Research article

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

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ABSTRACT

Steviol glycosides (SGs) in Stevia (*Stevia rebaudiana* Bertoni) leaves are 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, we investigate the effects of N rates on SGs production through hydroponic and plot experiments. The SGs yield was not significantly changed by N fertilization, but leaf SGs concentrations were significantly reduced due to the "dilution effect". Additionally, N addition decreased leaf carbon (C)/N ratio and soluble sugar concentration, accompanied with the inhibited phosphoenolpyruvate carboxylase and L-phenylalanine ammonia_lyase activities. A significant positive correlation between leaf SGs concentrations, C/N ratio and soluble sugar concentration was observed. Overall, we suggest that N-driven Stevia growth negatively affects SGs concentrations. The leaf C/N ratio and soluble sugar changes indicated the occurrence of metabolic reprogramming.

1. Introduction

As a perennial herb of Asteraceae family, Stevia (*Stevia rebaudiana* Bertoni) is known as a healthcare "source of sugar", which is superior to cane sugar, beet sugar or other intensive sweeteners. The sweetness 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 $\sim 1/300$ 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 many countries and regions (Additives and Food, 2010; Yadav and Guleria,

2012). Most recent studies have focused on the breeding of new Stevia varieties, improving SGs extraction technologies from leaves or investigating the SGs 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] management) plays an essential role in the regulation of plant growth and 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 phenolic substances. Structurally, SGs are tetracyclic

diterpenoids 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 was no significant impact on terpenoid compounds (Koricheva et al., 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 is 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 SGs 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 SGs biosynthetic pathway. SGs originate from the glycolysis products, pyruvate and glyceraldehyde 3-phosphate, in the chloroplast. These enter the 2-methyl-D-erythritol 4-phosphate (MEP) pathway and the generated isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are converted to geranylgeranyl diphosphate (GGDP). The resulting *ent*-kaurene is then transported to the endoplasmic reticulum, where the SGs are synthesized through the action of *ent*-kaurene synthase (KS), *ent*-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, SGs biosynthesis is similar to other terpenoids or phenolic compounds that derived from the hexose or phosphoenolpyruvate (PEP) (Kallscheuer, 2018). Such commonality could link SGs synthesis to leaf C metabolism but this relationship has only been tentatively suggested by Barbet-Massin et al. (2015). Further, it is still unclear how N could influence this C metabolism linked SGs synthesis.

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

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'.

2.1.1. 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 nutrient solution was prepared by adding various nutrients to tap-water and 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 emerged. The seedlings were treated with different levels of N (N deficiency: 0.6 mM (N concentration in tap water); Low-N: 1.6 mM, Intermediate-N: 4.6 mM and High-N (HN): 10.6 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 $MnCl_2·4H_2O$, 0.5 μM molybdenum (Mo) as $(NH_4)_6Mo_7O_{24}·4H_2O$, 60 μM boron (B) as H_3BO_3, 1 μM zinc (Zn) as $ZnSO_4·7H_2O$ and 0.4 μM copper (Cu) as $CuSO_4·5H_2O$.

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.

2.1.2. 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 seedling 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 leaf, stem and root of a single plant were separately ground 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 freezer.

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 were 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

The leaf total N content was measured following the H_2SO_4–H_2O_2

digestion method of Kjeldahl (Nelson and Sommers, 1972) while the leaf total C content was measured following the wet-combustion method (Hafsi et al., 2003).

2.5. Measurement of leaf total soluble sugar and starch

Leaf total soluble sugar content was determined according to Zhang et al. (2013), with modifications. A total of 0.05 g of dry leaf powder and 10 mL of deionized water were mixed and boiled for 30 min at 100 °C and centrifuged at 10,000 g for 10 min. The supernatant was transferred to a new glass tube. The supernatant was mixed with anthrone in ethyl acetate (2 g 100 mL^{-1}) and sulphuric acid and the absorbance was determined at 630 nm after boiling for 1 min.

The starch was extracted and measured from the residue, following the method of Fernandes et al. (2012). After the gelatinization of the residues with 2 mL distilled water in boiling water, 2 mL 9.2 N and 4.6 N pre-cooled chloric acid were added to decompose the starch into glucose, which could be further reacted with anthrone and sulphuric acid and detected following the method used for soluble sugar. Starch was calculated by multiplying the soluble sugar content by a factor of 0.9 (Rovalino-Córdova et al., 2018).

2.6. RNA isolation and quantitative real time PCR (RT-qPCR)

Fresh leaf samples were harvested and immediately frozen in liquid nitrogen, and then stored at −70 °C until RNA isolation. The total RNA was extracted with TRIzol reagent (Invitrogen, 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 mortar 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 supernatant 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 added to terminate the reaction before heating at 100 °C for 10 min. After cooling, 2.1 mL of 30% HCl and 0.6 mL of 0.1% resorcin in 95% ethanol were added to the mixtures before heating at 100 °C for 30 min. The enzyme activity was calculated based on the values read at 480 nm.

Following the method of Tovar et al. (2002), L-phenylalanine ammonia_lyase (PAL, EC 4.3.1.5) was extracted into sodium borate (Na$_2$B$_4$O$_7$) buffer (0.2 M, pH 8.8). The homogenate was centrifuged at 4 °C and 12,000×g for 15 min. The supernatant (400 μL) was then diluted with an equal volume of 100 mM Na$_2$B$_4$O$_7$ buffer and then pre-incubated at 40 °C for 5 min. The reaction was started by the addition of 40 μL phenylalanine to the reaction mixture and then incubated at 40 °C for 20 min before stopped by adding 5 N HCl. The absorbance was read at 290 nm, where one unit of PAL was defined as the amount of the enzyme that increased 0.1 of absorbance per minute at 290 nm.

2.8. Statistical analysis

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

Graphical vector analysis (GVA) was used to clarify the effect of N administration on the total SGs and soluble sugar concentrations, according to Koricheva (1999). Data was standardized by taking control as 100 and then plotted three-dimensionally. The relative values of compound content, concentration and biomass were plotted on the x, y and z axis, respectively. The centre of the diagram represents the reference point (control) and the calculated effect was classified according to the quadrant the sample located, as shown in Supplementary Fig. 1.

3. Results

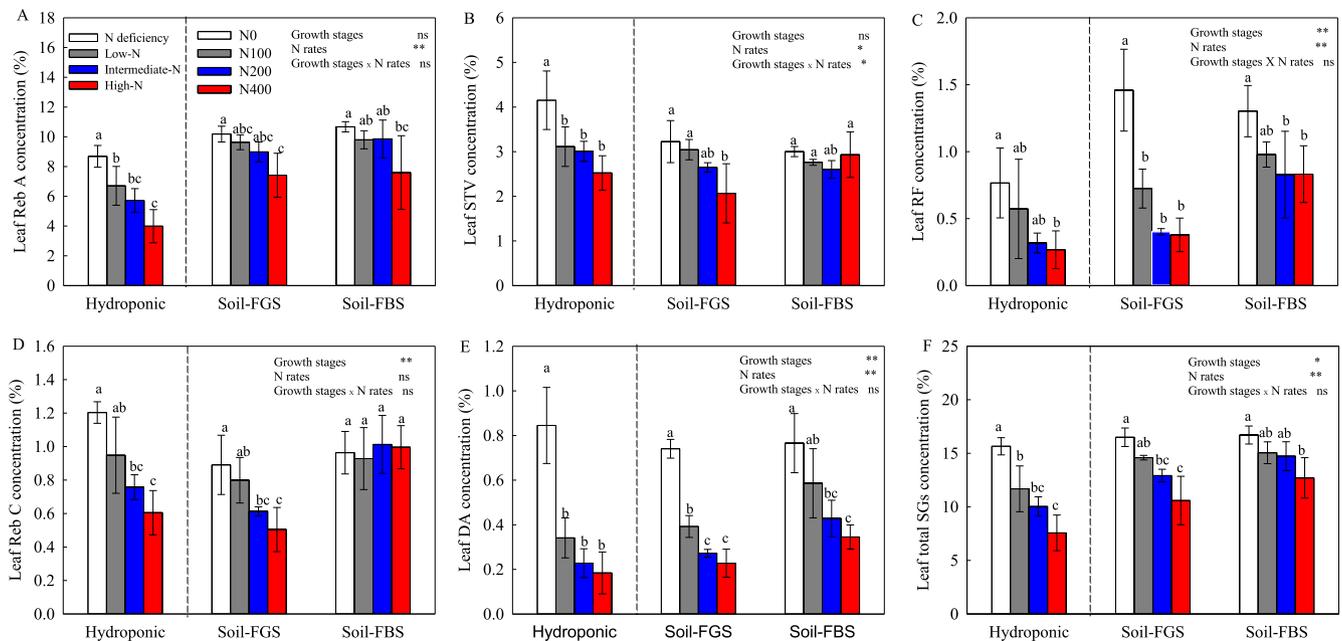### 3.1. Effects of N administration rates on stevia plants growth under both hydroponic and field conditions

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 aboveground biomass was increased by 31.6%–89.5% with N nutrients. The highest leaf or stem dry weight was 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 changes in plant height, total leaf area and aboveground biomass (Supplementary Table 3). The highest Stevia biomass was seen with N400 treatment 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 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 concentration by 25.4%, 35.9% and 51.7%, respectively. SGs concentrations in stem tissues of Stevia plants were significantly lower than those in leaves but exhibited no significant difference between different N treatments (Supplementary Fig. 2).

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

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 steviol 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.

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 increased 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 to the relationship between N administration rates and leaf SGs concentrations. GVA results suggested that reduced total SGs concentrations with N administrations were 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 and F). A negative correlation between leaf biomass formation and SGs concentrations was observed under various conditions, which again indicated that N increased growth "diluted" SGs levels (Fig. 3).

3.4. Effect of N administration rates and growth stages on the transcription level of genes associated with SGs synthesis

The expression of SGs 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, transcription 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 transcription patterns of SGs biosynthetic genes also changed with growth stage. *SrKAH1* and *SrKS1* were up-regulated at FBS compared to FGS, especially under N0 treatment. The transcription of *SrUGT85C2* was significantly down-regulated at FBS.

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 to the lowest C/N ratios under high N conditions (Fig. 5A and 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).

Interestingly, our GVA results showed that the reduced leaf soluble sugar concentration with N fertilization arose from the dilution effect (Supplementary Fig. 3). Nevertheless, further correlation analysis revealed significant positive correlations between leaf SGs concentrations 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 stages or cultivation methods. Similarly, the transcription levels of *SrPEPC*, *SrSPS* and *SrPAL* were also negatively regulated by N administration (Fig. 7B).
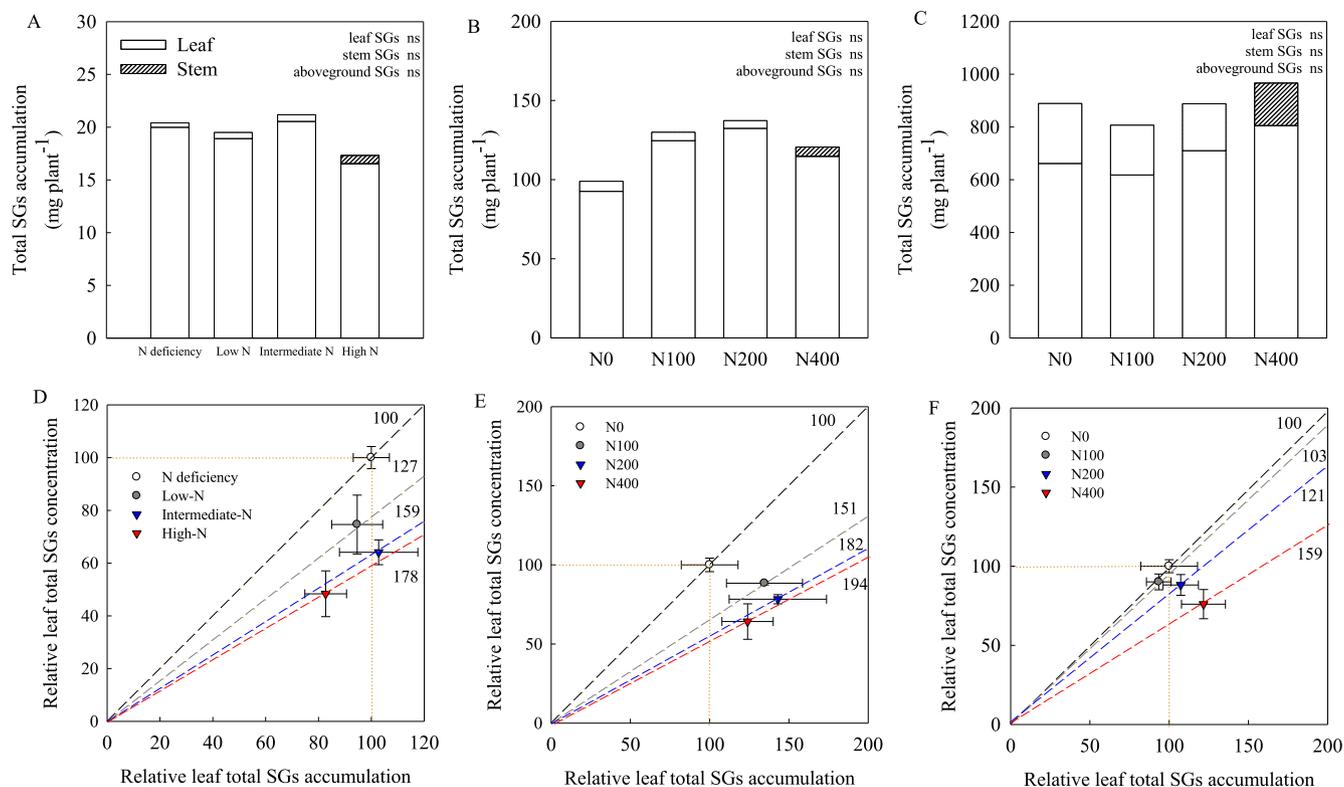
Fig. 2. The accumulation of total steviol glycosides (SGs) in Stevia plants (A, B, C) and graphical vector analysis (GVA) of leaf SGs contents (D, E, F) under hydroponic culture (A, D) and soil culture at fast growth stage (FGS, B, E) or flower bud stage (FBS, C, F).
ns represents no significant difference was observed between treatments. The number in GVA (D, E, F) represent the relative leaf biomass.
For hydroponic experiment, Stevia seedlings were grown in a greenhouse and supplied with nutrient solutions of different N levels (0.6 mM N
as N deficiency, 1.6 mM N
as Low-N, 4.6 mM N
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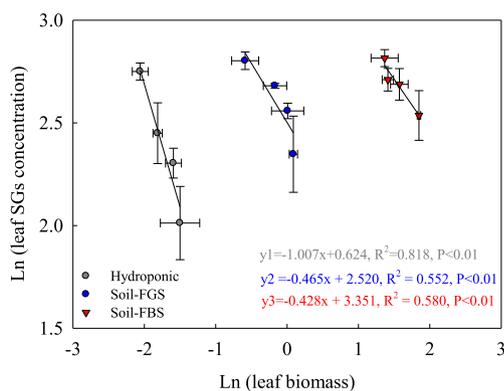
Fig. 3. The linear relationship between leaf biomass and steviol glycosides concentration of Stevia plants supplied with different rates of N.
Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples at flower-bud stage.

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 or content of SGs in Stevia plants were investigated by both hydroponic and plot experiments. We found that N administration reduced the SGs concentrations in Stevia leaves and reflected reduced transcription of genes associated with SGs synthesis (Figs. 1 and 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 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 are accompanied by a decrease in the concentration of SGs. Crucially, the total SGs accumulation was not significantly changed with N administration (Figs. 1 and 2). As N increased biomass, whilst the total SGs 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 (Figs. 2 and 3). Others have noted trade-offs between plant growth and secondary metabolites contents such like 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). These indicated that N administrations do not significantly affect SGs accumulation, and the corresponding transcriptional changes (Fig. 4) have an ineffective regulation of SGs production in this case. However, the synthesis of SGs can be regulated by other factors. For example, previous studies have
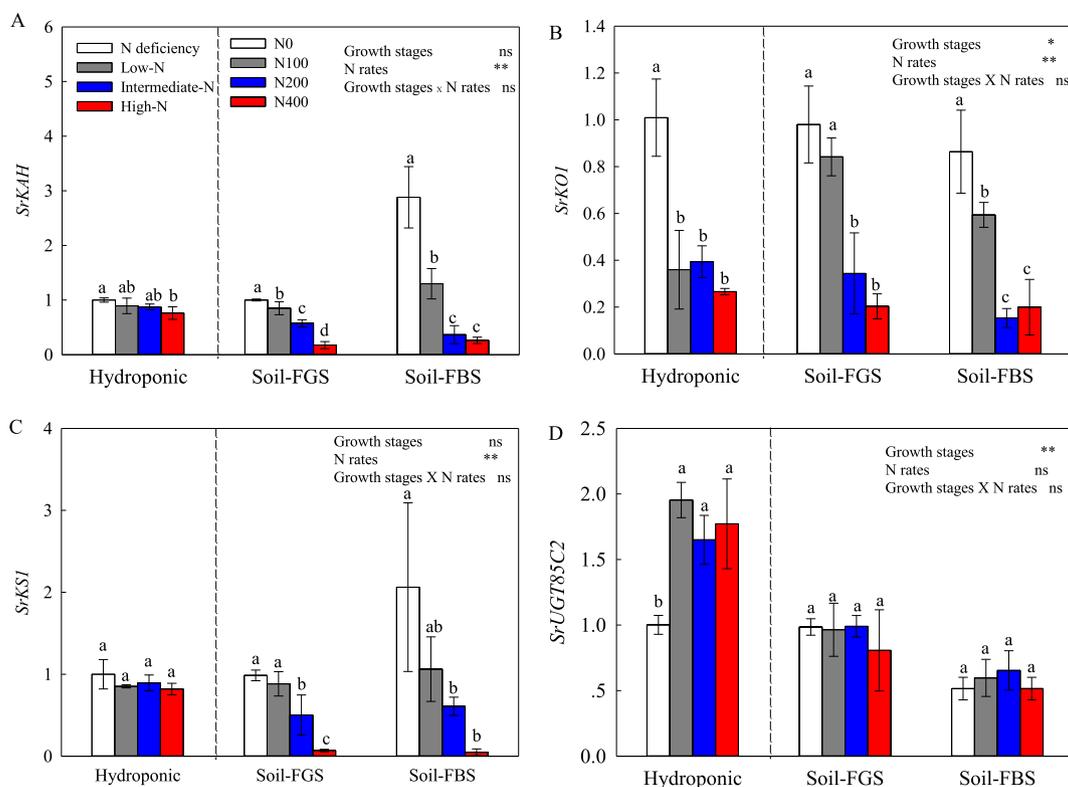
Fig. 4. Effect of N administration rates and growth stages on the relative gene expressions of *SrKAH* (A), *SrKO1* (B), *SrKS1* (C) and *SrUGT85C2* (D). Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples at flower-bud stage*
and ** indicate significant difference at 0.05 and 0.01 probability levels, respectively; ns means non-significant difference.
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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.

demonstrated that the SGs concentration and accumulation on a leaf basis can be critically enhanced by manipulating photoperiod or red RED light (Ceunen and Geuns, 2013b; Ceunen et al., 2012). Besides, arbuscular mycorrhiza fungi can promote SGs production via nutritional (phosphorus) or non-nutritional (carbohydrates and jasmonic acid) mechanisms (Mandal et al., 2013; Tavarini et al., 2018). Extensive studies have also illustrated the regulation role of environmental stresses such like drought and salt stress on SG synthesis (Gupta et al., 2016; Karimi et al., 2015; Zeng et al., 2013). Indeed, the growth

differentiation balance hypothesis (GDBH) has been proposed to explain the responses of secondary metabolites to environmental changes (Koricheva et al., 1998). GDBH suggest that plant growth inhibited by environmental stresses (including nutrient deficiency) 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). Such is not in contradiction to a dilution effect with increased
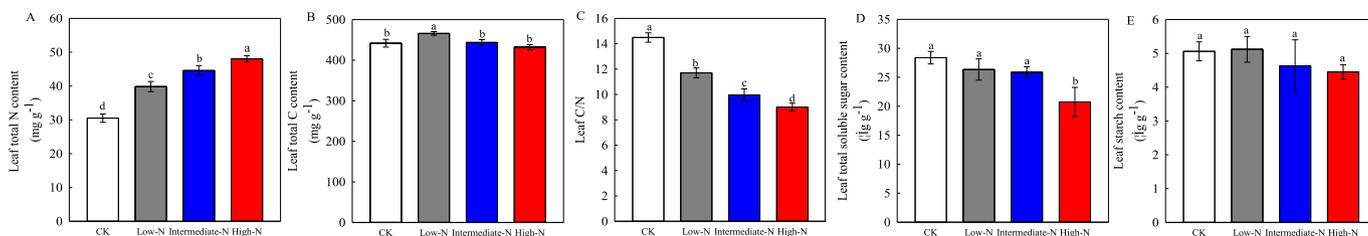
Fig. 5. Effect of different N concentrations on the total N content (A), total C content (B), C/N ratio (C), total soluble sugar content (D) and starch content (E) in Stevia leavesStevia
seedlings were hydroponically grown in greenhouse and supplied with different N levels (0.6 mM N
as N deficiency, 1.6 mM N
as Low-N, 4.6 mM N
as Intermediate-N, and 10.6 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.

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 ± 5.30 ab	26.39 ± 1.75b	1.25 ± 0.16a	19.88 ± 0.34b
	N200	22.63 ± 0.37b	437.69 ± 10.83 ab	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 ± 0.65 ab	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: no N fertilization; 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.

biomass but implies the involvement of active regulatory processes. Taken together, we propose that elevated N drives metabolism towards plant growth, including the synthesis of cytokinins and auxins (Mittelstraβ et al., 2006), which thereafter resulted in biomass diluted SGs concentration, rather than directly 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 concentrations (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 centre 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 are 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 transcription 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 plays 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
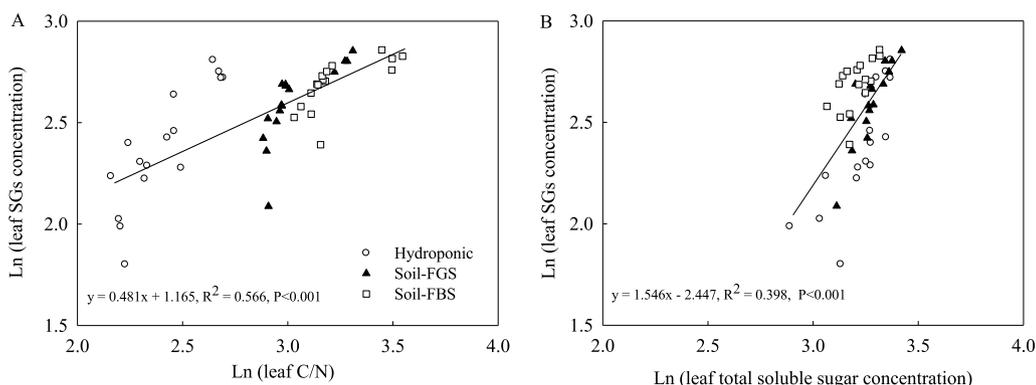
Fig. 6. The relationship between leaf SGs content and leaf C/N ratio (A) and leaf total soluble sugar content (B). The lines represent linear regressions with equation in the diagram. Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil cultured samples at fast growth stage; Soil-FBS: soil cultured samples at flower-bud stage.
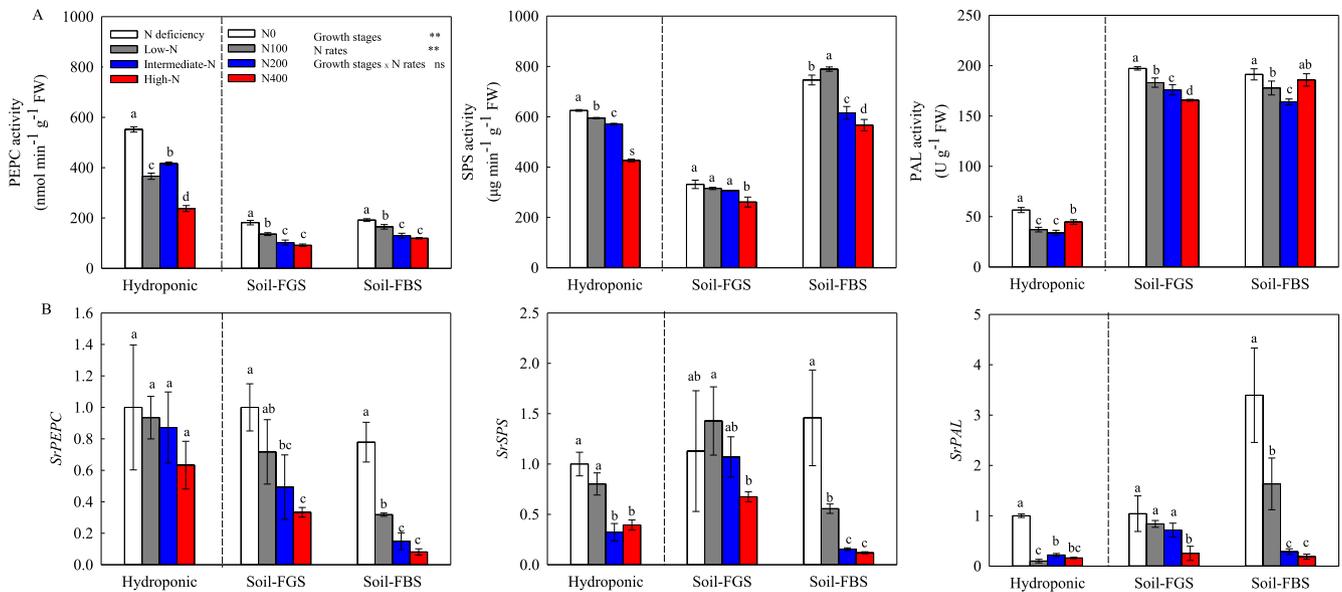
Fig. 7. Effect of N administration rates and growth stages on the activities of PEPCase, SPS and PAL (A) and the relative gene expression of *SrPEPC*, *SrSPS* and *SrPAL* (B). FGS: fast growth stage; FBS: flower-bud stage.
Hydroponic: experiments conducted under hydroponic conditions; Soil-FGS: soil 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.

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 et al., 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 SGs biosynthetic mechanism is clearly merited.

5. 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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Conflicts of interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.008.

References

Additives, E. P. o. F., Food, N. S. a. t., 2010. Scientific opinion on the safety of steviol glycosides for the proposed uses as a food additive. EFSA J 8, 1537. https://doi.org/10.2903/j.efsa.2010.1537.

Aharoni, A., Galili, G., 2011. Metabolic engineering of the plant primary–secondary metabolism interface. Curr. Opin. Biotechnol. 22, 239–244. https://doi.org/10.1016/j.copbio.2010.11.004.

Baque, M.A., Elgirban, A., Lee, E.-J., Paek, K.-Y., 2011. Sucrose regulatedenhanced induction of anthraquinone, phenolics, flavonoids biosynthesis and activities of anti-oxidant enzymes in adventitious root suspension cultures of *Morinda citrifolia* (L.). Acta Physiol. Plant. 34, 405–415. https://doi.org/10.1007/s11738-011-0837-2.

Barbet-Massin, C., Giuliano, S., Alletto, L., Dayde, J., Berger, M., 2015. Nitrogen limitation alters biomass production but enhances steviol glycoside concentration in *Stevia rebaudiana* Bertoni. PLoS One 10, e0133067. https://doi.org/10.1371/journal.pone.0133067.

Bursac Kovacevic, D., Barba, F.J., Granato, D., Galanakis, C.M., Herceg, Z., Dragovic-Uzelac, V., Putnik, P., 2018. Pressurized hot water extraction (PHWE) for the green recovery of bioactive compounds and steviol glycosides from *Stevia rebaudiana* Bertoni leaves. Food Chem. 254, 150–157. https://doi.org/10.1016/j.foodchem.2018.01.192.

Ceunen, S., Geuns, J., 2013a. Glucose, sucrose, and steviol glycoside accumulation in *Stevia rebaudiana* grown under different photoperiods. Biol. Plant. 57, 390–394. https://doi.org/10.1007/s10535-012-0289-6.

Ceunen, S., Geuns, J.M., 2013b. Influence of photoperiodism on the spatio-temporal accumulation of steviol glycosides in *Stevia rebaudiana* (Bertoni). Plant Sci. 198, 72–82. https://doi.org/10.1016/j.plantsci.2012.10.003.

Ceunen, S., Werbrouck, S., Geuns, J.M., 2012. Stimulation of steviol glycoside accumulation in Stevia rebaudiana by red LED light. J. Plant Physiol. 169, 749–752. https://doi.org/10.1016/j.jplph.2012.01.006.

Ding, L., Wang, K.J., Jiang, G.M., Biswas, D.K., Xu, H., Li, L.F., Li, Y.H., 2005. Effects of nitrogen deficiency on photosynthetic traits of maize hybrids released in different years. Ann. Bot. 96, 925–930. https://doi.org/10.1093/aob/mci244.

Fernandes, B., Dragone, G., Abreu, A.P., Geada, P., Teixeira, J., Vicente, A., 2012. Starch determination in Chlorella vulgaris—a comparison between acid and enzymatic methods. J. Appl. Phycol. 24, 1203–1208. https://doi.org/10.1007/s10811-011-9761-5.

Ghasemzadeh, A., Jaafar, H.Z., 2011. Effect of CO_2 enrichment on synthesis of some primary and secondary metabolites in ginger (Zingiber officinale Roscoe). Int. J. Mol. Sci. 12, 1101–1114. https://doi.org/10.3390/ijms12021101.

Ghorbani, T., Kahrizi, D., Saeidi, M., Arji, I., 2017. Effect of sucrose concentrations on Stevia rebaudiana Bertoni tissue culture and gene expression. Cell. Mol. Biol. 63, 33–37. https://doi.org/10.14715/cmb/2017.63.8.8.

Gibon, Y., Pyl, E.T., Sulpice, R., Lunn, J.E., Hohne, M., Gunther, M., Stitt, M., 2009. Adjustment of growth, starch turnover, protein content and central metabolism to a decrease of the carbon supply when Arabidopsis is grown in very short photoperiods. Plant Cell Environ. 32, 859–874. https://doi.org/10.1111/j.1365-3040.2009.01965.x.

Glynn, C., Herms, D.A., Orians, C.M., Hansen, R.C., Larsson, S., 2007. Testing the growth-differentiation balance hypothesis: dynamic responses of willows to nutrient availability. New Phytol. 176, 623–634. https://doi.org/10.1111/j.1469-8137.2007.02203.x.

Guo, Q., Major, I.T., Howe, G.A., 2018. Resolution of growth-defense conflict: mechanistic insights from jasmonate signaling. Curr. Opin. Plant Biol. 44, 72–81. https://doi.org/10.1016/j.pbi.2018.02.009.

Gupta, P., Sharma, S., Saxena, S., 2016. Effect of abiotic stress on growth parameters and steviol glycoside content in Stevia rebaudiana (Bertoni) raised in vitro. J. Appl. Res. Med. Aromat. Plants 3, 160–167. https://doi.org/10.1016/j.jarmap.2016.03.004.

Hafsi, M., Pfeiffer, W., Monneveux, P., 2003. Flag leaf senescence, carbon content and carbon isotope discrimination in durum wheat grown under semi-arid conditions. Cereal Res. Commun. 161–168.

Hu, W., Coomer, T.D., Loka, D.A., Oosterhuis, D.M., Zhou, Z., 2017. Potassium deficiency affects the carbon-nitrogen balance in cotton leaves. Plant Physiol. Biochem. 115, 408–417. https://doi.org/10.1016/j.plaphy.2017.04.005.

Hu, W., Lv, X., Yang, J., Chen, B., Zhao, W., Meng, Y., Wang, Y., Zhou, Z., 2016. Oosterhuis, D. M., Effects of potassium deficiency on antioxidant metabolism related to leaf senescence in cotton (Gossypium hirsutum L.). Field Crop. Res. 191, 139–149. https://doi.org/10.1016/j.fcr.2016.02.025.

Ibrahim, M.H., Jaafar, H.Z., Rahmat, A., Rahman, Z.A., 2011. Effects of nitrogen fertilization on synthesis of primary and secondary metabolites in three varieties of kacip Fatimah (Labisia pumila Blume). Int. J. Mol. Sci. 12, 5238–5254. https://doi.org/10.3390/ijms12085238.

Ibrahim, M.H., Jaafar, H.Z., Rahmat, A., Rahman, Z.A., 2010. The relationship between phenolics and flavonoids production with total non structural carbohydrate and photosynthetic rate in Labisia pumila Benth. under high CO_2 and nitrogen fertilization. Molecules 16, 162–174. https://doi.org/10.3390/molecules16010162.

Ibrahim, M.H., Jaafar, H.Z., 2011. The relationship of nitrogen and C/N ratio with secondary metabolites levels and antioxidant activities in three varieties of malaysian kacip fatimah (Labisia pumila Blume). Molecules 16, 5514–5526. https://doi.org/10.3390/molecules16075514.

Interdonato, R., Rosa, M., Nieva, C.B., González, J.A., Hilal, M., Prado, F.E., 2011. Effects of low UV-B doses on the accumulation of UV-B absorbing compounds and total phenolics and carbohydrate metabolism in the peel of harvested lemons. Environ. Exp. Bot. 70, 204–211. https://doi.org/10.1016/j.envexpbot.2010.09.006.

Kafle, G.G., Midmore, D.J., Gautam, R., 2017. Effect of nutrient omission and pH on the biomass and concentration and content of steviol glycosides in stevia (Stevia rebaudiana (Bertoni) Bertoni) under hydroponic conditions. J. Appl. Res. Med. Aromat. Plants 7, 136–142. https://doi.org/10.1016/j.jarmap.2017.08.001.

Kallscheuer, N., 2018. Engineered microorganisms for the production of food additives approved by the European Union-a systematic analysis. Front. Microbiol. 9, 1746. https://doi.org/10.3389/fmicb.2018.01746.

Karimi, M., Ahmadi, A., Hashemi, J., Abbasi, A., Tavarini, S., Guglielminetti, L., et al., 2015. The effect of soil moisture depletion on Stevia (Stevia rebaudiana Bertoni) grown in greenhouse conditions: growth, steviol glycosides content, soluble sugars and total antioxidant capacity. Sci. Hortic. 183, 93–99. https://doi.org/10.1016/j.scienta.2014.11.001.

Kim, M.J., Zheng, J., Liao, M.H., Jang, I.C., 2018. Overexpression of SrUGT76G1 in Stevia alters major steviol glycosides composition towards improved quality. Plant Biotechnol. J. 17, 1037–1047. https://doi.org/10.1111/pbi.13035.

Kirakosyan, A., Kaufman, P., Warber, S., Zick, S., Aaronson, K., Bolling, S., Chul Chang, S., 2004. Applied environmental stresses to enhance the levels of polyphenolics in leaves of hawthorn plants. Physiol. Plantarum 121, 182–186. https://doi.org/10.1111/j.1399-3054.2004.00332.x.

Koricheva, J., 1999. Interpreting phenotypic variation in plant allelochemistry: problems with the use of concentrations. Oecologia 119, 467–473. https://doi.org/10.1007/s004420050809.

Koricheva, J., 2002. The carbon-nutrient balance hypothesis is dead, long live the carbon-nutrient balance hypothesis? Oikos 98, 537–539. https://doi.org/10.1034/j.1600-0706.2002.980319.x.

Koricheva, J., Larsson, S., Haukioja, E., Keinänen, M., 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos 212–226. https://doi.org/10.2307/3546833.

Kováčik, J., Klejdu, s, B., Bačkor, M., Repčák, M., 2007. Phenylalanine ammonia-lyase activity and phenolic compounds accumulation in nitrogen-deficient Matricaria chamomilla leaf rosettes. Plant Sci. 172, 393–399. https://doi.org/10.1016/j.plantsci.2006.10.001.

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., Ah-Hen, K., 2012. Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: a comprehensive review on the biochemical, nutritional and functional aspects. Food Chem. 132, 1121–1132. https://doi.org/10.1016/j.foodchem.2011.11.140.

Liu, W., Zhu, D.-W., Liu, D.-H., Geng, M.-J., Zhou, W.-B., Mi, W.-J., Yang, T.-W., Hamilton, D., 2010. INFLUENCE OF NITROGEN ON THE PRIMARY AND SECONDARY METABOLISM AND SYNTHESIS OF FLAVONOIDS IN C HRYSANTHEMUM MORIFOLIUM RAMAT. J. Plant Nutr. 33, 240–254. https://doi.org/10.1080/01904160903434287.

MacDonald, M.J., D'Cunha, G.B., 2007. A modern view of phenylalanine ammonia lyase. Biochem. Cell Biol. 85, 273–282. https://doi.org/10.1139/O07-018.

Mandal, S., Evelin, H., Giri, B., Singh, V.P., Kapoor, R., 2013. Arbuscular mycorrhiza enhances the production of stevioside and rebaudioside-A in Stevia rebaudiana via nutritional and non-nutritional mechanisms. Appl. Soil Ecol. 72, 187–194. https://doi.org/10.1016/j.apsoil.2013.07.003.

Masclaux-Daubresse, C., Daniel-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L., Suzuki, A., 2010. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann. Bot. 105, 1141–1157. https://doi.org/10.1093/aob/mcq028.

Massad, T.J., Dyer, L.A., Vega, C.G., 2012. Costs of defense and a test of the carbon-nutrient balance and growth-differentiation balance hypotheses for two co-occurring classes of plant defense. PLoS One 7, e47554. https://doi.org/10.1371/journal.pone.0047554.

Mittelstraß, K., Treutter, D., Pleßl, M., Heller, W., Elstner, E., Heiser, I., 2006. Modification of primary and secondary metabolism of potato plants by nitrogen application differentially affects resistance to Phytophthora infestans and Alternaria solani. Plant Biol. 8, 653–661. https://doi.org/10.1055/s-2006-924085.

Nelson, D., Sommers, L., 1972. A simple digestion procedure for estimation of ammonium in Kjeldahl soils. J. Environ. Qual. 1, 423–425. https://doi.org/10.2134/jeq1972.00472425000100040020x.

Olsen, K.M., Lea, U.S., Slimestad, R., Verheul, M., Lillo, C., 2008. Differential expression of four Arabidopsis PAL genes, PAL1 and PAL2 have functional specialization in abiotic environmental-triggered flavonoid synthesis. J. Plant Physiol. 165, 1491–1499. https://doi.org/10.1016/j.jplph.2007.11.005.

Osakabe, Y., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L.S., 2013. Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. J. Exp. Bot. 64, 445–458. https://doi.org/10.1093/jxb/ers354.

Osuna, D., Usadel, B., Morcuende, R., Gibon, Y., Blasing, O.E., Hohne, M., Gunter, M., Kamlage, B., Trethewey, R., Scheible, W.R., Stitt, M., 2007. Temporal responses of transcripts, enzyme activities and metabolites after adding sucrose to carbon-deprived Arabidopsis seedlings. Plant J. 49, 463–491. https://doi.org/10.1111/j.1365-313X.2006.02979.x.

Pal, P.K., Kumar, R., Guleria, V., Mahajan, M., Prasad, R., Pathania, V., Gill, B.S., Singh, D., Chand, G., Singh, B., Singh, R.D., Ahuja, P.S., 2015. Crop-ecology and nutritional variability influence growth and secondary metabolites of Stevia rebaudiana Bertoni. BMC Plant Boil 15, 67. https://doi.org/10.1186/s12870-015-0457-x.

Payyavula, R.S., Singh, R.K., Navarre, D.A., 2013. Transcription factors, sucrose, and sucrose metabolic genes interact to regulate potato phenylpropanoid metabolism. J. Exp. Bot. 64, 5115–5131. https://doi.org/10.1093/jxb/ert303.

Philippaert, K., Pironet, A., Mesuere, M., Sones, W., Vermeiren, L., Kerselaers, S., Pinto, S., Segal, A., Antoine, N., Gysemans, C., 2017. Steviol glycosides enhance pancreatic beta-cell function and taste sensation by potentiation of TRPM5 channel activity. Nat. Commun. 8, 14733. https://doi.org/10.1038/ncomms14733.

Ramakrishna, A., Ravishankar, G.A., 2011. Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal. Behav. 6, 1720–1731. https://doi.org/10.4161/psb.6.11.17613.

Riipi, M., Ossipov, V., Lempa, K., Haukioja, E., Koricheva, J., Ossipova, S., Pihlaja, K., 2002. Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? Oecologia 130, 380–390. https://doi.org/10.1007/s00442-001-0826-z.

Rovalino-Córdova, A.M., Fogliano, V., Capuano, E., 2018. A closer look to cell structural barriers affecting starch digestibility in beans. Carbohydr. Polym. 181, 994–1002. https://doi.org/10.1016/j.carbpol.2017.11.050.

Royer, M., Larbat, R., Le Bot, J., Adamowicz, S., Robin, C., 2013. Is the C:N ratio a reliable indicator of C allocation to primary and defence-related metabolisms in tomato? Phytochemistry 88, 25–33. https://doi.org/10.1016/j.phytochem.2012.12.003.

Ruan, J., Haerdter, R., Gerendas, J., 2010. Impact of nitrogen supply on carbon/nitrogen allocation: a case study on amino acids and catechins in green tea [Camellia sinensis (L.) O. Kuntze] plants. Plant Biol. 12, 724–734. https://doi.org/10.1111/j.1438-8677.2009.00288.x.

Schluter, U., Mascher, M., Colmsee, C., Scholz, U., Brautigam, A., Fahnenstich, H., Sonnewald, U., 2012. Maize source leaf adaptation to nitrogen deficiency affects not only nitrogen and carbon metabolism but also control of phosphate homeostasis. Plant Physiol. 160, 1384–1406. https://doi.org/10.1104/pp.112.204420.

Shi, L., Cao, S., Shao, J., Chen, W., Zheng, Y., Jiang, Y., Yang, Z., 2014. Relationship between sucrose metabolism and anthocyanin biosynthesis during ripening in Chinese bayberry fruit. J. Agric. Food Chem. 62, 10522–10528. https://doi.org/10.1021/jf503317k.

Tavarini, S., Pagano, I., Guidi, L., Angelini, L.G., 2015a. Impact of nitrogen supply on growth, steviol glycosides and photosynthesis in Stevia rebaudiana Bertoni. Plant Biosyst. 150, 953–962. https://doi.org/10.1080/11263504.2014.993743.

Tavarini, S., Passera, B., Martini, A., Avio, L., Sbrana, C., Giovannetti, M., et al., 2018. Plant growth, steviol glycosides and nutrient uptake as affected by arbuscular mycorrhizal fungi and phosphorous fertilization in Stevia rebaudiana Bert. Ind. Crops Prod. 111, 899–907. https://doi.org/10.1016/j.indcrop.2017.10.055.

Tavarini, S., Sgherri, C., Ranieri, A.M., Angelini, L.G., 2015b. Effect of nitrogen

fertilization and harvest time on steviol glycosides, flavonoid composition, and antioxidant properties in *Stevia rebaudiana* Bertoni. J. Agric. Food Chem. 63, 7041–7050. https://doi.org/10.1021/acs.jafc.5b02147.

Tovar, M.J., Romero, M.P., Girona, J., Motilva, M.J., 2002. l-Phenylalanine ammonia-lyase activity and concentration of phenolics in developing olive (*Olea europaea* L cv Arbequina) fruit grown under different irrigation regimes. J. Sci. Food Agric. 82, 892–898. https://doi.org/10.1002/jsfa.1122.

Vranová, E., Coman, D., Gruissem, W., 2013. Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu. Rev. Plant Biol. 64, 665–700. https://doi.org/10.1146/annurev-arplant-050312-120116.

Wang, C.-L., Liang, Z.-S., Li, D.-R., Yang, J.-L., 2012. Salvianolic acids production in *Salvia miltiorrhiza* Bunge was regulated by both soluble sugars accumulation and growth substances salicylic acid and methyl jasmonate. J. Med. Plants Res. 6, 2666–2673. https://doi.org/10.5897/JMPR.

Wang, J., Li, S., Xiong, Z., Wang, Y., 2016. Pathway mining-based integration of critical enzyme parts for de novo biosynthesis of steviol glycosides sweetener in *Escherichia coli*. Cell Res. 26, 258. https://doi.org/10.1038/cr.2015.111.

Winter, H., Huber, S.C., 2010. Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. Crit. Rev. Plant Sci. 19, 31–67.

https://doi.org/10.1080/07352680091139178.

Yadav, S.K., Guleria, P., 2012. Steviol glycosides from Stevia: biosynthesis pathway review and their application in foods and medicine. Crit. Rev. Food Sci. 52, 988–998. https://doi.org/10.1080/10408398.2010.519447.

Yang, Y., Huang, S., Han, Y., Yuan, H., Gu, C., Wang, Z., 2015. Environmental cues induce changes of steviol glycosides contents and transcription of corresponding biosynthetic genes in Stevia rebaudiana. Plant Physiol. Biochem. 86, 174–180. https://doi.org/10.1016/j.plaphy.2014.12.004.

Zakhartsev, M., Medvedeva, I., Orlov, Y., Akberdin, I., Krebs, O., Schulze, W.X., 2016. Metabolic model of central carbon and energy metabolisms of growing *Arabidopsis thaliana* in relation to sucrose translocation. BMC Plant Biol. 16, 262. https://doi.org/10.1186/s12870-016-0868-3.

Zeng, J., Chen, A., Li, D., Yi, B., Wu, W., 2013. Effects of salt stress on the growth, physiological responses, and glycoside contents of *Stevia rebaudiana* Bertoni. J. Agric. Food Chem. 61, 5720–5726. https://doi.org/10.1021/jf401237x.

Zhang, G., Duan, C., Wang, Y., Wang, Y., Ji, K., Xu, H., et al., 2013. The expression pattern of β-glucosidase genes (VvBGs) during grape berry maturation and dehydration stress. Plant Growth Regul. 70, 105–114. https://doi.org/10.1007/s10725-012-9782-3.