.167	0.411 ± 0.591	0.442 ± 0.053
Threonic acid	1.000 ± 0.004	0.929 ± 0.192	**0.542 ± 0.180**	0.869 ± 0.253	1.000 ± 0.118	1.540 ± 0.103	0.997 ± 0.435	**1.883 ± 0.013**
Sugars & sugar phosphates								
Arabinose	1.000 ± 0.018	**0.485 ± 0.185**	0.929 ± 0.242	**0.325 ± 0.251**	1.000 ± 0.207	1.139 ± 0.089	1.208 ± 0.172	0.636 ± 0.053
Cellobiose	1.000 ± 0.366	1.642 ± 0.292	1.132 ± 0.163	0.625 ± 0.112	1.000 ± 0.089	1.109 ± 0.154	1.087 ± 0.304	1.202 ± 0.271
Digalactosylglycerol	1.000 ± 0.028	**2.060 ± 0.202**	0.861 ± 0.358	0.890 ± 0.572	1.000 ± 0.719	1.754 ± 0.126	1.488 ± 0.627	1.069 ± 0.144
Erythritol	1.000 ± 0.217	2.433 ± 0.283	1.935 ± 0.482	1.495 ± 0.430	1.000 ± 0.051	**1.673 ± 0.044**	1.105 ± 0.155	1.345 ± 0.164
Fructose	1.000 ± 0.353	1.538 ± 0.434	**3.435 ± 0.095**	**3.160 ± 0.101**	1.000 ± 0.141	**3.704 ± 0.261**	***7.350 ± 0.212***	1.676 ± 0.227
Galactinol	1.000 ± 0.029	0.598 ± 0.228	0.955 ± 0.505	0.843 ± 0.381	1.000 ± 0.450	0.224 ± 0.260	**0.018 ± 0.298**	**0.008 ± 0.407**
Galactose	1.000 ± 0.128	1.179 ± 0.093	2.231 ± 0.278	1.715 ± 0.299	1.000 ± 0.221	1.970 ± 0.174	1.592 ± 0.217	**0.052 ± 0.400**
Galactosylglycerol	1.000 ± 0.298	1.177 ± 0.470	1.094 ± 0.416	1.106 ± 0.168	1.000 ± 0.430	1.152 ± 0.174	1.667 ± 0.115	0.414 ± 0.129
Glucose	1.000 ± 0.312	1.256 ± 0.291	0.894 ± 0.591	**0.377 ± 0.141**	1.000 ± 0.254	0.831 ± 0.058	1.582 ± 0.385	0.615 ± 0.038
Glycerol	1.000 ± 0.169	**1.870 ± 0.077**	1.863 ± 0.224	1.490 ± 0.140	1.000 ± 0.254	1.194 ± 0.144	1.918 ± 0.204	2.563 ± 0.442
Glycerol-3-phosphate	1.000 ± 0.117	0.792 ± 0.127	0.609 ± 0.325	**0.289 ± 0.290**	1.000 ± 0.365	0.546 ± 0.079	0.589 ± 0.328	0.415 ± 0.251
Inositol_chiro	1.000 ± 0.496	1.028 ± 0.141	1.087 ± 0.262	0.949 ± 0.013	1.000 ± 0.426	1.183 ± 0.017	1.271 ± 0.125	1.392 ± 0.187
Inositol_myo	1.000 ± 0.380	1.340 ± 0.325	1.231 ± 0.105	1.240 ± 0.065	1.000 ± 0.098	1.270 ± 0.142	1.511 ± 0.146	1.724 ± 0.263
Maltose	1.000 ± 0.164	1.396 ± 0.183	1.115 ± 0.347	0.378 ± 0.445	1.000 ± 0.250	1.304 ± 0.180	1.291 ± 0.076	0.744 ± 0.201
Melezitose	1.000 ± 0.223	1.038 ± 0.023	0.763 ± 0.248	**0.353 ± 0.235**	1.000 ± 0.250	1.106 ± 0.059	1.363 ± 0.506	0.915 ± 0.145
Raffinose	1.000 ± 0.757	0.468 ± 0.875	0.383 ± 0.462	0.146 ± 0.977	1.000 ± 0.149	0.413 ± 0.134	0.060 ± 0.476	**0.006 ± 0.177**
Rhamnose	1.000 ± 0.233	1.452 ± 0.218	**3.400 ± 0.097**	**3.018 ± 0.057**	1.000 ± 0.608	1.098 ± 0.262	2.416 ± 0.338	0.454 ± 0.224
Sucrose	1.000 ± 0.610	0.837 ± 0.314	1.120 ± 0.458	0.169 ± 0.681	1.000 ± 0.370	1.471 ± 0.315	0.149 ± 0.059	**0.060 ± 0.249**
Threitol	1.000 ± 0.172	0.570 ± 0.087	0.615 ± 0.088	0.724 ± 0.389	1.000 ± 0.108	**2.281 ± 0.250**	1.632 ± 0.141	1.260 ± 0.064
Trehalose	1.000 ± 0.413	0.234 ± 0.302	0.164 ± 0.578	0.094 ± 0.281	1.000 ± 0.678	0.751 ± 0.258	0.146 ± 0.174	0.240 ± 0.272
Xylitol	1.000 ± 0.145	0.745 ± 0.193	0.900 ± 0.285	1.442 ± 0.212	1.000 ± 0.150	0.597 ± 0.075	1.339 ± 0.350	1.459 ± 0.281
Other compounds								
Phosphate	1.000 ± 0.255	0.698 ± 0.067	0.485 ± 0.074	0.622 ± 0.127	1.000 ± 0.312	1.216 ± 0.215	0.749 ± 0.178	0.723 ± 0.070
Un_12.53_306	1.000 ± 0.242	0.353 ± 0.578	***0.009 ± 0.346***	**0.009 ± 0.515**	1.000 ± 0.146	***0.006 ± 0.194***	0.558 ± 0.517	***0.010 ± 0.072***
Un_16.17_306.2	1.000 ± 0.167	0.873 ± 0.118	1.164 ± 0.428	1.300 ± 0.216	1.000 ± 0.278	1.297 ± 0.067	1.401 ± 0.323	2.115 ± 0.060
Un_18.69_394.2	1.000 ± 0.222	0.970 ± 0.189	0.985 ± 0.181	1.239 ± 0.215	1.000 ± 0.192	0.794 ± 0.113	1.446 ± 0.049	1.465 ± 0.097
Un_21.94_361.2	1.000 ± 0.066	1.228 ± 0.234	0.507 ± 0.419	***0.294 ± 0.046***	1.000 ± 0.269	0.823 ± 0.083	1.285 ± 0.324	1.006 ± 0.019

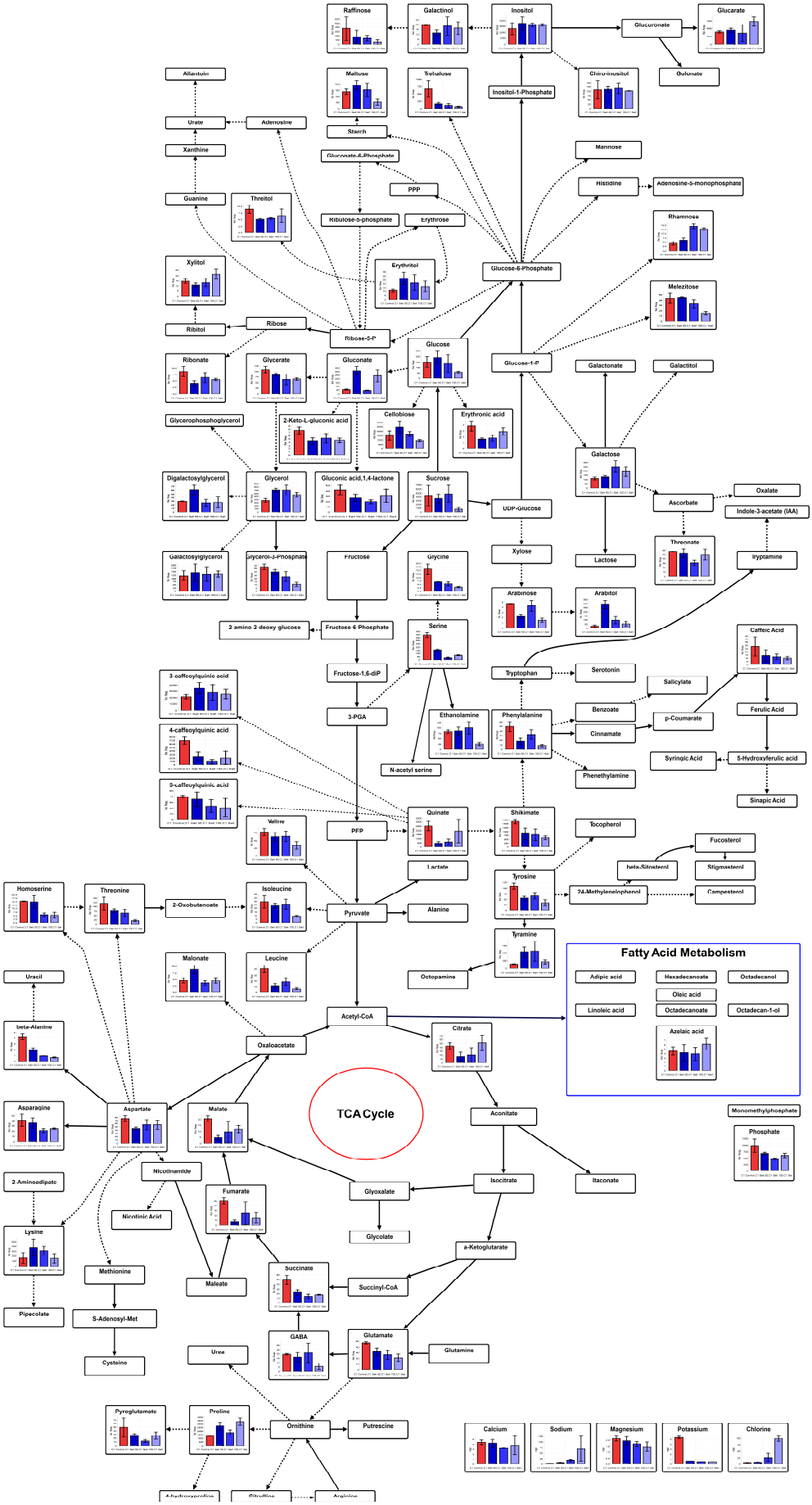
Un_22.12_394.2	1.000	±	0.386	0.982	±	0.094	0.518	±	0.309	1.596	±	0.101	1.000	±	0.476	1.368	±	0.173	1.301	±	0.348	1.852	±	0.086
Un_22.2_361.2	1.000	±	0.116	1.207	±	0.182	0.418	±	0.603	***0.220***	***±***	***0.088***	1.000	±	0.262	0.866	±	0.094	5.787	±	0.497	1.374	±	0.107
Un_22.7_466.3	1.000	±	0.458	0.656	±	0.306	0.253	±	0.442	0.464	±	0.114	1.000	±	0.294	0.769	±	0.126	1.677	±	0.285	0.607	±	0.244
Un_23.03_333.2	1.000	±	0.181	**2.763**	**±**	**0.176**	1.653	±	0.463	1.375	±	0.247	1.000	±	0.475	1.116	±	0.136	1.238	±	0.192	2.695	±	0.257
Un_23.3_333.2	1.000	±	0.339	2.653	±	0.165	1.234	±	0.479	0.594	±	0.380	1.000	±	0.422	1.934	±	0.147	**5.156**	**±**	**0.142**	**4.958**	**±**	**0.201**
Un_23.43_391.2	1.000	±	0.148	2.115	±	0.220	3.905	±	0.722	**3.303**	**±**	**0.195**	1.000	±	0.583	1.030	±	0.101	1.080	±	0.460	1.070	±	0.167
Un_23.9_319.2	1.000	±	0.223	0.750	±	0.167	0.650	±	0.149	0.541	±	0.255	1.000	±	0.102	0.618	±	0.261	0.764	±	0.179	0.867	±	0.141
Un_24.09_290.2	1.000	±	0.288	2.471	±	0.299	1.394	±	0.085	1.741	±	0.380	1.000	±	0.606	1.587	±	0.206	2.690	±	0.207	3.479	±	0.161
Un_24.2_319.2	1.000	±	0.246	**0.304**	**±**	**0.341**	0.317	±	0.376	0.203	±	0.635	1.000	±	0.221	0.728	±	0.266	0.679	±	0.375	**0.177**	**±**	**0.097**
Un_24.27_319.2	1.000	±	0.248	0.338	±	0.336	0.348	±	0.382	0.204	±	0.625	1.000	±	0.206	0.722	±	0.256	0.597	±	0.355	**0.162**	**±**	**0.079**
Un_24.81_345.2	1.000	±	0.076	**0.296**	**±**	**0.416**	**0.093**	**±**	**0.527**	**0.222**	**±**	**0.354**	1.000	±	0.346	1.262	±	0.220	0.867	±	0.176	1.243	±	0.147
Un_26.76_549.4	1.000	±	0.888	0.367	±	0.190	0.100	±	0.443	0.582	±	0.486	1.000	±	0.525	**19.803**	**±**	**0.131**	**6.157**	**±**	**0.421**	**30.739**	**±**	**0.229**
Un_26.96_433.3	1.000	±	0.435	0.493	±	0.246	**0.063**	**±**	**0.507**	0.234	±	0.353	1.000	±	0.395	1.188	±	0.240	1.228	±	0.197	1.641	±	0.458
Un_27.55_351.2	1.000	±	0.351	**13.241**	**±**	**0.319**	3.006	±	0.388	1.682	±	0.287	1.000	±	0.564	2.617	±	0.161	0.806	±	0.963	0.265	±	0.303
Un_27.87_334.2	1.000	±	0.277	0.499	±	0.385	**0.071**	**±**	**0.581**	0.183	±	0.534	1.000	±	0.354	0.831	±	0.186	0.595	±	0.482	0.638	±	0.323
Un_28.58_361.2	1.000	±	0.155	1.522	±	0.151	0.937	±	0.052	1.414	±	0.205	1.000	±	0.344	1.031	±	0.141	1.825	±	0.217	1.686	±	0.166

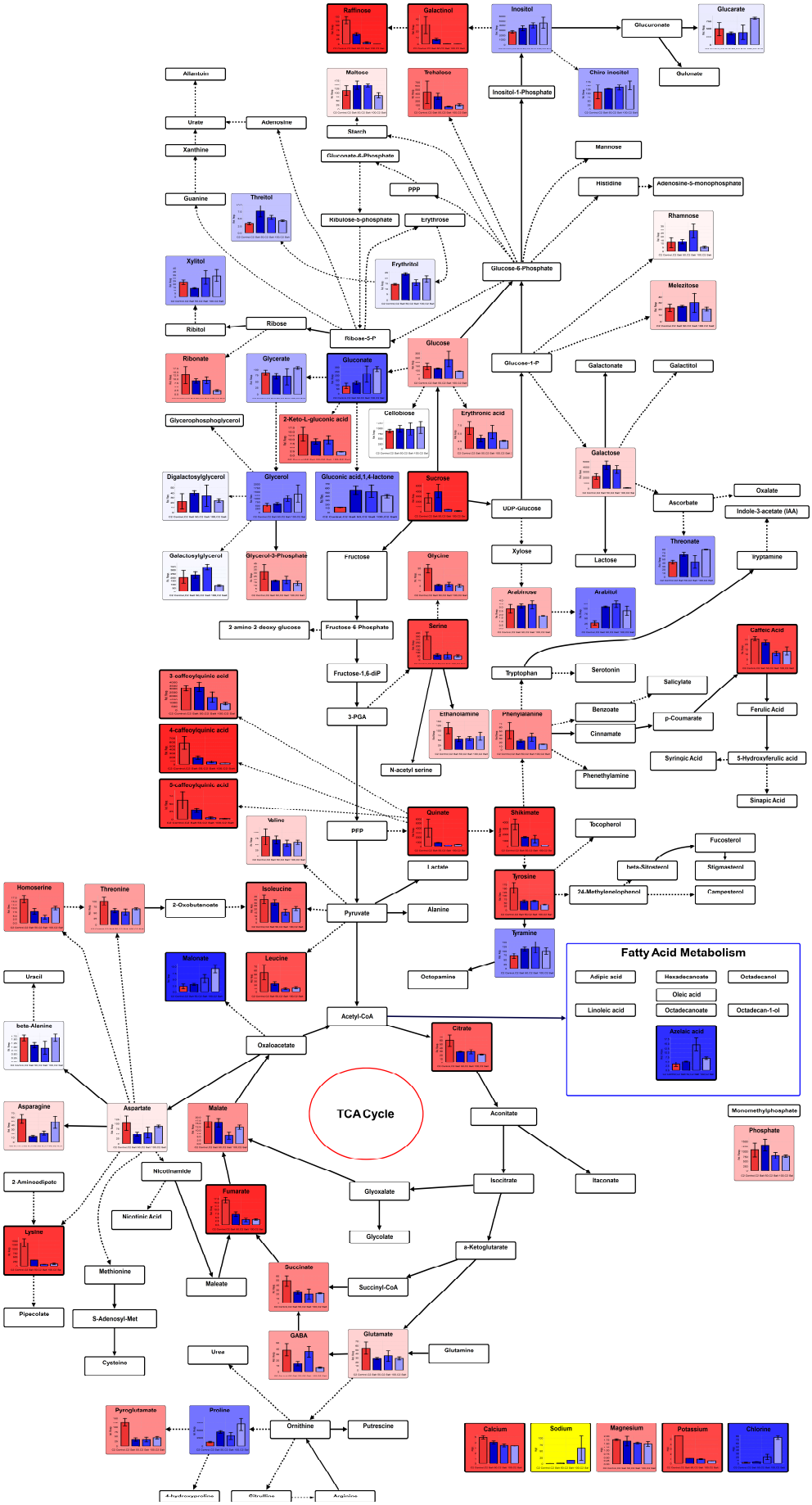


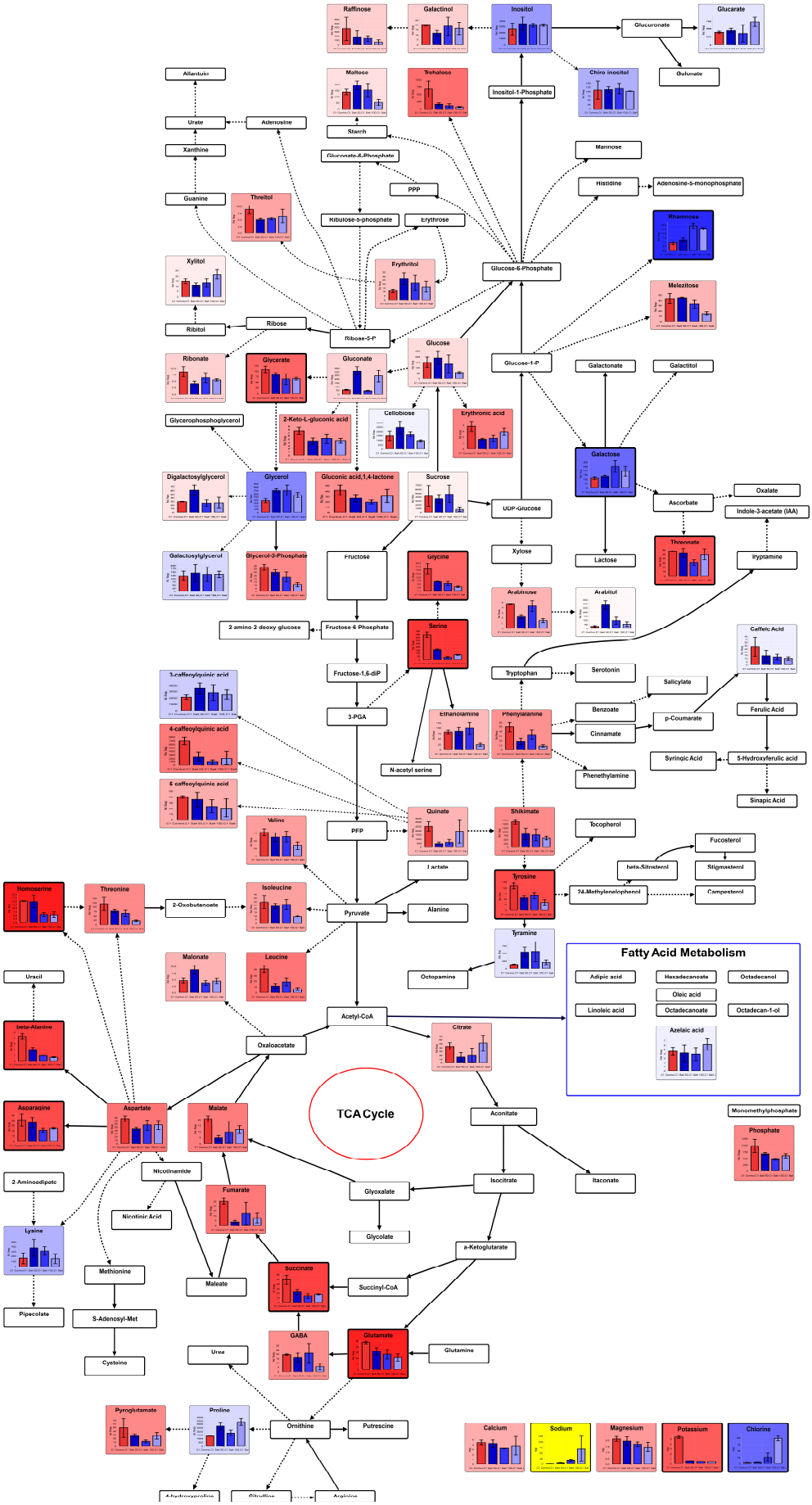












Highlights

- Fengtian showed increased levels of steviol glycosides, particularly rebaudioside A.
- Salinity stress reduced stevia plant height and biomass, particularly in cultivar Shoutian-2.
- Fengtian maintained higher K^+/Na^+ ratios as compared to Shoutian-2.
- Amino acids and amines were the major osmotica in stevia under salinity stress.
- Fengtian accumulated higher levels of proline and gluconate.

Contributions

M.D., N.A. and U.R. conceived and designed the experiments. M.D. performed the Stevia growth, salinity and ionomic experiments. D.D. and N.J. performed the metabolite profiling analysis. D.C. performed the steviol glycoside measurements and N.A. and M.D. performed the statistical analysis. M.D., N.A., C.H., D.D., N.J., D.C., D.M. and U.R. analysed and interpreted the data. All authors read and approved the manuscript.