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Nitrogen drives plant growth to the detriment of leaf sugar and steviol glycoside metabolisms in stevia (*Stevia rebaudiana* Bertoni)

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Abstract

Steviol glycosides (SGs) in Stevia (*Stevia rebaudiana* Bertoni) leaves are economically important due to their high sweetness and low calorific value. The yield of SGs is dependent on fertilization regimes, but the relationship between nitrogen (N) administration and SGs synthesis is still unclear. In this study, both hydroponic and plot experiments were conducted to investigate the effects of N rates on SGs production in Stevia leaves. N addition resulted in a significant reduction of leaf SGs contents which was linked to the down-regulation of SGs synthesis related genes. However, the total SGs yield when expressed on per plant was not significantly changed by N. Graphical vector and linear regression analyses confirmed that N-promoted growth compensated for reduced SGs content when considered leaf level. Additionally, N addition decreased leaf carbon (C)/N ratio (approximate 24.6%-32.0%) and soluble sugar concentrations (approximate 3.2%-17.3%), accompanied with the inhibited phosphoenolpyruvate carboxylase and L-phenylalanine ammonia_lyase activities. A significant positive correlation between leaf SGs concentration, C/N ratio and soluble sugar status was observed. Overall, we suggest that N-driven plant growth has negative effects on Stevia SG concentration, C/N ratio and sugar metabolism when considered on a leaf basis. Changes in leaf C/N ratio and soluble sugar indicated the occurrence of metabolic reprogramming. This has implications for Stevia growth and harvesting practice.

Keywords

Stevia rebaudiana Bertoni; steviol glycosides; nitrogen; dilution effect; sugar

Abbreviations

STV, stevioside; Reb A, rebaudioside A; Reb C, rebaudioside C; Reb F, rebaudioside F; DA , dulcoside; SGs, steviol glycosides; KS, kaurene synthase; KO, kaurene oxidase; KAH, ent-kaurenoic acid 13-hydroxylase; UGTs, UDP-dependent glycosyltransferases; PEP, phosphoenolpyruvate; PEPCase, phosphoenolpyruvate carboxylase; SPS, sucrose phosphate synthase; PAL, L-phenylalanine ammonia_lyase; FBS, flower-bud stage; FGS, fast growth stage; CNBH, Carbon-Nutrient Balance Hypothesis; GDBH, Growth Differentiation Balance Hypothesis.

1. Introduction

As a perennial herb of Asteraceae family, Stevia (*Stevia rebaudiana* Bertoni) is known as a health -care "source of sugar", which is superior to cane sugar, beet sugar or other intensive sweeteners. The sweetness is arises from diterpene steviol glycosides (SGs) found especially in the leaf tissue. SGs are 250-450 times sweeter than sucrose, but with a calorific value ~1/300 that of sucrose (Lemus-Mondaca et al. 2012). SGs in Stevia plants, including stevioside (STV), rebaudioside (Reb A, C, F) and dulcoside A (DA), can account for 4-20% of the leaf dry weight (Winter and Huber 2010). The adjuvant effects of SGs which include reducing blood sugar, preventing hypertension, hyperlipidemia, caries and improving human immunity, have been recognized and exploited in recent years (Philippaert et al. 2017; Yadav and Guleria 2012). Therefore, the worldwide demand for SGs is rapidly increasing, especially after those were authorized use as food additives in the European Union (Additives and Food 2010). Most recent studies have focused on the breeding of new Stevia varieties, improving SGs extraction technologies from leaves or investigating the SG biosynthesis pathway (Bursac Kovacevic et al. 2018; Wang et al. 2016). However, there has been little focus on the possible role of different means of agronomic cultivation in improving the production of SGs.

Amongst these agronomic practices, soil fertilization (especially nitrogen [N] managements) plays an essential role in regulating plant growth and the production of active secondary compounds (Ibrahim et al. 2011). Thus, proteins, nucleic acids and chlorophyll, benefit from N to improve plant growth, yield and primary metabolism. However, the impact of N on secondary metabolism is different. Numerous studies have shown that N fertilization favors alkaloid production but inhibits the synthesis of phenolic compounds (Aharoni and

Galili 2011; Ibrahim et al. 2011; Masclaux-Daubresse et al. 2010). This reflects a difference between the N-containing alkaloid and carbon (C)-containing of phenolic substances. Structurally, SGs is tetracyclic diterpenoid belonging to C- containing substance class but the relationship between N and terpenoids is more complicated than that of phenolic compounds. A metanalysis of woody plants confirmed the negative effect of N fertilization on phenolic synthesis but there were no significant impact on terpenoids compounds (Koricheva 1998). In Stevia, N fertilization had been shown to increase biomass (Ruan et al. 2010; Tavarini et al. 2015a) but the influence on SGs synthesis was unclear. For example, Tavarini et al. (2015a) reported that the leaf concentrations of stevioside (STV) and rebaudioside A (Reb A) were increased after N administration. Similarly, Pal et al. (2015) showed a positive effect of N fertilization on leaf SG concentrations. However, opposite N effects were seen under different growth stages or conditions (Barbet-Massin et al. 2015; Kafle et al. 2017).

Regulatory insights are now aided by elucidation of SG biosynthetic pathway. SGs originate from the glycolysis products, pyruvate and glyceraldehyde 3-phosphate, in the chloroplast. These enter either the 2-cmethyl-D-erythritol 4-phosphate (MEP) or mevalonate (MVA) pathway and the generated isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are converted to geranylgeranyl diphosphate (GGDP). The resulting ent-kauriene is then transported to the endoplasmic reticulum, where the SGs are synthesized through the action of kaurene synthase (KS), kaurene oxidase (KO), ent-kaurenoic acid 13-hydroxylase (KAH) and UDP-dependent glycosyltransferases (UGTs) (Kim et al. 2018; Wang et al. 2016; Yadav and Guleria 2012). Therefore, SG biosynthesis is similar to other terpenoids or phenolic compounds that derived from the hexose or phosphoenolpyruvate (PEP)

92 (Kallscheuer 2018). Such commonality could link SGs synthesis to leaf C metabolism but this
93 relationship has only been tentatively suggested by (Barbet-Massin et al. 2015) Further, it is
94 still unclear how N could influence this C metabolism linked SGs synthesis.

95 In this study, we tested the effect of N administration through both hydroponic and plot
96 experiments on the SGs synthesis. The leaf C status including the total C, soluble sugar and
97 starch contents together with the related enzyme activities were also measured. We reveal as
98 suppressive effect of N on both SGs and C metabolism when measured on a per leaf basis.;
99 although this effect was hidden when considered at a plant levels due to increased growth. Such
100 a dominance of N over SG and C metabolism appears to reflect transcriptional changes in the
101 plant.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

To examine the effect of N on the production of SGs in Stevia plants, both hydroponic and plot experiments were performed using similar sized cutting seedlings of Stevia (*Stevia rebaudiana* Bertoni) cultivar 'Zhongshan No. 8'.

Hydroponic Experiment

Stevia seedlings were transplanted to plastic pots containing 1000 mL aerated quarter-strength nutrient solution. After 4 days, the solutions were changed to half-strength nutrient solution and after an additional 4 days, to full-strength nutrient solution (the concentrations of other nutrients are shown below). Then the Stevia plants were divided into four groups (8 seedlings per treatment) when 6 pairs of leaves were emerged. The seedlings were treated with different levels of N (N deficiency: 0 mM; Low-N: 1 mM, intermediate-N: 4 mM and high-N (HN): 10 mM) as $(NH_4)_2SO_4$ and $Ca(NO_3)_2$ at the ratio of 1:1 while the other nutrients were maintained at full-strength as defined below.

The composition of full-strength nutrient solution was as follows: Macronutrients: 4 mM N as $(NH_4)_2SO_4$ and $Ca(NO_3)_2$; 1 mM phosphorus (P) as KH_2PO_4, 6 mM potassium (K) as K_2SO_4 and KH_2PO_4 and 2 mM magnesium (Mg) as $MgSO_4$. Micronutrients: 35 μM iron (Fe) as Fe–EDTA, 10 μM manganese (Mn) as $MnCl_2{\cdot}4H_2O$, 0.5 μM molybdenum (Mo) as $(NH4)_6Mo_7O_{24}{\cdot}4H_2O$, 60 μM boron (B) as H_3BO_3, 1 μM zinc (Zn) as $ZnSO_4{\cdot}7H_2O$ and 0.4 μM copper (Cu) as $CuSO_4{\cdot}5H_2O$.

The nutrient solutions were changed at 3-day intervals and the pH was adjusted daily to 6.00± 0.10 with 0.1 mol L^{-1} HCl or 0.1 mol L^{-1} NaOH. The plants were grown in a greenhouse

at 30/25 °C (day/night) with relative humidity of 70 ± 10% and photoperiod of 14 h d^{-1} (> 300 μmol m^{-2} s^{-1}). The Stevia seedlings were harvested after 2 weeks of treatment.

Plot Experiment

The plot experiment was conducted at Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (Jiangsu, China, latitude 32°03' N, longitude 118°49' E). The cut seedlings were transplanted on June 6, 2018. The field was divided into 16 plots with equal area (2.5 m^2). The four treatments at different N rates were arranged in a randomized block design with 4 replicates for each treatment. Stevia plants received 0 (N0), 100 (N100), 200 (N200), or 400 (N400) kg N ha^{-1} in the form of urea which were administered at different growth stages: June 6 (transplanting, 50%), July 5 (fast growing stage, 30%) and August 5 (late fast growing stage, 20%). For all treatments, 75 kg P_2O_5 ha^{-1} (as $Ca(H_2PO_4)_2$) and 90 kg K_2O ha^{-1} (as KCl) were administered as the base fertilizer. The Stevia plants were harvested at fast growth stage (July 14) and flower-bud stage (September 10).

The properties of the soil at 0-30 cm were measured and found to be: pH 6.72 (soil: H_2O= 1: 2.5), organic matter 27.72 mg g^{-1}, total N 1.528 mg g^{-1}, alkali-hydrolyzable N 105.075 mg kg^{-1}, Olsen-P 63.97 mg kg^{-1}, and NH_4OAc-K 347.72 mg kg^{-1}.

2.2 Sampling and Processing

Stevia seedlings were washed briefly with distilled water. The leaves, stems and roots were separated by hand and then the leaves were photographed and the leaf areas were determined using Image J. The plant parts were baked in an oven at 105 °C for 30 min and subsequently at 70 °C to constant weight after which the dry weights of all seedlings parts were determined.

The dry samples of leave, stem and root of single plant were separately grinded and mixed before storage, to avoid the influence of leaf positions (Ceunen and Geuns 2013b). Similarly, fresh leaf samples were also evenly mixed and ground in liquid nitrogen before stored in -80°C refrigerator.

2.3 Extraction of Steviol Glycosides (SGs) and HPLC Analysis

SGs were extracted according to the methods described by Ceunen and Geuns (2013a) with minor modification. Approximately 0.10 g leaf or 0.50 g stem samples was ground in a mortar and pestle and then extracted in 10 mL of 80% ethanol at 100 °C for 1 h and then centrifuged at 12,000× g for 10 min. The supernatant was taken and moved into a new centrifuge tube, which was dried by rotary evaporation. The dry residue was dissolved in 1 ml of distilled water and filtered through a 0.22 mm filter prior to HPLC analysis.

HPLC analyses were performed using a Sapphire C18 sorbent column (4.6×250 mm). The temperature was set at 25 °C and the samples (10 μL) were eluted with acetonitrile: sodium phosphate buffer (32:68) over a period of 20 min (Yang et al. 2015). The SGs which were analyzed included Reb A, STV, Rebaudioside F and C (1Reb F and C) and dulcoside A (DA) with the retention times of 7.415 min, 7.923 min, 9.415 min, 10.198 min and 11.040 min, respectively. SGs were detected by monitoring the UV at A210 nm with a mobile phase flow rate of 1 mL min^{-1}. The samples were quantified against standard curves of Reb A, STV, Reb F, Reb C and DA (99.99% pure, Chroma Dex, USA).

2.4. Measurement of Leaf Total N and Total C content

168 The leaf total N content was measured following the H_2SO_4–H_2O_2 digestion method of
169 Kjeldahl (Nelson and Sommers 1972) while the leaf total C content was measured following
170 the wet-combustion method (Hafsi et al. 2003).
171
172 *2.5. Measurement of Leaf Total Soluble Sugar and Starch*
173 Leaf total soluble sugar content was determined according to Zhang et al. (2013), with
174 modifications. A total of 0.05 g of dry leaf powder and 10 mL of deionized water were mixed
175 and boiled for 30 min at 100 °C and centrifuged at 10,000 *g* for 10 min. The supernatant was
176 transferred to a new glass tube. The supernatant was mixed with anthrone in ethyl acetate (2 g
177 100 mL^{-1}) and sulphuric acid and the absorbance was determined at 630 nm after boiling for 1
178 min.
179 The starch was extracted and measured from the residue, following the method of
180 Fernandes et al. (2012). After the gelatinization of the residues with 2 mL distilled water in
181 boiling water, 2 mL 9.2 N and 4.6 N pre-cooled chloric acid were added to decompose the
182 starch into glucose, which could be further reacted with anthrone and sulphuric acid and
183 detected following the method used for soluble sugar. Starch was calculated by multiplying the
184 soluble sugar content by a factor of 0.9 (Rovalino-Córdova et al. 2018).
185
186 *2.6. RNA Isolation and Quantitative Real Time PCR (RT-qPCR)*
187 Fresh leaf samples were harvested and immediately frozen in liquid nitrogen, and then stored
188 at -70 °C until RNA isolation. The total RNA was extracted with TRIzol reagent (Invitrogen,
189 USA) according to the manufacturer's instructions. cDNA was synthesized using the Prime

ScriptTMRT reagent Kit with DNA Eraser (Takara, Dalian, China). Reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) was performed using the ABI 7500 Real-Time PCR system, and the products were labeled using SYBR Green master mix (SYBR R Premix Ex TaqTM II (TliRNaseH Plus); TaKaRa, Dalian, China). The primers for RT- qPCR were as described by Yang et al. (2015), and actin gene was used as internal standard. Gene identifiers are listed in Supplementary Table 1. The relative gene expression was calculated with the $2^{-\triangle\triangle Ct}$ method.

2.7. Enzyme Activities.

The extraction and measurement of phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31) was based on Hu et al. (2017). Samples of 0.5 g fresh Stevia leaves were ground in a moreter and pestle with 1 mL extraction buffer (50 mM Hepes-KOH, pH= 7.4, with 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, 12 mM $MgCl_2$, 2 mM benzamidine and 2 mM e-amino-n-caproic acid). The homogenate was centrifuged at 4 °C for 20 min at 12, 000 g and the supernatant was used for enzyme activity measurement. PEPCase activity was measured based on the decreased rate of NADH absorbance at 340 nm after adding of the esupernatant sample and PEP to the reaction solution (50 mM Tris-HCl, pH= 7.6) with $NaHCO_3$, NADH, $MgCl_2$, DTT and malate dehydrogenase.

The determination of sucrose phosphate synthase (SPS, E.C. 2.4.1.14) activity was according to Hu et al. (2016). Samples of 100 μL enzyme solutions were mixed with the 450 μL reaction buffers (50 mM of extraction buffer, 10 mM of $MgCl_2$, 50 mM of UDP-glucose and 50 mM of fructose-6-P) and incubated at 25 °C for 10 min. Then, 150 μL NaOH (2 N) was

212 added to terminate the reaction before heating at 100 °C for 10 min. After cooling, 2.1 mL of
213 30% HCl and 0.6 mL of 0.1% resorcin in 95% ethanol were added to the mixtures before
214 heating at 100 °C for 30 min. The enzyme activity was calculated based on the values read at
215 480 nm.

216 Following the method of Tovar et al. (2002), L-phenylalanine ammonia_lyase (PAL, EC
217 4.3.1.5) was extracted into sodium borate ($Na_2B_4O_7$) buffer (0.2 M, pH 8.8). The homogenate
218 was centrifuged at 4 °C and 12,000 × g for 15 min. The supernatant (400 μL) was then diluted
219 with an equal volume of 100 mM $Na_2B_4O_7$ buffer and then pre-incubated at 40 °C for 5 min.
220 The reaction was started by the addition of 40 μL phenylalanine to the reaction mixture and
221 then incubated at 40 °C for 20 min before stopped by adding 5 N HCl. The absorbance was
222 read at 290 nm, where one unit of PAL was defined as the amount of the enzyme that increased
223 0.1 of absorbance per minute at 290 nm.

224

225 *2.8. Statistical Analysis*

226 One-way analysis of variance (ANOVA) was applied to assess differences in each parameter
227 with the treatments using the SPSS 16.0 statistical software package. Each means was based
228 on 4 experimental replicates and calculated standard deviations (SD) are reported. Significance
229 was tested at the 5% level.

230 Graphical vector analysis (GVA) was used to clarify the effect of N administration on the
231 total SGs and soluble sugar concentrations, according to Koricheva (1999). Data was
232 standardized by taking control as 100 and then plotted three-dimensionally. The relative values
233 of compound content, concentration and biomass were plotted on the x, y and z axis,

234 respectively. The centre of the diagram represents the reference point (control) and the
235 calculated effect was classified according to the quadrant the sample located, as shown in
236 Supplementary Fig. 1.

3. Results

3.1. Effects of N Administration Rates on Stevia Plants Growth under both Hydroponic and Field Conditions

Both plant growth and development were affected by N nutrition. N addition significantly enhanced Stevia growth and biomass formation under hydroponic cultures (Supplementary Table 2). Compared to N deficient conditions, plant heights were 33.4%-80.9% higher, leaf areas were 46.8%-95.8% larger and above ground biomass was increased by 31.6%-89.5% with N nutrients. The highest leaf and stem dry weight were observed with high-N treatment.

The responses of plant growth to growth stages and N rates were tested in plot experiments. Growth stages and N fertilization regimes both induced significant increases in plant height, total leaf area and aboveground biomass formation (Supplementary Table 3). The highest Stevia biomass was seen with N400 at the flower-bud stage (FBS).

3.2 Effects of N Administration Rates on the SGs content under both Hydroponic and Soil Cultures

The concentrations of steviol glycosides (SGs) were also altered by N levels. As shown in Fig. 1, the Reb A, STV, Reb F, Reb C and DA concentrations in Stevia leaves were all at their highest levels at the lowest N. Compared with N deficiency, low-N, intermediate- N and high-N treatments reduced total SGs contents by 25.4%, 35.9% and 51.7%, respectively. SGs concentrations in stem tissues of Stevia plants were significantly lower than that in leaves but exhibited no significant between different N treatments (Supplementary Fig. 2).

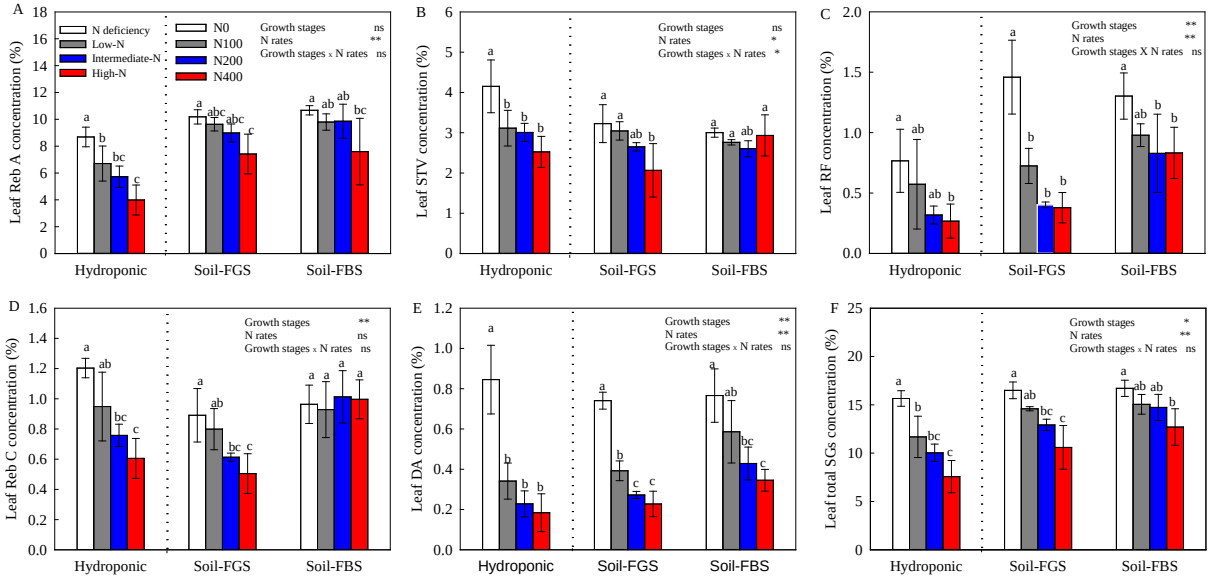


Fig. 1 Effect of N administration rates and growth stages on the leaf concentrations of rebaudioside A (Reb A, A), stevioside (STV, B), rebaudioside F (Reb F, C), rebaudioside C (Reb C, D), dulcoside A (DA, E) and total stevia glycosides (SGs).

FGS: fast growth stage; FBS: flower-bud stage.

Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples at flower-bud stage

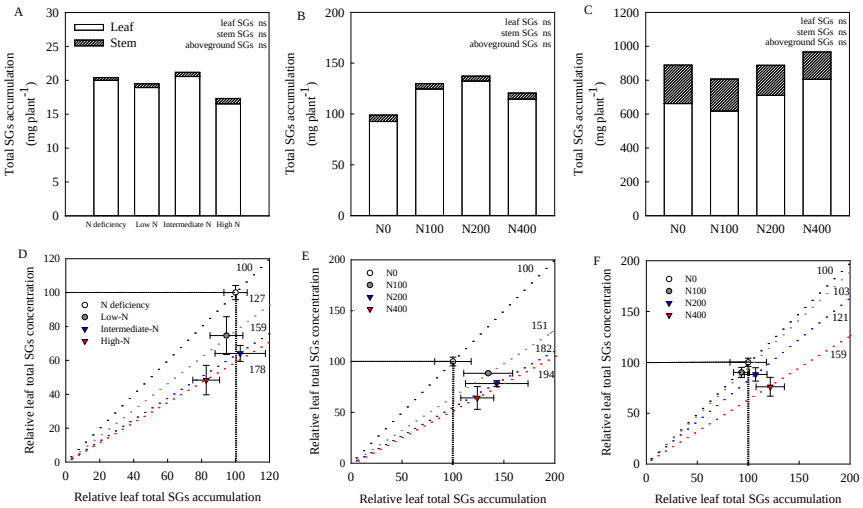
* and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively; ns means non-significant difference. Plot experiments were conducted and supplied with different N levels (N0: no N fertilization; N100: 100 kg N ha^{-1}; N200: 200 kg N ha^{-1}; N400: 400 kg N ha^{-1}). Data represent means of four replicates and the bars indicate the SD. Significant differences ($P < 0.05$) between different N rates at the same growth stage are indicated by different letters.

The SGs concentrations in soil grown Stevia leaves and stems were also affected by N fertilization (Fig. 1). At the fast growth stage (FGS), the SGs concentrations decreased with increased N. The leaf concentrations of Reb A, STV, Reb F, Reb C and DA of Stevia plants subjected to N400 treatment were respectively 27.2%, 36.0%, 74.1%, 43.4% and 69.3% lower when compared to N0 (Fig. 1). At flower bud stage (FBS), N fertilization also had a negative effect on leaf total SGs concentrations, but there was no significant change in leaf STV and Reb C. Once again, SGs concentrations in Stevia stem tissues were negatively regulated by N and their concentrations were significantly lower than in leaf tissues (Supplementary Fig. 1).

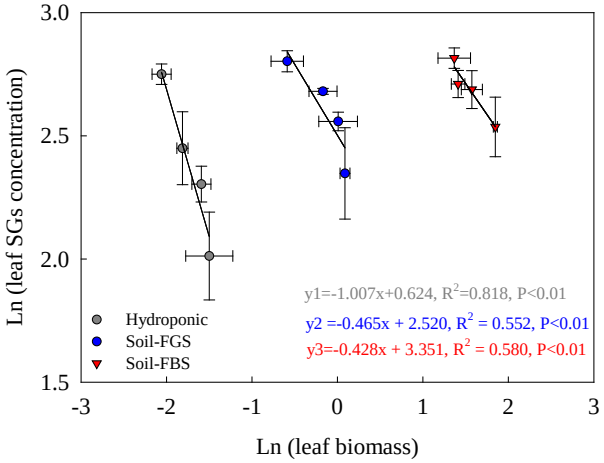
No significant changes in leaf SGs status were observed at different growth stages. However, the SGs concentrations in the stems were significantly higher at FBS than that at FGS (Supplementary Fig. 2).

3.3. Effects of N Administration Rates on the SGs accumulations under both Hydroponic and Soil Experiments

The accumulation of SGs in Stevia plants under different growth conditions or treatments were calculated. As shown in Fig. 2A, B and C, SGs production was most prominent in the leaf and was higher than stems regardless of growth stage or N rate. Although SGs accumulation was increase with the growth stages, no significant differences were observed with different N fertilization rates. Given these results, we performed graphical vector analysis (GVA) to understand the contribution of growth effects in the relationship between N administration rates and leaf SG concentrations. GVA results suggested that reduced total SGs concentrations with N administration was a result of reduced synthesis under hydroponic culture (Fig. 2D). However, when grown in soil, the growth associated reduction (dilution effect) occurred at FGS and FBS (Fig. 2E, F). A negative correlation between leaf biomass formation and SGs concentration was observed under various conditions, which again indicated that N increased growth “diluted” SGs levels (Fig. 3).



299 Fig. 2 The accumulation of total stevia glycosides (SGs) in Stevia plants (A, B, C) and graphical vector
300 analysis (GVA) of leaf SGs contents (D, E, F) under hydroponic culture (A, D) and soil culture at fast growth
301 stage (FGS, B, E) or flower bud stage (FBS, C, F).
302 ns represent no significant difference was observed between treatments. The number in GVA (D, E, F)
303 represent the relative leaf biomass.
304 For hydroponic experiment, Stevia seedlings were growth in a greenhouse and supplied with nutrient
305 solutions of different N levels (0 mM N as N deficiency, 1 mM N as Low-N, 4 mM N as intermediate-N,
306 and 10 mM N as high-N). Plot experiments were conducted and supplied with different N levels (N0: no N
307 fertilization; N100: 100 kg N ha^{-1}; N200: 200 kg N ha^{-1}; N400: 400 kg N ha^{-1}).
308



309 Fig. 3 The linear relationship between leaf biomass and stevia glycosides concentration of of Stevia plants
310 supplied with different rates of N.
311 Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast
312 growth stage; Soil-FBS: soil cultured samples at flower-bud stage
313

3.4. Effect of N Administration Rates and Growth Stages on the Transcription Level of Genes Associated With SGs Synthesis

The expression of SG synthesis- related genes (*SrKAH*, *SrKO1*, *SrKS1* and *SrUGT85C2*) in Stevia leaves were also influenced by growth stage and N administration regimes. The transcription of *SrKAH*, *SrKO1* and *SrKS1* was down-regulated by N fertilization, regardless of the cultivation styles or growth stages. However, expression of *SrUGT85C2* was hardly altered by N administration rates. Some down-regulation in *SrUGT85C2* was exhibited under N deficiency conditions compared with that under N addition (Fig. 4D). The expression patterns of SG biosynthetic genes also changed with growth stage. *SrKAH1* and *SrKS1* were up-regulated at FBS compared to FGS, especially under N0 treatment. The expression of *SrUGT85C2* was significantly down-regulated at FGS.

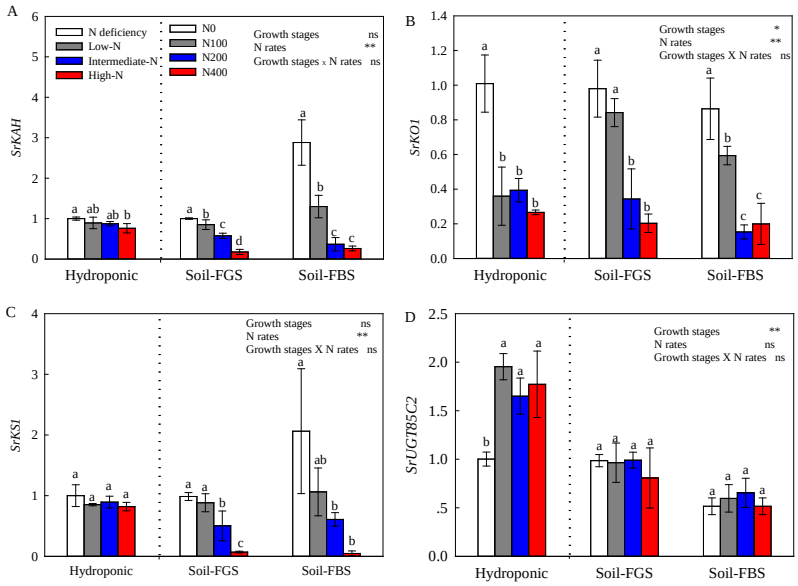


Fig. 4 Effect of N administration rates and growth stages on the relative gene expressions of *SrKAH* (A), *SrKO1* (B), *SrKS1* (C) and *SrUGT85C2* (D).

Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples at flower-bud stage

* and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively; ns means non-significant difference.

For hydroponic experiment, Stevia seedlings were growth in a greenhouse and supplied with nutrient

solutions of different N levels (0 mM N as N deficiency, 1 mM N as Low-N, 4 mM N as Intermediate-N, and 10 mM N as High-N). Plot experiments were conducted and supplied with different N levels (N0: no N fertilization; N100: 100 kg N ha^{-1}; N200: 200 kg N ha^{-1}; N400: 400 kg N ha^{-1}). Data represent means of four replicates and the bars indicate the SD. Significant differences ($P < 0.05$) between different N rates at the same growth stage are indicated by different letters.

3.5. Effect of N Administration Rates and Growth Stages on the C-N Status and C Metabolism in the Stevia Leaves

With the increased N administration rates, leaf total N concentrations were gradually higher, leading in the lowest C/N ratios under high N conditions (Fig. 5A, B, Table 1). The trend for increased N with increasing N administration rate was not as pronounced at FBS than at FGS. However, leaf total C content was strikingly higher at FBS compared with FGS. Leaf carbohydrates concentrations were also altered by N administration rates. Leaf soluble sugar contents were gradually decreased while starch contents were not significantly changed after the administration of N administration (Fig. 5, Table 1).

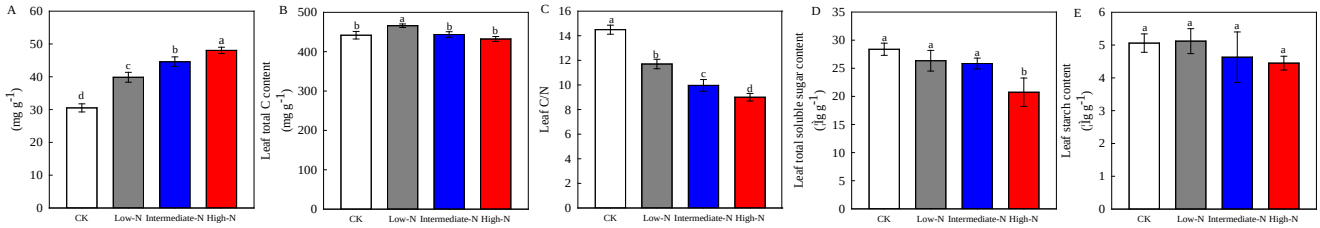


Fig. 5 Effect of different N concentrations on the total N content (A), total C content (B), C/N ratio (C), total soluble sugar content (D) and starch content (E) in Stevia leaves
Stevia seedlings were hydroponically growth in greenhouse and supplied with different N levels (0 mM N as N deficiency, 1 mM N as Low-N, 4 mM N as Intermediate-N, and 10 mM N as High-N). Data represent means of four replicates and the bars indicate the SD. Significant differences ($P < 0.05$) between treatments are indicated by different letters.

Interestingly, our GVA results showed that the reduced leaf soluble sugar concentration with N fertilization was arose through the dilution effect (Supplementary Fig. 3). Nevertheless,

Table 1 Effect of N fertilization and growth stages on the contents of total N, total C, total soluble sugar and starch as well as the ratio of C/N of Stevia leaves

Growth stages	Treatment	Total N (mg g^{-1})	Total C (mg g^{-1})	Total soluble sugar (μg g^{-1})	starch (μg g^{-1})	C/N
FGS	N0	17.11±1.00c	450.34±7.88a	29.22±1.21a	1.24±0.09a	26.36±1.17a
	N100	22.03±0.33b	437.91±5.30ab	26.39±1.75b	1.25±0.16a	19.88±0.34b
	N200	22.63±0.37b	437.69±10.83ab	26.25±0.46b	1.15±0.14a	19.34±0.27bc
	N400	23.29±0.38a	434.23±6.06b	24.17±1.76b	1.25±0.12a	18.16±0.25c
FBS	N0	14.26±0.71b	469.77±3.65b	26.61±1.62a	1.69±0.16a	33.00±1.63a
	N100	19.93±0.92a	472.59±5.6b	25.76±0.65ab	1.50±0.10a	23.75±1.21b
	N200	20.77±1.56a	477.74±4.64b	22.72±1.13bc	1.51±0.11a	23.07±1.48b
	N400	21.71±1.39a	486.04 ±3.06a	23.89±1.06c	1.23±0.16b	22.45±1.53b
Growth stages		34.39**	200.00**	11.212**	22.744**	100.47**
N rates		67.78**	0.805 ns	11.207**	2.872 ns	87.79**
Growth periods× N rates		0.308 ns	6.775**	2.204 ns	3.596*	2.16 ns

For each growth stage, data are mean ± SD of four replications. Different letters in the same column indicate a significant difference ($P < 0.05$, Duncan's multiple range test). N0: 0 kg N ha^{-1}; N100: 100 kg N ha^{-1}; N200: 200 kg N ha^{-1}; N400: 400 kg N ha^{-1}.

FGS: fast growth stage; FBS: flower-bud stage.

* and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively; ns means non-significant difference

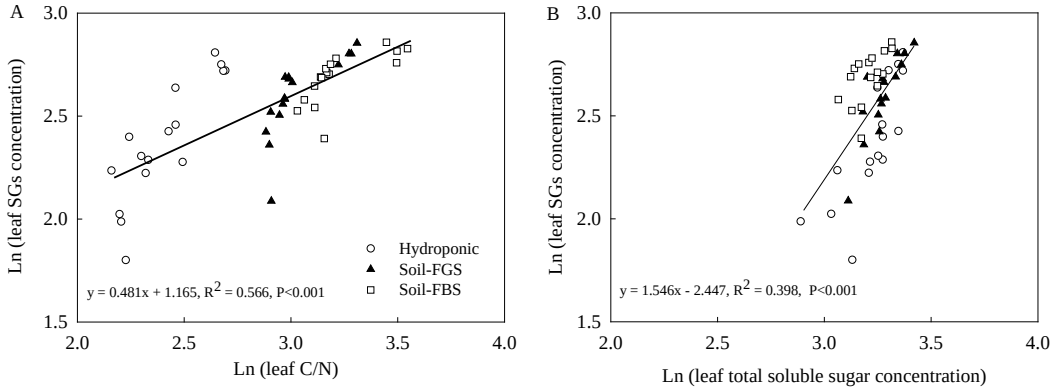


Fig. 6 The relationship between leaf SGs content and leaf C/N ratio (A) and leaf total soluble sugar content (B). The lines represent linear regressions with equation in the diagram. Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples at flower-bud stage

further correlation analysis revealed significant positive correlation between leaf SGs concentration and C/N ratio or total soluble sugar concentration under different N administration regimes (Fig. 6).

Additionally, the activities of key enzymes; some related to the C metabolism, were assessed with different N treatments (Fig. 7A). The PEPase, SPS and PAL activities were all inhibited by the N fertilization, regardless of growth stage or cultivation method. Similarly, the transcription levels of *SrPEPC*, *SrSPS* and *SrPAL* were also negative regulated by N administration (Fig. 7B).

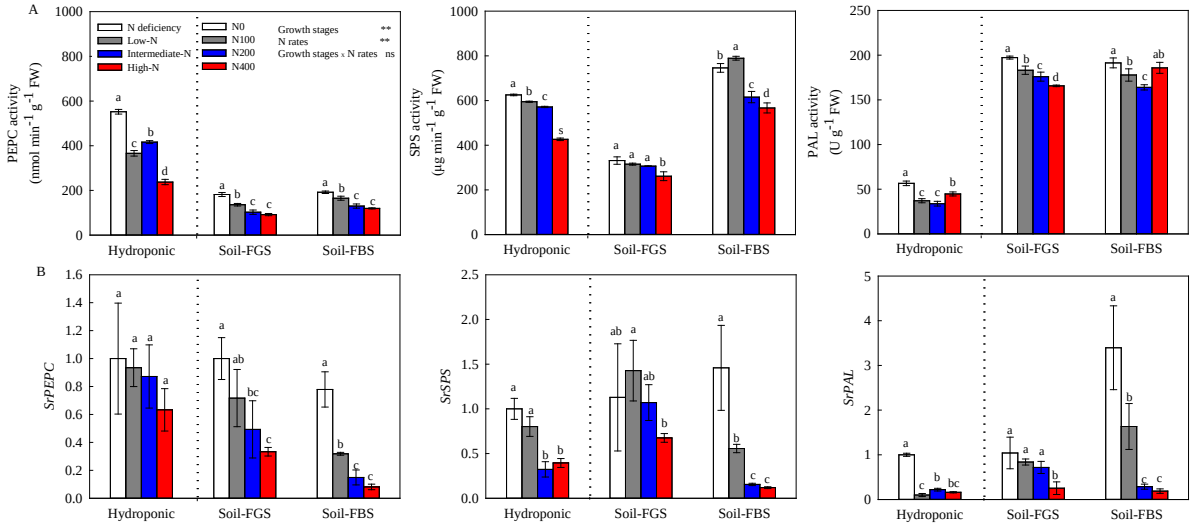


Fig. 7 Effect of N administration rates and growth stages on the activities of PEPCase, SPS and PAL (A) and the relative gene expression of *SrPEPC*, *SrSPS* and *SrPAL* (B). FGS: fast growth stage; FBS: flower-bud stage.

Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples at flower-bud stage

* and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively; ns means non-significant difference. Plot experiments were conducted and supplied with different N levels (N0: no N fertilization; N100: 100 kg N ha^{-1}; N200: 200 kg N ha^{-1}; N400: 400 kg N ha^{-1}). Data represent means of four replicates and the bars indicate the SD. Significant differences ($P < 0.05$) between different N rates at the same growth stage are indicated by different letters.

4. Discussion

With the growing economic importance of SGs, the relationship between their production and factors such like N administration needs to be established. Previous studies have documented the influence of N fertilization on SGs synthesis in stevia leaves but it is difficult to draw a causal conclusion due to the diverse experimental methods and environmental cultivation conditions (Barbet-Massin et al. 2015; Tavarini et al. 2015a). In this study, the relationship between N rates and the concentration and contents of SGs in Stevia plants was investigated by both hydroponic and plot experiments. We found that N administration reduced the SGs concentrations in stevia leaves and reflected reduced the transcription of genes associated with SGs synthesis (Fig. 1, Fig. 4). This was not reflected at a whole-plant level as total biomass was increased (Fig. 2). Crucially, this reduction appeared to reflect a wider reprogramming in metabolism as patterns of SGs accumulation were reflected in leaf C/N ratios as well as soluble sugar concentrations.

Studying of the relationship between plant nutrition and secondary metabolism is crucial, especially in crop species. The positive effects of N on alkaloids production and the opposing effects on phenolic compounds (lignin, flavonoids, etc.), are well-established but this is not the case with terpenoids including SGs. Our results showed that elevated leaf N levels correlated with a decrease in the concentration of SGs. Crucially, the total SGs accumulation was not significantly changed (Fig. 1, Fig. 2). As N increased biomass, whilst the total SG yield was not affected; at a leaf level there was a “dilution effect” in N-SGs relationship. This was validated in our result through GVA or correlation analysis (Fig. 2 and 3). Others have noted balances between plant biomass formation and secondary metabolites synthesis. Thus, trade-

offs between plant growth and secondary metabolism has been demonstrated in the phenolic compounds of birch leaf (Riipi et al. 2002), flavones contents in *Pentaclethra macroloba* (Massad et al. 2012) and phenylpropanoid concentrations in willows (Glynn et al. 2007).

Additionally, the growth differentiation balance hypothesis (GDBH) has been proposed to explain the responses of secondary metabolites to environmental variations (Koricheva 1998). GDBH suggested that plant growth inhibited by environmental stresses would allocate more resources to secondary metabolism in order to increase plant defence. Therefore an increase of defensive compounds (phenolic and other compounds) is accompanied by the decrease of plant growth under stresses (Kirakosyan et al. 2004; Ramakrishna and Ravishankar 2011). Similarly, the negative relationship between biomass formation and SGs concentration has also been reported in drought and salty stressed Stevia plants (Zeng et al. 2013). Such is not in contradiction to a dilution effect with increased biomass but implies the involvement of active regulatory processes. This is also the implication of (e.g.) our observed reduced SG biosynthetic gene expression. Taken together, we propose that elevated N diverts metabolism towards plant growth, including the synthesis of cytokinins and auxins (Mittelstraß et al. 2006), but against other pathways secondary metabolite including SGs through transcriptional changes.

Some common features were also investigated to reveal the regulation mechanism related to SGs synthesis. Our results exhibited the consistent changes of SGs concentrations and C/N ratio or soluble sugar concentration in Stevia leaves (Fig. 6). The function of C/N ratio in the regulation of C-based secondary metabolites is central to the carbon-nutrient balance hypothesis (CNBH), which indicates C allocations to defence compounds (Royer et al. 2013).

Although the applicability of this hypothesis has been questioned and proved to be defective (Koricheva 2002), a positive correlation between leaf C/N ratio and SGs concentration (Fig. 6 A) was also observed in our current study. This relationship was not affected by factors such as cultivation styles and growth period, suggesting a causal relationship. In another study, Ibrahim and Jaafar (2011) established the relationship between C/N ratio and total phenolics or flavonoids contents in *Labisia pumila* (Blume) exposed to different N fertilization rates. Furthermore, the increased leaf C/N ratio with higher environmental CO_2 would also contribute to accelerated secondary metabolism (Ghasemzadeh and Jaafar 2011).

The enhancement of secondary metabolic pathways is inseparable from the improved production of non-structural carbohydrates. Sugars are located at the center of plant C metabolism and integrate C assimilation and distribution (Zakhartsev et al. 2016). In another study, Osuna et al. (2007) demonstrated that sucrose supply would alter the expression levels of genes involved in carbohydrate synthesis, glycolysis, and respiration. The correlation between sugar and secondary metabolism has been reported under various conditions, such as growth stages (Shi et al. 2014), abiotic stresses (Interdonato et al. 2011), CO_2 concentration (Ghasemzadeh and Jaafar 2011), and N fertilizations (Ibrahim et al. 2010; Osakabe et al. 2013). Addition of sucrose improves the contents of secondary metabolites, such as anthraquinone, phenolics and flavonoids in *Morinda citrifolia* (Baque et al. 2011) and tanshinone in *Salvia miltiorrhiza* Bunge (Wang et al. 2012). Treatment with sucrose induced the expression of transcription factors that associated with phenylpropanoid metabolism in potato and *Arabidopsis* (Osuna et al. 2007; Payyavula et al. 2013). Interestingly, exogenous feeding of sucrose significantly up-regulated the expression levels of genes associated with SGs synthesis

(Ghorbani et al. 2017). Moreover, the bio-synthesis of SGs is based on glycolysis products and pyruvate as substrates (Vranová et al. 2013). This represents a good indication of tightly association between sugar metabolism and SGs synthesis, as exhibited in our study (Fig. 6B).

Osakabe et al. (2013) reported the synchronous decreased soluble sugar and phenolic substances contents in Stevia leaves after N administration. N deficiency shifted plant metabolic profiles to down- regulate nitrate reduction and amino acid assimilation but increases the ratio of carbohydrates into the cell wall and secondary metabolites (Schluter et al. 2012). We noted that the reduced available sugar content under high N conditions affected secondary metabolites including SGs. In line with this, the synchronous decreased soluble sugar content and secondary metabolites under high N conditions were also documented in *Labisia Pumila* Benth (Ibrahim et al. 2010) and *Chrysanthemum Morifolium* Ramat (Liu et al. 2010). We defined a key regulatory note that could regulate the relative flux through different N or C routes. A most important enzyme during glycolysis, PEPCase play key roles in reducing C skeletons to the TCA cycle and thereafter allocate more C to the gluconeogenesis or shikimate pathway (Gibon et al. 2009; Guo et al. 2018). The enzyme activities of PEPCase together with the expression level of *PEPC* (Fig. 7) were inhibited by N fertilization, as also reported by Ding et al. (2005). Interestingly, PAL, the enzyme involved in phenylpropanoid metabolism and subsequent phenolic synthesis (Kováčik 2007; MacDonald and D'Cunha 2007) was also inhibited by N administration (Fig. 5). N depletion resulted in remarkably increased contents of PAL-catalytic products (Olsen et al. 2008). Similarly, in Stevia plants, the negative regulation on secondary metabolites by N has also been reported on flavonoids (Tavarini et al. 2015b). All these findings suggested that the response of primary C metabolism to N

administration play critical intermediate roles in the N-SGs relationship. A mechanistic study of how N, C metabolism impact on SG biosynthetic mechanism is clearly merited.

6. Conclusion

Our study showed that when N supply is sufficient, plants would prefer growth than C-related secondary metabolism, the negative correlation between N and SGs concentrations was inseparable from the dilution effect caused by Stevia growth. Furthermore, the reduction of C/N ratio and soluble sugar concentration caused by N administration also contributed greatly to the changes of SGs.

Contribution

Yuming Sun, Menglan Hou and Suzhen Huang designed and performed the experiment; Yongheng Yang and Ting Zhang analyzed the content of steviol glycosides in stevia plants; Yuming Sun and Xiaoyang Xu analyzed the data; Yuming Sun and Haiying Tong wrote the paper. Luis A. J. Mur improved the language and English writing of the entire manuscript. All authors gave final approval for its publication.

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Conflict of interest

The authors declare that they have no conflict of interest to this work.

Supplementary Materials

Supplementary Table 1 Primers Used for qRT-PCRs in this Study

Supplementary Table 2 Effect of different N rates on the growth parameters of Stevia (*Stevia rebaudiana* Bertoni) seedlings grown under hydroponic conditions

Supplementary Table 3 Effect of N fertilization rates and growth periods on the growth parameters of Stevia (*Stevia rebaudiana* Bertoni) plants

Supplementary Fig. 1 Interpretation of the graphical vector analysis (GVA) results.

Supplementary Fig. 2 Effect of N administration rates and growth stages on the stem concentrations of rebaudioside A (Reb A, A), stevioside (STV, B), rebaudioside F (Reb F, C),

515 rebaudioside C (Reb C, D), dulcoside A (DA, E) and total stevia glycosides (SGs).

516 **Supplementary Fig. 3** Graphical vector analysis (GVA) of leaf soluble sugar of Stevia plants
517 supplied with different rates of N under hydroponic culture (A) and soil culture at fast growth
518 stage (FGS, B) or flower bud stage (FBS, C).

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