Impact of nitrogen supply on growth, steviol glycosides and photosynthesis in *Stevia rebaudiana* Bertoni

Running title: Nitrogen supply in stevia

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Abstract

This work investigated the agronomic, physiological and biochemical response of *Stevia rebaudiana* Bertoni grown under different nitrogen (N) rates. A pot trial in open air conditions was set up in 2012 with the aim to evaluate the effect of four N rates, on the biometric and productive characteristics, steviol glycoside (SGs) content as well as on leaf gas exchanges, chlorophyll fluorescence, photosynthetic pigments, Rubisco activity, and N use efficiency. N deficiency caused a decrease in leaf N content, chlorophylls and photosynthetic CO_2 assimilation, resulting in a lower dry matter accumulation as well as in reduced SGs production. The application of 150 kg N ha^{-1} seems to be the most effective treatment to improve rebaudioside A content, rebaudioside A/stevioside ratio, photosynthetic CO_2 assimilation, stomatal conductance, N use efficiency, Rubisco and PSII efficiency. The results demonstrate that using an appropriate N rate it is possible to modulate the SGs biosynthesis, with a significant increase in the rebaudioside A content and, consequently, in the ratio between rebaudioside A and stevioside. This finding is of pivotal importance in order to obtain a raw material designed to meet consumer needs and bio-industry requirements for high quality, high Rebaudioside A content, safe and environmental friendly products.

Keywords: chlorophyll fluorescence; gas exchange; nitrogen use efficiency; photosynthesis; *Stevia rebaudiana*; steviol glycosides.

Introduction

Increasing health-consciousness among consumers is the leading cause for an increasing demand for natural non-nutritive sweeteners as a substitute for sucrose and other intensive sweeteners such as saccharine, acesulfame and aspartame. *Stevia rebaudiana* Bertoni (stevia) is an herbaceous species native to the North Eastern Paraguay, characterized by a high content, in its leaves, of diterpenoid glycosides - steviol glycosides (SGs). The SGs are non-cariogenic and non-caloric sweeteners, possessing a 250-300 times higher sweetening property than sucrose (Crammer & Ikan 1986). They can be used in treatment of patients suffering from carbohydrate metabolic diseases such as diabetes mellitus, obesity, hypertension (Lee et al. 2001; Gregersen et al. 2004; Anton et al. 2010). The SGs include stevioside, rebaudiosides A-G, steviolbioside, rubusoside, dulcoside A and account for about 4-20 % of the dry weight of the leaves (Gardana et al. 2010; Chaturvedula & Prakash 2011). Stevioside and rebaudioside A are the major SGs and their concentrations vary quite widely depending on the genotype and production environment (Ramesh et al. 2006). Rebaudioside A (Reb A) is reported not only to exhibit sweetness more pronouncedly than the other SGs, but also to show a palatable taste profile, having less of the metallic/liquorice taste, often associated with SGs. Consequently, the Reb A /stevioside ratio can be considered a good qualitative measure of sweetness (Yadav et al. 2011). Stevia is a relatively new crop for Europe, where stevia is still in its infancy and there is a lack of practical experiences on its cultivation and agronomy. So, the development of optimal agro-techniques is of paramount importance to obtain a raw material designed to meet consumer needs and bio-industry requirements for high quality, high Reb A content, safe, and fully traceable products. To date, little is known about the effect of levels of nitrogen (N) fertilization on stevia growth (Ramesh et al. 2006; Aladakatti et al. 2012) and no information are reported on the relationship between the N and SGs content as well as on the main physiological characteristics related to the photosynthetic process.

It is well known as, among all plant nutrients, N is one the key limiting factors for crop development and quality, plant biomass production, photosynthesis and, finally, economic yield. Over the last four decades, N fertilization has been an essential agronomic practice for increasing crop yield and quality (Glass 2003). However, the high energy cost of synthesizing N fertilizers and its high mobility in the soil-plant-atmosphere system made N use a great contributor of agriculture-related pollution through leaching, volatilization and denitrification (Limaux et al. 1999; Giambalvo et al. 2010). In intensive agricultural production systems, as much as 50% of the N applied to the field is not used by the crop plant (Cameron et al. 2013). The surplus N may be lost to the aqueous and atmospheric environments where it can become a serious pollutant and cause an important

reduction of food quality (Cameron et al. 2013). On the other hand, an excessive nitrogen fertilization could lead to risks of nitrate accumulation at leaf level. Therefore, the optimization of the nitrogen fertilization is gaining greater attention to avoid irrational fertilization management of the crop, minimizing the potential N losses, and to combine high yields with relatively low environmental impacts and product safety.

Metabolic processes based on proteins, leading to increased vegetative and reproductive growth and yield, are totally dependent upon the adequate supply of nitrogen (Lawlor 2002). Indeed, N is a key factor regulating photosynthesis because it is a major component of Rubisco and other photosynthetic enzymes and structures (Ripullone et al. 2003). In fact, more than half of the total leaf N is allocated to the photosynthetic apparatus (Makino & Osmond 1991). As general trend, N shortage reduces the leaf production, individual leaf area and total leaf area, resulting in a reduced area for light interception for photosynthesis (Vos & Biemond 1992; Toth et al. 2002) and, consequently, in a marked decrease in plant CO_2 uptake. Photosynthetic capacity and total amount of leaf N per unit of leaf area are usually correlated (Walcroft et al. 1997). A positive correlation between N fertilization rate and chlorophyll content is well documented for a number of plant species and has been investigated for most major crops including corn, rice, wheat (Bojovic & Stojanovic 2005; Fritshi & Ray 2007; Houles et al. 2007). Despite the large number of studies on the interactions among nitrogen, photosynthesis and productivity for many plants, very little is reported concerning the effects of N fertilization on the production of diterpenoid glycosides and on CO_2 gas exchange and related physiological aspects in *Stevia rebaudiana*. So, in this context, the knowledge of nitrogen requirement of stevia is an important tool in order to achieve high crop yields, high quality level, rational use of fertilizers, low environmental impact, suitable adaptation and mitigation strategies able to encourage responsible sustainable development. Consequently a pot trial in open air conditions was set up in 2012 with the aim to define the agronomic, biochemical and physiological response of stevia to different N rates.

Materials and methods

Plant material and experimental conditions

A pot trial was carried out at the Experimental Centre of Department of Agriculture, Food and Environment (DAFE) of the University of Pisa, located to San Piero a Grado, Pisa, Italy (43°40'N; 10°19'E, 5 m above sea level), during the 2012 growing season. A selected clone (RG) of *Stevia rebaudiana*, belonging to the DAFE germplasm collection was used. Seeds, harvested in November 2011, were selected and cleaned and then stored in controlled conditions at 5°C and 50% relative

humidity until January 2012. The seeds were placed to germinate in 150 mm diameter Petri dishes moistened with distilled water and put into a cabinet at alternating temperature of 20/30°C (16/8h) and light (16/8h dark/light; 10µmol photons s^{-1} m^{-2} photosynthetically active radiation) conditions. The seedlings obtained were initially transplanted into peat-moss medium to select the well-established plantlets and maintained into a greenhouse. After three weeks, the selected plantlets were transplanted into 20L pots containing sandy loam soil (field capacity 19.0%; wilting point 8.5%; bulk density 1.3 g cm^{-3}). The soil physical and chemical characteristics are presented in Table I. The pots were filled with soil up to 2 cm below the surface. One plant was cultivated in each pot. In April 2012 the plants were moved to an open-air facility that was protected from rain by a movable rain-out shelter. Mean maximum and minimum temperatures in the growing season were 26.9°C and 13.1°C, respectively.

Stevia plants were grown under different rates of nitrogen: N0 (without N fertilisation); N50 (50 kg N ha^{-1} equal to 0.4 g N pot^{-1}); N150 (150 kg N ha^{-1}, equal to 1.2 g N pot^{-1}) and N300 (300 kg N ha^{-1}, equal to 2.4 g N pot^{-1}). These N rate applications were chosen on the basis of the results obtained in a previous study, carried out in order to define the nutrient requirements of stevia plants, grown in open field conditions (Angelini & Tavarini 2014).

A complete randomized block design with 5 replicates (1 pot per replicate) for each treatment was used. The nitrogen was supplied as ammonium nitrate and it was split in four applications every 30 days during the vegetative growth.

In addition, a mix of microelements (MgO, Bo, Cu, Fe, Mn, Mo, Zn) was supplied, at the dose of 0.1 g L^{-1} per pot. A constant source of phosphorus and potassium was distributed to all treatments at the rate of 100 and 180 kg ha^{-1} respectively, before transplanting the plants. Water was supplied to all pots to facilitate transplanting recovery. During the trial the plants were maintained under optimal water supply through a drip irrigation system in order to maintain soil moisture to 75-80% of field capacity.

Biometric and productive characteristics

The plant harvest was manually accomplished at the end of the vegetative growth (29 August 2012). After harvest, the plant height, branch number, specific leaf weight (SLW) and the fresh total above-ground biomass were measured. Plants were air-dried in a ventilated oven at 30-40°C for dry weight determinations of the stems, leaves and the total above-ground biomass. For each pot, a representative sub-sample of dry leaves was ground to fine powder using a laboratory mill to

evaluate the N concentration and stevioside, rebaudioside A and rebaudioside C content by HPLC analysis.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange measurements on leaves were carried out at the end of the experiment using an open system (Walz, Effeltrich, Germany). For more details of the experimental procedures see Guidi et al. (1997). During the gas exchange measurements, the temperature in the assimilation chamber was maintained at 25±1.4°C, with a relative humidity 65±9%, and O_2 21%. Leaf photosynthetic CO_2 assimilation responses to irradiance were calculated using the Smith equation (Tenhunen et al. 1976) and determined at a CO_2 concentration of 380 µmol mol^{-1}. Photosynthetic CO_2 assimilation rate was recorded after stabilization at each light intensity. Gas exchange parameters (CO_2 photoassimilation, stomatal conductance and intercellular CO_2 concentration) were determined at light-saturated level (about 800 µmol $m^{-2}s^{-1}$).

The imaging technique was performed using an IMAGING-PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany) according to Guidi et al. (2007). The captured images were elaborated and the mean values of the total leaf area for each parameter was utilized. The current fluorescence yield (F_t) was continuously measured and the F_0 (minimal fluorescence in a dark-adapted leaf) images were recorded in a dark state. The maximum fluorescence yield F_m was determined with a saturating pulse of 8,000 µmol m^{-2} s^{-1} PPFD (Photosynthetic Photon Flux Density) for 1-2 s. Images of F_0 and F_m (maximal fluorescence in a dark-adapted leaf) were subtracted and divided [$(F_m - F_0)/F_m$] to generate the image of the maximum quantum efficiency of PSII photochemistry F_v/F_m (where F_v is the variable fluorescence = F_m - F_0). The current fluorescence yield (F_t) and the maximum light-adapted fluorescence (F_m') were determined in the presence of an actinic illumination of 800 µmol m^{-2} s^{-1}. Then Φ_{PSII} as the quotient $(F_m'-F_t)/F_m'$ (Genty et al. 1989) was computed. The coefficient of photochemical (q_P) quenching was calculated according to Schreiber et al. (1994). Correct F_0' (minimal fluorescence in a light-adapted leaf) determination requires the application of a far-red light, which would disturb the fluorescence imaging. Therefore, F_0' was computed using the approximation of Oxborough and Baker (1997): $F_0' = F_0/(F_v/F_m + F_0/F_m')$. Calculation of quenching due to non-photochemical dissipation of absorbed light energy (NPQ) was determined at each saturating pulse, according to the equation NPQ = $(F_m - F_m')/F_m'$ (Bilger & Bjorkman 1990).

To determine the light-response curve of apparent electron transport rate (ETR), the leaves were adapted to the desired irradiance for 5 min. Images of the fluorescence parameters were

displayed by means of a false colour code ranging from 0.00 (black) to 1.00 (purple). Chl fluorescence imaging analysis was carried out on well-expanded leaves of five stevia plants.

Photosynthetic pigment determination

Three discs of leaf tissue (1.0 cm^2 of leaf area) were used to determine the photosynthetic pigment content. The plant tissues were homogenised with 1 mL of acetone 80% and extracted in the dark for 48 hours, at 4°C ± 1, in an Eppendorf tube. At the end of extraction, the mixture was centrifuged at 7000 *g* for 5 min and the absorbance values of supernatant spectrophotometrically measured. The chlorophyll contents were calculated from equations proposed by Porra et al. (1989) whereas carotenoids content was calculated from equation proposed by Lichtenthaler (1987).

Ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) activity assay

The activity of Rubisco (EC 4.1.1.39) was measured spectrophotometrically at 340 nm and 30°C according to Ouerghi et al. (2000). Fresh leaf samples were ground in liquid nitrogen, in 100 mM Tricine-KOH (pH 8.0) containing 1 mM ethylene diamine tetraacetic acid (EDTA), 1% 2-mercaptoethanol (v/v), 1 mM phenylmethylsulfonyl (PMSF), and 5% polyvinylprrolidone (PVP) (w/w of sample FW), and centrifuged at 12,000 *g* for 20 min at 4°C. Rubisco activity was assayed in a reaction medium containing 100 mM Tris-bicine (pH 8.0), 10 mM $MgCl_2$, 0.2 mM EDTA, 5 mM dithiothreitol (DTT), 40 mM $NaHCO_3$, 4 mM ATP, 0.2 mM NADH, 0.2 mM ribulose 1,5-bisphosphate (RuBP), and one enzyme unit of 3-phosphoglycerate kinase (PGK) and glyceraldehyde 3-phosphate dehydrogenase (3-PGADH). The crude extract was added to the reaction medium, and the activity was monitored for 10 min. Enzyme activity was expressed as µmol CO_2 min^{-1} mg^{-1} protein. Protein determination was performed according to the method of Lowry et al. (1951). Each analysis was carried in triplicate.

Leaf nitrogen concentration (LNC) and photosynthetic nitrogen use efficiency (PNUE)

Leaf tissue nitrogen (N) was determined in triplicate by the Kjeldahl method (Jones 1998). Photosynthetic nitrogen use efficiency (PNUE) was calculated as A_{max} per unit of foliar N content (g N m^{-2}).

Steviol glycosides analysis

The procedure used was fully described in Tavarini and Angelini (2013). An aliquot of extract (20 µL) was injected into the HPLC system (Jasco PU980) coupled with a UV-visible wavelength

detector. A LiChrospher NH2 column, 5µm, 250 mm x 4.6 mm (Alltech Italia), in conjunction with LiChrospher Amino All-Guard and All-Guard Cartridge Holder (Alltech Italia) was used. To optimize the separation of glycosides the HPLC operating conditions reported by Hearn and Subedi (2009), were used: an isocratic mobile phase, acetonitrile/water (80/20), pH 5 adjusted with acetic acid, a flow rate of 1.0 mL min^{-1} and a run time of 20 min. The UV-visible detector was set to monitor at 210 nm, at ambient temperature. The accuracy of the method was determined by assessing the recovery and the appropriate relative standard deviation in 7 different leaf samples spiked with different amounts of the two steviol glycosides (stevioside and Reb A). Chromatograms were acquired online, and data were collected via a Jasco interface (Hercules 2000) and analysed using a Jasco Borwing 2000 data system. For each sample the compounds were identified by comparing their retention times with those of the external standards and quantified by calibration curves. For quantification purposes, pure stevioside (99.9% purity) and rebaudioside A (97.4% purity) were used. Calibration curves were obtained from standard solutions of 0.25, 0.5 and 1.0 g L^{-1} prepared for both stevioside and rebaudioside A in ethanol 70% (w/w). Rebaudioside C was quantified using the calibration curve of stevioside, after correction for molecular weights. Each analysis was carried out in triplicate.

Data analysis

Data were subjected to one-way analysis of variance (ANOVA) with the nitrogen concentration as source of variation. When the effect of the treatment was statistically significant ($P<0.05$, F-test), means were separated using Least Significant Difference *post-hoc* test at the 5% level (LSD0.05). Linear correlation analysis was performed by the Pearson's correlation test, in order to evaluate the relationship among the different parameters of *Stevia rebaudiana* plants grown under different nitrogen rates. All these statistical analyses were carried out with GraphPad Prism 6 statistical package

Results

Biometric and productive characteristics

An increase in plant height was found at increasing N doses, whereas the number of branches per plant was significantly lower as compared with the control (Table II). The beneficial effect of nitrogen was observed in the yield components. In fact, the leaf dry yield was about 6 g $plant^{-1}$ in plants grown without N but it significantly increased in N150 and N300 treatment, reaching 16 and

19 g plant^{-1} respectively (Table II). However, the lowest harvest index (HI = the ratio between leaf dry yield and total above-ground dry yield) was found in plants grown with the highest N dose, to indicate a greater stem production and development in comparison to the other N conditions. These results, suggest that in stevia, the increase of HI was associated with a decrease of plant height and plant lateral branches, with a partitioning of dry matter in favour of an increased leaf yield per plant.

Pigment concentration

Both Chl *a* and *b* contents were significantly higher in leaves of plants grown with 300 kg N ha^{-1} as compared to the other treatments and, consequently, a similar pattern was observed for total Chl content (Table III). This induced also a significant decrease in Chl *a/b* ratio in leaves of plant grown with the highest N concentration, suggesting that, in stevia, the Chl *a/b* ratio was mainly determined by the amount of Chl *b* rather than Chl *a*. As a general trend, it is possible to observe that the Chl *a/b* ratio decreased both in absence or in excess of nitrogen, which represented two different stress conditions for the plant. The carotenoid content was similar at 0 and 50 kg N ha^{-1} and it significantly increased at the higher N doses (Table III).

Leaf nitrogen concentration (LNC), specific leaf nitrogen (SLN) and photosynthetic nitrogen use efficiency (PNUE)

No differences in leaf nitrogen concentration (LNC) was found between plants grown with 0 or 50 kg ha^{-1} but LNC increased significantly in both N150 and N300 (Table IV), even if the highest value of specific leaf nitrogen (SLN) was found in leaves grown with the highest nitrogen dose. The highest PNUE value was found in plants grown with 150 kg N ha^{-1} while the lowest value was observed with the highest N dose.

Gas exchange, Rubisco and Chl fluorescence measurements

Light-saturated photosynthesis per unit area (A_{max}) increased linearly with N amount until 150 kg ha^{-1} (Figure 1A). At the highest N concentration (N300) A_{max} was similar to that of plants grown without nitrogen (N0). Stomatal conductance (Gs) and transpiration rate (E) were higher in N150 plants as compared to the other treatments, among which no differences were found (Figures 1B and 1C). Intercellular CO_2 concentration was similar in leaves grown with 150 kg N ha^{-1} and without nitrogen (Fig. 1D). Lower values were found in leaves grown with 50 kg N ha^{-1}.

The lowest Rubisco activity was found in leaves of stevia plants grown without nitrogen, followed by the N50-treated plants, while plants grown with 150 kg N ha^{-1} showed the highest value (Figure 2).

No significant differences were found in F_v/F_m ratio values in plants grown with nitrogen but this ratio significantly decreased in leaves grown without N (Figure 3A). N150 plants had higher actual PSII efficiency (Φ_{PSII}), as compared to the other N doses (Figure 3B) whereas plants grown without nitrogen showed the lowest Φ_{PSII} values. On the other hand,the mechanisms aimed to dissipate excexx of excitation energy as heat (NPQ) were higher in leaves of N0 and N50 plants and it decreased significantly in leaves of N150 and N300 plants (Figure 3C).

Steviol glycosides

Both stevioside and the total SGs content were significantly highest in the leaves of plants grown with 300 kg N ha^{-1}, followed by N150, N50 and N0 treatments (Figure 4). The plants grown with 300 kg ha^{-1} showed also the greatest rebaudioside C content, while this compound was at the same amount in plants grown with the other N rates. On the contrary, rebaudioside A was significantly highest in the leaves of plants grown with 150 kg ha^{-1}, with the lowest value in plant grown without nitrogen. Consequently, the best rebaudioside A/stevioside ratio was recorded for the plants grown under 150 kg N ha^{-1} (Figure 4).

Correlation among different parameters

Interestingly leaf pigment and total SGs content was significantly and positively correlated with leaf N concentration (Table V). A strong correlation was found also between chlorophyll *a* and *b*, as well as between both chlorophylls and leaf dry yield and PNUE. Chl *a*/*b* ratio and PNUE were also positively correlated. CO_2 photoassimilation in light-saturated conditions was significantly related with the rebaudioside A/stevioside ratio and PNUE. Leaf dry yield was significantly related with all parameters with the exception of A_{max}, rebaudioside A/stevioside ratio and HI. HI was significantly correlated only with total Chl and SGs content.

Discussion

This study was aimed to fill the lack of scientific information related on stevia response to different nitrogen regime, in terms of leaf biomass, SGs, CO_2 gas exchange and related physiological aspects. In N deficiency conditions, stevia plants showed a reduced size, the lowest branch number and the

lowest leaf dry yield. As expected, the yield production increased significantly with N applications as widely reported by other authors (Das et al. 2007; Blumenthal et al. 2008; Patil 2010; Aladakatti et al. 2012). The behaviour of stevia regarding to N availability is similar to other crops showing the highest SLW in plants grown under maximum N rate (300 kg N ha^{-1}) suggesting that leaf thickness was altered. Consequently, the highest specific leaf nitrogen significantly increased under the same conditions. However, in the plants subjected to the highest N dose, the photosynthetic nitrogen use efficiency was the lowest, to indicate a poor nitrogen utilization in relation to the carbon assimilation by the plant. Taking into account the PNUE data, the optimal dose appears to be 150 kg N ha^{-1}.

N deficiency affected several components of the carbon metabolism in stevia plants. The response of leaf photosynthesis is largely dependent on the leaf N content. Low levels of leaf N reduced both Chl *a* concentration and *A*max. The photosynthetic pigments (chlorophylls and carotenoids) significantly increased with N rates, while the Chl *a/b* ratio was lowest in N300-treated plants, followed by N0. According to Hikosaka and Terashima (1995), the Chl *a/b* ratio decreased with the increase in N availability. In our study, the results showed that, in stevia, the Chl *a/b* ratio decreased in response to strong N limitation (N0) or to N excess (N300), that can represent two limiting conditions for stevia plants.

Photosynthetic rate is generally closely correlated to foliar N concentration (Makino 2011). Our data support the hypothesis that the magnitude of photosynthetic rate increases as N supply increases. In fact, the leaf photosynthetic CO_2 assimilation was remarkably improved by 150 Kg N ha^{-1} rate and is the reason of the best N use efficiency found in these plants. This high photosynthetic rate is related to a high stomatal conductance indicating an optimal water use efficiency in plants grown at 150 kg N ha^{-1}. Conversely, plants under the highest N dose, showed a lower CO_2 photoassimilation rate, attributable to a decrease in stomatal conductance, even though no alteration in photosynthetic capacity of mesophyll cells was observed (i.e., values of Ci similar to control).

The relationship between photosynthesis and plant growth is not simple and it has been debated for many years (von Caemmerer & Evans 2010; Zhu et al. 2010; Parry et al. 2011). It is often believed that enhancing photosynthesis at the level of the single leaf would increase crop yields (Long et al. 2006). On the other hand, a lack of correlation between photosynthesis and plant yield has been frequently observed for different species, such as wheat and rice (Makino 2011). These different relationships are mainly due to natural genetic variation (that occurs in both crop and wild species under field conditions) in plant photosynthesis as well as to the interaction of

photosynthetic phenotypes with their environment (Flood et al. 2011). Our results underline that in stevia there is a lack of correlation between leaf yield (the economic yield of this species) and photosynthetic rate per unit of leaf area. However, leaf nitrogen concentration and chlorophyll content (Chl *a*, *b* and total) *versus* leaf dry yield positively correlated , as well as HI and total Chl content.

A number of studies have shown that Chl fluorescence is a good indicator of nutrient deficiency/excess (Tremblay et al. 2012; Donnini et al. 2013; Schmidt et al. 2013). Application of this technique in precision agriculture can help to avoid excess fertilizer application while assuring optimal productivity (Chaerle et al. 2007). The studies have focused on nitrogen (N) availability in consequence of its largest amount needed during plant development. Actually, most of the N (50–80%) in the leaf has a role in photosynthesis as component of the complexes involved in electron transport and, overall, for Rubisco enzyme (Langsdorf et al. 2000). Data from Chl fluorescence analysis evidenced as only the N deficiency (N0) induced a decrease in the maximum potential efficiency for PSII photochemistry, F_v/F_m ratio. In light conditions, however, the efficiency of PSII was higher in plants under 150 kg N ha^{-1} as compared to the other ones. To confirm an altered PSII activity in plants grown without N or in N deficiency (N50) the non-photochemical quenching parameter NPQ significantly increased indicating that need to increase the mechanisms involved in the dissipation of the excess of excitation energy as heat. On the other hand, Rubisco activity was significantly high in N150-treated stevia plants and the lowest value was recorded in plants grown without N. The high Rubisco activity accounts for the high CO_2 photoassimilation and the high Φ_{PSII}. Definitively, plants grown with 150 kg N ha^{-1} showed an improvement in the use of N and in the photosynthetic activity. These best crop performances observed in N150-treated stevia plants, were also reflected in an improvement of the quality of the production, since they revealed the highest rebaudioside A content and the Reb A/stevioside ratio. Stevioside is the substrate for the synthesis of rebaudioside A (Shibata et al. 1991) and an optimal N rate during stevia growth, probably leads to high rebaudioside A amount in plants, even though low levels of stevioside. Due to the increasing importance of stevia, some studies have reported on its nutrient requirements (Ramesh et al. 2006; Aladakatti et al. 2012), but none focused on the association between nutrient supply and SGs accumulation. Our results demonstrate that using an appropriate N rate it is possible to increase significantly the content of rebaudioside A and, consequently the ratio between rebaudioside A and stevioside. These results suggest that nitrogen fertilization could modulate the composition of steviol glycosides for its function of promoting the transformation of stevioside to rebaudioside A. So, the possibility to shift, through the N fertilisation, the biosynthesis of SGs

towards Rebaudioside A represents a finding of pivotal importance, in order to obtain plants characterized by high levels of this steviol glycoside.

The accumulation of steviol glycosides in cells of stevia both *in vivo* and *in vitro*, was related to the extent of the development of the membrane system of chloroplasts and the content of photosynthetic pigments (Ladygin et al. 2008). To confirm this statement, a positive correlation between total chlorophyll content and total SGs was found, indicating that the increase in chlorophyll content could directly affect steviol glycoside production in chloroplast of stevia as already observed by Jain et al. (2009). In addition, the significant correlation between the leaf yield and the content of the SGs was recorded, in agreement with Bondarev et al. (2003/4). Furthermore a positive correlation was also found between SGs and HI and between SGs and LNC.

This study underlines that N fertilizer had a consistent effect on stevia productivity and quality, since the importance of nitrogen on SGs content and leaf growth as well as on photosynthetic CO_2 assimilation. In stevia, nitrogen deficiency caused a decrease in plant growth, leaf N content, chlorophylls and photosynthetic CO_2 assimilation, resulting in a lower dry matter accumulation as well as in a reduced steviol glycoside production. Definitively, the application of 150 kg N ha^{-1} seems to be the most effective treatment to improve rebaudioside A content, rebaudioside A/stevioside ratio, photosynthetic CO_2 assimilation, stomatal conductance, photosynthetic nitrogen use efficiency, Rubisco activity and efficiency of PSII. Conversely, the crop quality were not been improved by greater N amounts, that may lead to problems of leaching and runoff of nitrates.

This study represents a first step towards the optimisation of the nitrogen fertilisation in stevia defining the best conditions to obtain positive effects on rebaudioside A content and rebaudioside A/stevioside ratio. These findings could play an important role in order to produce raw material characterized by high quality level, under optimal nitrogen conditions and in a sustainable manner. This is in line with market trends, which require final products more environmentally friendly compared to the existing alternatives, characterized at the same time by a high content of rebaudioside A and, consequently, a low or absent aftertaste due to the presence of stevioside.

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Figure Captions

Figure 1. CO_2 assimilation rate (A_{max}; A), stomatal conductance (Gs; B), transpiration rate (E; C) and intercellular CO_2 concentration (Ci; D) in leaves of *Stevia rebaudiana* plants grown under different nitrogen rates. The data represent the mean of 3 replicates.

Figure 2. Rubisco activity in leaves of *Stevia rebaudiana* plants grown under different nitrogen rates. The data represent the mean of 3 replicates.

Figure 3. Maximal (F_v/F_m; A) and actual photochemical PSII efficiency (Φ_{PSII}; B) and non photochemical quenching (NPQ; C) in leaves of *Stevia rebaudiana* plants grown under different nitrogen rates. The data represent the mean of 9 replicates.

Figure 4. Effect of different nitrogen rates on stevioside, rebaudoside A, rebaudioside C and total SGs content (A) and on the rebaudioside A/stevioside ratio (B) in leaves of *Stevia rebaudiana* plants. The data represent the mean of 3 replicates.

Table I. Mean physical and chemical characteristics (± standard deviation) of the soil used for pot trials, before transplanting, before vegetative re-growth.

Physical and chemical characteristics	Value
Sand (2-0.05mm, %)	71.97
Silt (0.05-0.02 mm, %)	23.75
Clay (<0.002 mm, g kg^{-1})	4.28
pH (H_2O 1:2.5 soil:water suspension; McLean method)	8.08
Organic matter (Walkley–Black method, g kg^{-1})	1.47
Total nitrogen (Kjeldhal method, g kg^{-1})	0.56
Available phosphorus (Olsen method, mg kg^{-1})	11.84
Exchangeable potassium (Thomas method, mg kg^{-1})	150.36
Cation exchange capacity (method $BaCl_2$, pH 8.1, meq/100 g)	14.82

Table II. Biometric characteristics and yield components in *Stevia rebaudiana* plants grown under different nitrogen rates. The data represent the mean of five replicates.

Treatment	Plant height (cm)	Branches (number plant^{-1})	Leaf dry yield (g plant^{-1})	Harvest index (HI)
N0	64.00	1.33	6.28	0.63
N50	76.00	4.00	8.70	0.63
N150	85.00	4.00	15.83	0.60
N300	88.67	3.00	18.83	0.56
$LSD_{0.05}$	16.728	1.476	2.306	0.040

LSD, least significant difference.

Table III. Photosynthetic pigments in leaves *Stevia rebaudiana* plants grown under different nitrogen concentration. The data represent the mean of six replicates.

Treatment	Chl *a* (μg cm^{-2})	Chl *b* (μg cm^{-2})	Chl tot (μg cm^{-2})	Chl *a*/ Chl *b*	Carotenoids (μg cm^{-2})
N0	18.93	10.04	28.00	1.89	5.24
N50	20.48	9.88	29.86	2.07	5.58
N150	22.98	10.81	32.69	2.13	7.16
N300	36.34	22.35	56.67	1.63	7.72
$LSD_{0.05}$	3.793	2.420	5.146	0.276	1.397

LSD, least significant difference.

Table IV. Leaf nitrogen concentration (LNC), specific leaf nitrogen (SLN) and photosynthetic nitrogen use efficiency (PNUE) in *Stevia rebaudiana* plants grown under different nitrogen concentration. The data represent the mean of three replicates.

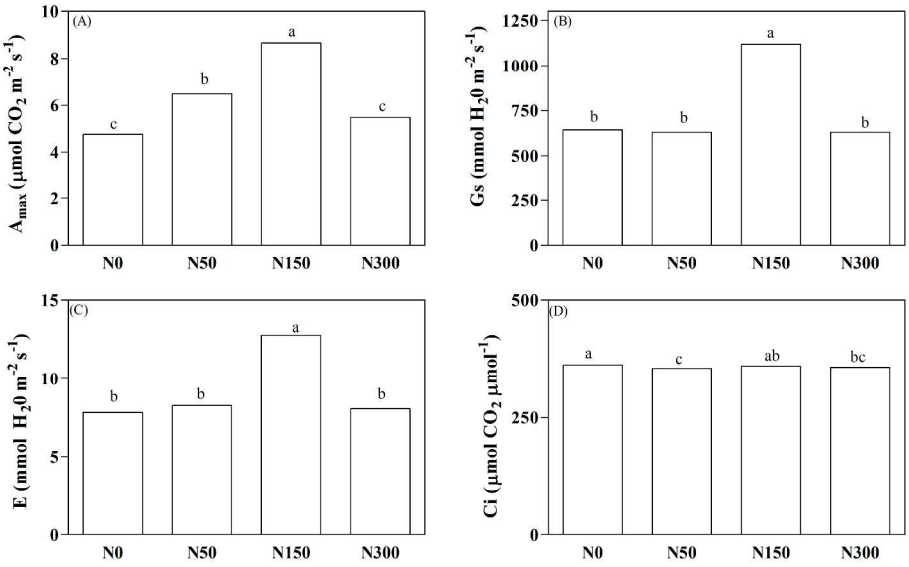
Treatment	LNC ($mg\ g^{-1}$)	SLN ($g\ N\ m^{-2}$)	PNUE ($\mu mol\ CO_2\ \mu mol\ N^{-1}\ s^{-1}$)
N0	5.68	0.23	0.286
N50	5.95	0.31	0.291
N150	7.55	0.31	0.397
N300	8.21	0.39	0.196
$LSD_{0.05}$	0.938	0.046	0.036

LSD, least significant difference.

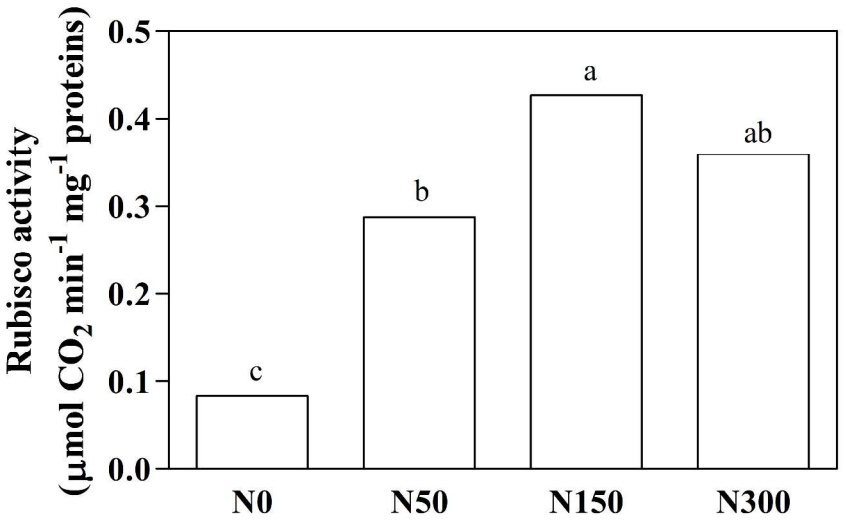
Table V. Simple correlation coefficients and significance levels among the different characters of *Stevia rebaudiana* plants grown under different nitrogen rates.

Character	LNC	Chl *a* content	Chl *b* content	Chl *tot* content	Chl *a/b* ratio	A_{max}	Total SG content	Reb A / stevioside	Leaf dry yield	PNUE	HI
LNC	1	0.709[a]	0.641[a]	0.621[a]	0.032[b]	0.107 [b]	0.832[a]	0.256 [b]	0.968[a]	0.134 [b]	0.357 [b]
Chl *a* content		1	0.901[a]	0.764[a]	0.183 [b]	0.038 [b]	0.351 [b]	0.0002 [b]	0.589[c]	0.564[c]	0.266 [b]
Chl *b* content			1	0.868[a]	0.292 [b]	0.052 [b]	0.335 [b]	0.008 [b]	0.561[c]	0.672[a]	0.333 [b]
Chl *tot* content				1	0.228 [b]	0.035 [b]	0.448[d]	0.002 [b]	0.722[a]	0.482[c]	0.422[d]
Chl *a/b* ratio					1	0.251 [b]	0.051 [b]	0.155 [b]	0.004 [b]	0.540[c]	0.222 [b]
A_{max}						1	0.274 [b]	0.816[a]	0.133 [b]	0.469[d]	0.001 [b]
Total SG content							1	0.445[d]	0.843[a]	0.034 [b]	0.515[c]
Reb A / stevioside								1	0.328 [b]	0.287 [b]	0.048 [b]
Leaf dry yield									1	0.079 [b]	0.326 [b]
PNUE										1	0.124 [b]
HI											1

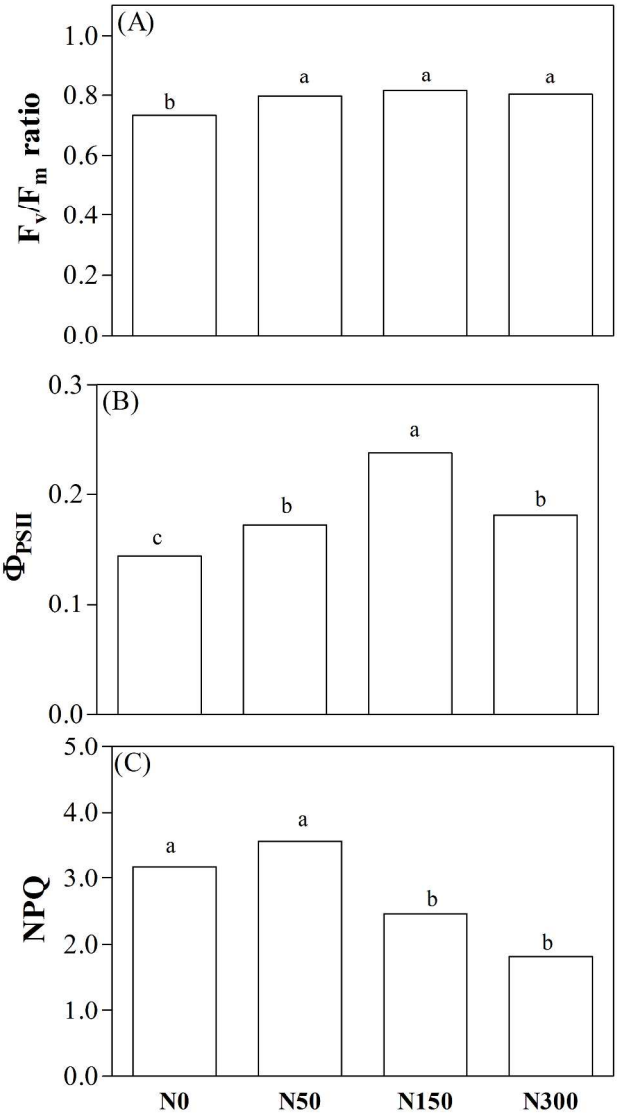
[a] Significant at $P \leq 0.001$.
[b] ns, not significant.
[c] Significant at $P \leq 0.01$.
[d] Significant at $P \leq 0.05$.



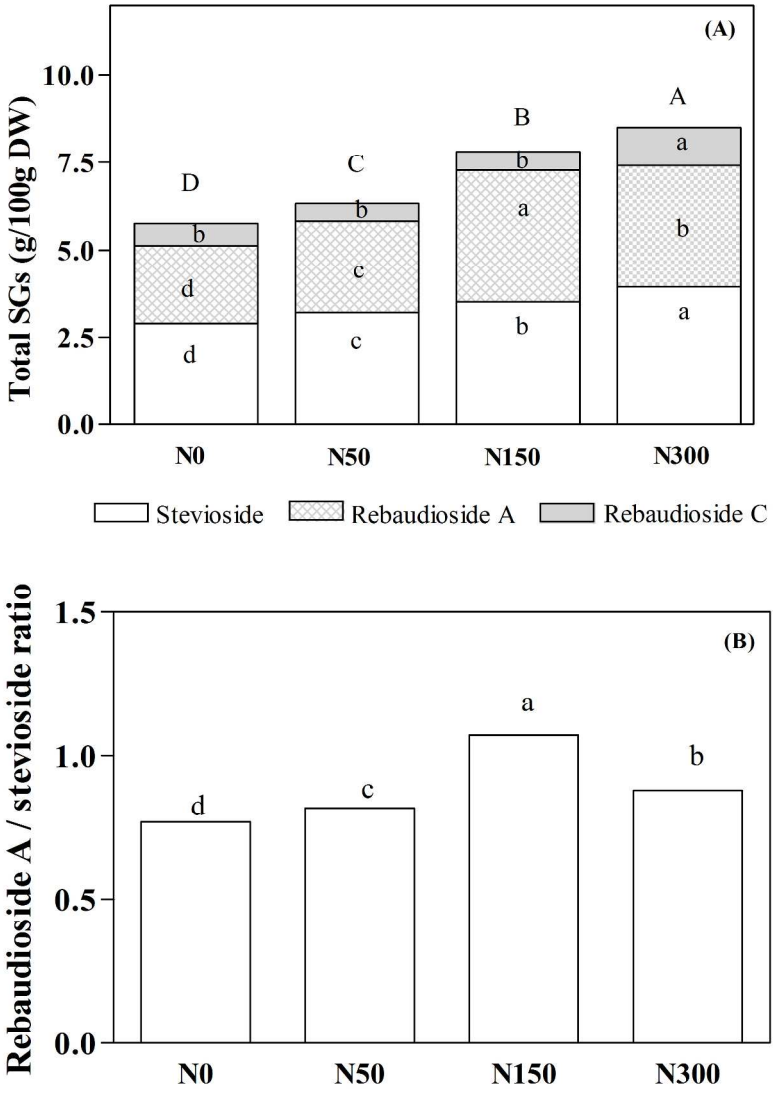
CO2 assimilation rate (Amax; A), stomatal conductance (Gs; B), transpiration rate (E; C) and intercellular CO2 concentration (Ci; D) in leaves of Stevia rebaudiana plants grown under different nitrogen rates. The data represent the mean of 3 replicates
406x249mm (300 x 300 DPI)



Rubisco activity in leaves of Stevia rebaudiana plants grown under different nitrogen rates. The data represent the mean of 3 replicates
382x226mm (300 x 300 DPI)



Maximal (Fv/Fm; A) and actual photochemical PSII efficiency (ΦPSII; B) and non photochemical quenching (NPQ; C) in leaves of Stevia rebaudiana plants grown under different nitrogen rates. The data represent the mean of 9 replicates
230x366mm (300 x 300 DPI)



Effect of different nitrogen rates on stevioside, rebaudoside A, rebaudioside C and total SGs content (A) and on the rebaudioside A/stevioside ratio (B) in leaves of Stevia rebaudiana plants. The data represent the mean of 3 replicates.
268x347mm (300 x 300 DPI)