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1 **Nitrogen drives plant growth to the detriment of leaf sugar and steviol glycoside**

2 **metabolisms in stevia (*Stevia rebaudiana* Bertoni)**

3

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20 **Abstract**

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

38 **Keywords**

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

40 **Abbreviations**

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

48 **1. Introduction**

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

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

70 Galili 2011; Ibrahim et al. 2011; Masclaux-Daubresse et al. 2010). This reflects a difference
71 between the N-containing alkaloid and carbon (C)-containing of phenolic substances.
72 Structurally, SGs is tetracyclic diterpenoid belonging to C- containing substance class but the
73 relationship between N and terpenoids is more complicated than that of phenolic compounds.
74 A metanalysis of woody plants confirmed the negative effect of N fertilization on phenolic
75 synthesis but there were no significant impact on terpenoids compounds (Koricheva 1998). In
76 Stevia, N fertilization had been shown to increase biomass (Ruan et al. 2010; Tavarini et al.
77 2015a) but the influence on SGs synthesis was unclear. For example, Tavarini et al. (2015a)
78 reported that the leaf concentrations of stevioside (STV) and rebaudioside A (Reb A) were
79 increased after N administration. Similarly, Pal et al. (2015) showed a positive effect of N
80 fertilization on leaf SG concentrations. However, opposite N effects were seen under different
81 growth stages or conditions (Barbet-Massin et al. 2015; Kafle et al. 2017).

82 Regulatory insights are now aided by elucidation of SG biosynthetic pathway. SGs
83 originate from the glycolysis products, pyruvate and glyceraldehyde 3-phosphate, in the
84 chloroplast. These enter either the 2-cmethyl-D-erythritol 4-phosphate (MEP) or mevalonate
85 (MVA) pathway and the generated isopentenyl diphosphate (IPP) and dimethylallyl
86 diphosphate (DMAPP) are converted to geranylgeranyl diphosphate (GGDP). The resulting
87 ent-kauriene is then transported to the endoplasmic reticulum, where the SGs are synthesized
88 through the action of kaurene synthase (KS), kaurene oxidase (KO), ent-kaurenoic acid 13-
89 hydroxylase (KAH) and UDP-dependent glycosyltransferases (UGTs) (Kim et al. 2018; Wang
90 et al. 2016; Yadav and Guleria 2012). Therefore, SG biosynthesis is similar to other terpenoids
91 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.

102 **2. Materials and Methods**

103 *2.1 Plant Materials and Growth Conditions*

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

107 *Hydroponic Experiment*

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

116 The composition of full-strength nutrient solution was as follows: Macronutrients: 4 mM
117 N as $(NH_4)_2SO_4$ and $Ca(NO_3)_2$; 1 mM phosphorus (P) as KH_2PO_4, 6 mM potassium (K) as
118 K_2SO_4 and KH_2PO_4 and 2 mM magnesium (Mg) as $MgSO_4$. Micronutrients: 35 μM iron (Fe)
119 as Fe–EDTA, 10 μM manganese (Mn) as $MnCl_2 \cdot 4H_2O$, 0.5 μM molybdenum (Mo) as
120 $(NH4)_6Mo_7O_{24} \cdot 4H_2O$, 60 μM boron (B) as H_3BO_3, 1 μM zinc (Zn) as $ZnSO_4 \cdot 7H_2O$ and 0.4
121 μM copper (Cu) as $CuSO_4 \cdot 5H_2O$.

122 The nutrient solutions were changed at 3-day intervals and the pH was adjusted daily to
123 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

124 at 30/25 °C (day/night) with relative humidity of 70 ± 10% and photoperiod of 14 h d^{-1} (> 300

125 μmol m^{-2} s^{-1}). The Stevia seedlings were harvested after 2 weeks of treatment.

126 *Plot Experiment*

127 The plot experiment was conducted at Institute of Botany, Jiangsu Province and Chinese

128 Academy of Sciences (Jiangsu, China, latitude 32°03' N, longitude 118°49' E). The cut

129 seedlings were transplanted on June 6, 2018. The field was divided into 16 plots with equal

130 area (2.5 m^2). The four treatments at different N rates were arranged in a randomized block

131 design with 4 replicates for each treatment. Stevia plants received 0 (N0), 100 (N100), 200

132 (N200), or 400 (N400) kg N ha^{-1} in the form of urea which were administered at different

133 growth stages: June 6 (transplanting, 50%), July 5 (fast growing stage, 30%) and August 5 (late

134 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

135 ha^{-1} (as KCl) were administered as the base fertilizer. The Stevia plants were harvested at fast

136 growth stage (July 14) and flower-bud stage (September 10).

137 The properties of the soil at 0-30 cm were measured and found to be: pH 6.72 (soil: H_2O=

138 1: 2.5), organic matter 27.72 mg g^{-1}, total N 1.528 mg g^{-1}, alkali-hydrolyzable N 105.075 mg

139 kg^{-1}, Olsen-P 63.97 mg kg^{-1}, and NH_4OAc-K 347.72 mg kg^{-1}.

140

141 *2.2 Sampling and Processing*

142 Stevia seedlings were washed briefly with distilled water. The leaves, stems and roots were

143 separated by hand and then the leaves were photographed and the leaf areas were determined

144 using Image J. The plant parts were baked in an oven at 105 °C for 30 min and subsequently at

145 70 °C to constant weight after which the dry weights of all seedlings parts were determined.

146 The dry samples of leave, stem and root of single plant were separately grinded and mixed
147 before storage, to avoid the influence of leaf positions (Ceunen and Geuns 2013b). Similarly,
148 fresh leaf samples were also evenly mixed and ground in liquid nitrogen before stored in -80°C
149 refrigerator.
150

151 *2.3 Extraction of Steviol Glycosides (SGs) and HPLC Analysis*

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

158 HPLC analyses were performed using a Sapphire C18 sorbent column (4.6×250 mm). The
159 temperature was set at 25 °C and the samples (10 μL) were eluted with acetonitrile: sodium
160 phosphate buffer (32:68) over a period of 20 min (Yang et al. 2015). The SGs which were
161 analyzed included Reb A, STV, Rebaudioside F and C (1Reb F and C) and dulcoside A (DA)
162 with the retention times of 7.415 min, 7.923 min, 9.415 min, 10.198 min and 11.040 min,
163 respectively. SGs were detected by monitoring the UV at A210 nm with a mobile phase flow
164 rate of 1 mL min^{-1}. The samples were quantified against standard curves of Reb A, STV, Reb
165 F, Reb C and DA (99.99% pure, Chroma Dex, USA).
166

167 *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

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

197

198 *2.7. Enzyme Activities.*

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

208 The determination of sucrose phosphate synthase (SPS, E.C. 2.4.1.14) activity was
209 according to Hu et al. (2016). Samples of 100 μL enzyme solutions were mixed with the 450
210 μL reaction buffers (50 mM of extraction buffer, 10 mM of $MgCl_2$, 50 mM of UDP-glucose
211 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.

237 **3. Results**

238 *3.1. Effects of N Administration Rates on Stevia Plants Growth under both Hydroponic and*

239 *Field Conditions*

240 Both plant growth and development were affected by N nutrition. N addition significantly

241 enhanced Stevia growth and biomass formation under hydroponic cultures (Supplementary

242 Table 2). Compared to N deficient conditions, plant heights were 33.4%-80.9% higher, leaf

243 areas were 46.8%-95.8% larger and above ground biomass was increased by 31.6%-89.5%

244 with N nutrients. The highest leaf and stem dry weight were observed with high-N treatment.

245 The responses of plant growth to growth stages and N rates were tested in plot experiments.

246 Growth stages and N fertilization regimes both induced significant increases in plant height,

247 total leaf area and aboveground biomass formation (Supplementary Table 3). The highest

248 Stevia biomass was seen with N400 at the flower-bud stage (FBS).

249

250 *3.2 Effects of N Administration Rates on the SGs content under both Hydroponic and Soil*

251 *Cultures*

252 The concentrations of steviol glycosides (SGs) were also altered by N levels. As shown in

253 Fig. 1, the Reb A, STV, Reb F, Reb C and DA concentrations in Stevia leaves were all at their

254 highest levels at the lowest N. Compared with N deficiency, low-N, intermediate- N and high-

255 N treatments reduced total SGs contents by 25.4%, 35.9% and 51.7%, respectively. SGs

256 concentrations in stem tissues of Stevia plants were significantly lower than that in leaves but

257 exhibited no significant between different N treatments (Supplementary Fig. 2).

258

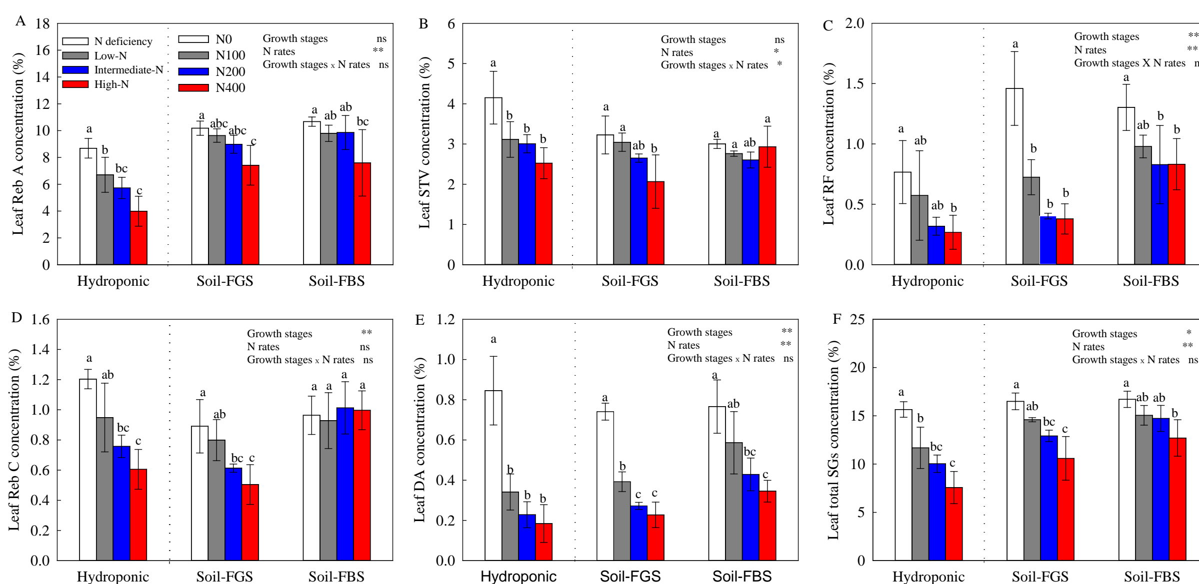


Fig. 1 Effect of N administration rates and growth stages on the leaf concentrations of rebaudioside A (Reb A, A), stevioside (STV, B), rebaudioside F (Reb F, C), rebaudioside C (Reb C, D), dulcoside A (DA, E) and total stevia glycosides (SGs).
FGS: fast growth stage; FBS: flower-bud stage.
Hydroponic: 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).

279 No significant changes in leaf SGs status were observed at different growth stages. However,
280 the SGs concentrations in the stems were significantly higher at FBS than that at FGS
281 (Supplementary Fig. 2).
282
283 *3.3. Effects of N Administration Rates on the SGs accumulations under both Hydroponic and*
284 *Soil Experiments*
285 The accumulation of SGs in Stevia plants under different growth conditions or treatments were
286 calculated. As shown in Fig. 2A, B and C, SGs production was most prominent in the leaf and
287 was higher than stems regardless of growth stage or N rate. Although SGs accumulation was
288 increase with the growth stages, no significant differences were observed with different N
289 fertilization rates. Given these results, we performed graphical vector analysis (GVA) to
290 understand the contribution of growth effects in the relationship between N administration rates
291 and leaf SG concentrations. GVA results suggested that reduced total SGs concentrations with
292 N administration was a result of reduced synthesis under hydroponic culture (Fig. 2D).
293 However, when grown in soil, the growth associated reduction (dilution effect) occurred at
294 FGS and FBS (Fig. 2E, F). A negative correlation between leaf biomass formation and SGs
295 concentration was observed under various conditions, which again indicated that N increased
296 growth "diluted" SGs levels (Fig. 3).
297
298

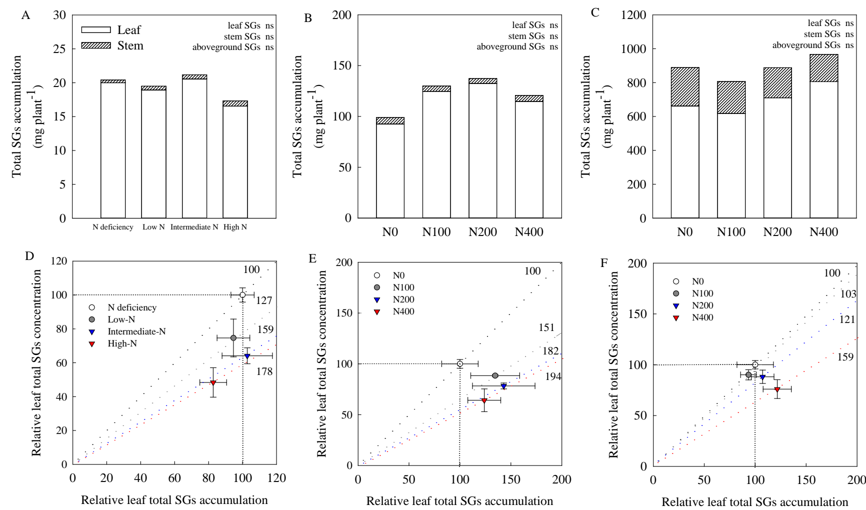


Fig. 2 The accumulation of total stevia glycosides (SGs) in Stevia plants (A, B, C) and graphical vector analysis (GVA) of leaf SGs contents (D, E, F) under hydroponic culture (A, D) and soil culture at fast growth stage (FGS, B, E) or flower bud stage (FBS, C, F).

ns represent no significant difference was observed between treatments. The number in GVA (D, E, F) represent the relative leaf biomass.

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}).

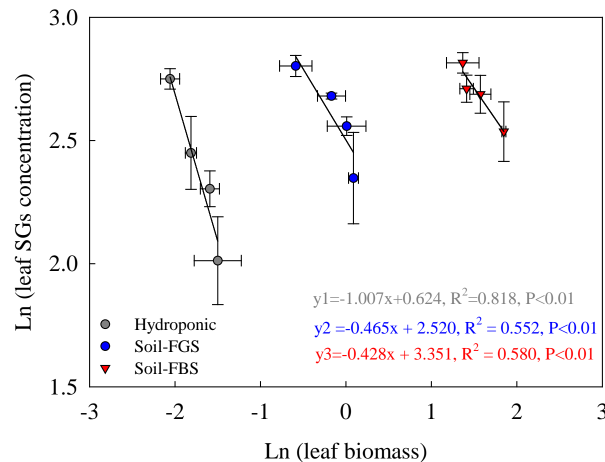


Fig. 3 The linear relationship between leaf biomass and stevia glycosides concentration of of Stevia plants supplied with different rates of N.

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

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.

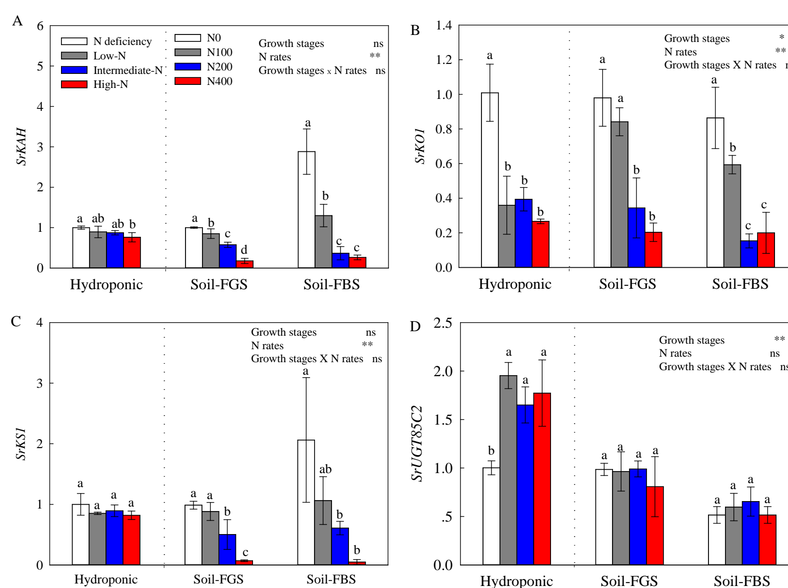


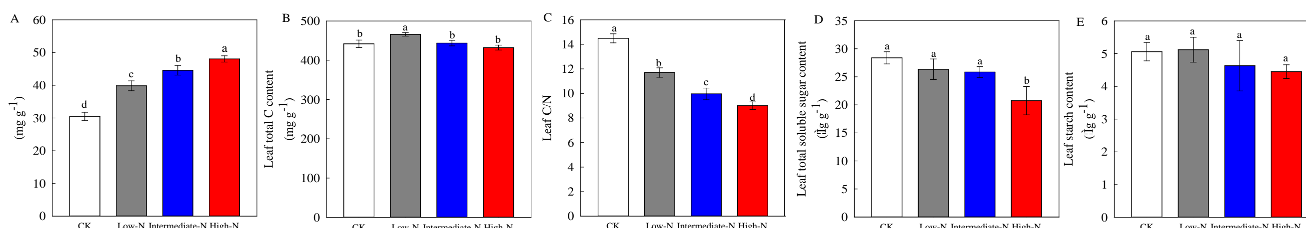
Fig. 4 Effect of N administration rates and growth stages on the relative gene expressions of *SrKAH* (A), *SrKO1* (B), *SrKS1* (C) and *SrUGT85C2* (D).
Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples 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

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

337

338 *3.5. Effect of N Administration Rates and Growth Stages on the C-N Status and C Metabolism*
339 *in the Stevia Leaves*

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



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

353

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

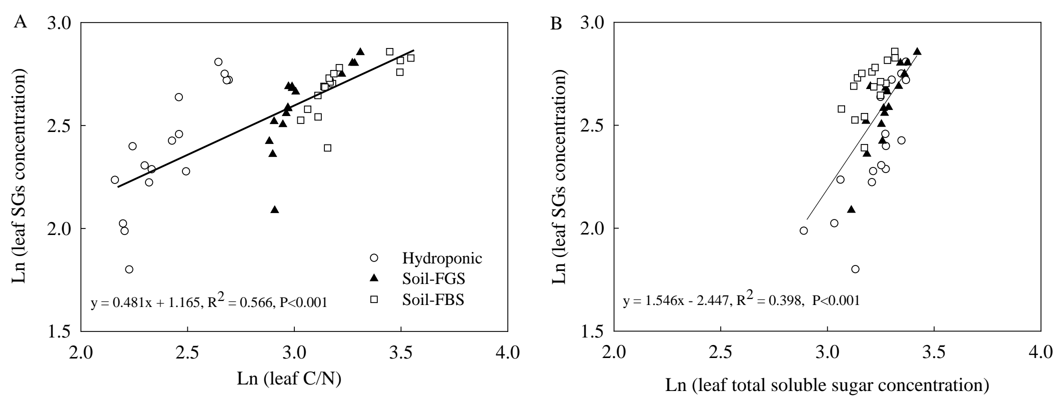
356

357 Table 1 Effect of N fertilization and growth stages on the contents of total N, total C, total
358 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

359

360 For each growth stage, data are mean ± SD of four replications. Different letters in the same column indicate
361 a significant difference (P < 0.05, Duncan's multiple range test). N0: 0 kg N ha^{-1}; N100: 100 kg N ha^{-1}; N200:
362 200 kg N ha^{-1}; N400: 400 kg N ha^{-1}.
363 FGS: fast growth stage; FBS: flower-bud stage.
364 * and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively; ns means non-
365 significant difference
366



367 **Fig. 6** The relationship between leaf SGs content and leaf C/N ratio (A) and leaf total soluble sugar content
368 (B). The lines represent linear regressions with equation in the diagram. Hydroponic: experiments conducted
369 under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured
370 samples at flower-bud stage
371

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

375 Additionally, the activities of key enzymes; some related to the C metabolism, were
376 assessed with different N treatments (Fig. 7A). The PEPase, SPS and PAL activities were all
377 inhibited by the N fertilization, regardless of growth stage or cultivation method. Similarly, the
378 transcription levels of *SrPEPC*, *SrSPS* and *SrPAL* were also negative regulated by N
379 administration (Fig. 7B).
380

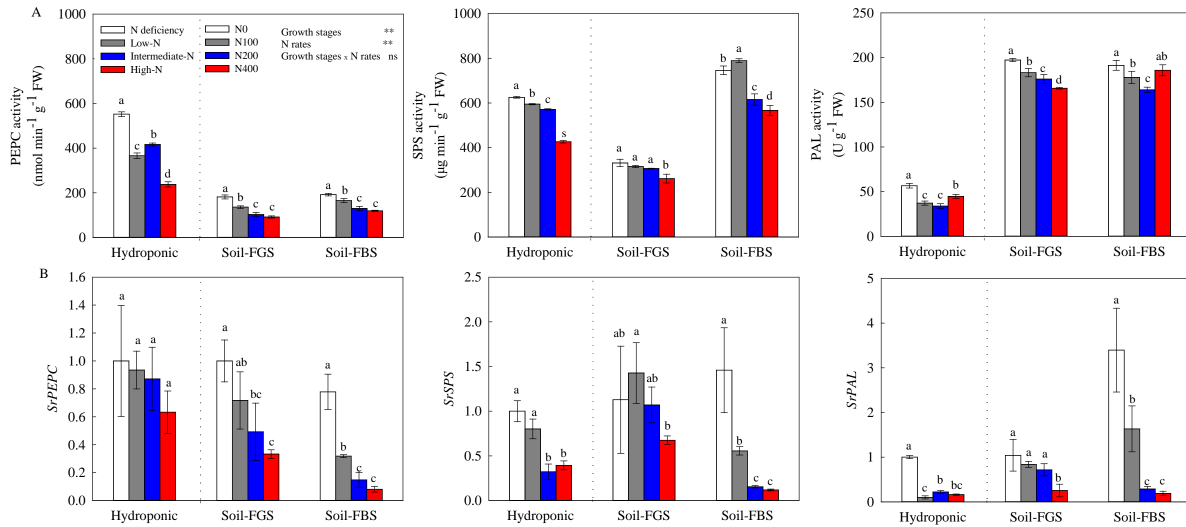


Fig. 7 Effect of N administration rates and growth stages on the activities of PEPCase, SPS and PAL (A) and the relative gene expression of *SrPEPC*, *SrSPS* and *SrPAL* (B). FGS: fast growth stage; FBS: flower-bud stage.

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.

393 **4. Discussion**

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

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

415 offs between plant growth and secondary metabolism has been demonstrated in the phenolic
416 compounds of birch leaf (Riipi et al. 2002), flavones contents in *Pentaclethra macroloba*
417 (Massad et al. 2012) and phenylpropanoid concentrations in willows (Glynn et al. 2007).
418 Additionally, the growth differentiation balance hypothesis (GDBH) has been proposed to
419 explain the responses of secondary metabolites to environmental variations (Koricheva 1998).
420 GDBH suggested that plant growth inhibited by environmental stresses would allocate more
421 resources to secondary metabolism in order to increase plant defence. Therefore an increase of
422 defensive compounds (phenolic and other compounds) is accompanied by the decrease of plant
423 growth under stresses (Kirakosyan et al. 2004; Ramakrishna and Ravishankar 2011). Similarly,
424 the negative relationship between biomass formation and SGs concentration has also been
425 reported in drought and salty stressed Stevia plants (Zeng et al. 2013). Such is not in
426 contradiction to a dilution effect with increased biomass but implies the involvement of active
427 regulatory processes. This is also the implication of (e.g.) our observed reduced SG
428 biosynthetic gene expression. Taken together, we propose that elevated N diverts metabolism
429 towards plant growth, including the synthesis of cytokinins and auxins (Mittelstraß et al. 2006),
430 but against other pathways secondary metabolite including SGs through transcriptional
431 changes.
432 Some common features were also investigated to reveal the regulation mechanism related
433 to SGs synthesis. Our results exhibited the consistent changes of SGs concentrations and C/N
434 ratio or soluble sugar concentration in Stevia leaves (Fig. 6). The function of C/N ratio in the
435 regulation of C-based secondary metabolites is central to the carbon-nutrient balance
436 hypothesis (CNBH), which indicates C allocations to defence compounds (Royer et al. 2013).

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

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

459 (Ghorbani et al. 2017). Moreover, the bio-synthesis of SGs is based on glycolysis products and
460 pyruvate as substrates (Vranová et al. 2013). This represents a good indication of tightly
461 association between sugar metabolism and SGs synthesis, as exhibited in our study (Fig. 6B).
462 Osakabe et al. (2013) reported the synchronous decreased soluble sugar and phenolic
463 substances contents in Stevia leaves after N administration. N deficiency shifted plant
464 metabolic profiles to down- regulate nitrate reduction and amino acid assimilation but increases
465 the ratio of carbohydrates into the cell wall and secondary metabolites (Schluter et al. 2012).
466 We noted that the reduced available sugar content under high N conditions affected secondary
467 metabolites including SGs. In line with this, the synchronous decreased soluble sugar content
468 and secondary metabolites under high N conditions were also documented in *Labisia Pumila*
469 Benth (Ibrahim et al. 2010) and *Chrysanthemum Morifolium* Ramat (Liu et al. 2010). We
470 defined a key regulatory note that could regulate the relative flux through different N or C
471 routes. A most important enzyme during glycolysis, PEPCase play key roles in reducing C
472 skeletons to the TCA cycle and thereafter allocate more C to the gluconeogenesis or shikimate
473 pathway (Gibon et al. 2009; Guo et al. 2018). The enzyme activities of PEPCase together with
474 the expression level of *PEPC* (Fig. 7) were inhibited by N fertilization, as also reported by
475 Ding et al. (2005). Interestingly, PAL, the enzyme involved in phenylpropanoid metabolism
476 and subsequent phenolic synthesis (Kováčik 2007; MacDonald and D'Cunha 2007) was also
477 inhibited by N administration (Fig. 5). N depletion resulted in remarkably increased contents
478 of PAL-catalytic products (Olsen et al. 2008). Similarly, in Stevia plants, the negative
479 regulation on secondary metabolites by N has also been reported on flavonoids (Tavarini et al.
480 2015b). All these findings suggested that the response of primary C metabolism to N

481 administration play critical intermediate roles in the N-SGs relationship. A mechanistic study

482 of how N, C metabolism impact on SG biosynthetic mechanism is clearly merited.

483

484

485 **6. Conclusion**

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

491 **Contribution**

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

497

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501 Resources (JSPKLB201810).

502

503 **Conflict of interest**

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

505

506 **Supplementary Materials**

507 **Supplementary Table 1** Primers Used for qRT-PCRs in this Study
508 **Supplementary Table 2** Effect of different N rates on the growth parameters of Stevia (*Stevia*
509 *rebaudiana* Bertoni) seedlings grown under hydroponic conditions
510 **Supplementary Table 3** Effect of N fertilization rates and growth periods on the growth
511 parameters of Stevia (*Stevia rebaudiana* Bertoni) plants
512 **Supplementary Fig. 1** Interpretation of the graphical vector analysis (GVA) results.
513 **Supplementary Fig. 2** Effect of N administration rates and growth stages on the stem
514 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).

519

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