1 **Effect of nitrogen fertilization and harvest time on steviol glycosides, flavonoid composition**

2 **and antioxidant properties in *Stevia rebaudiana* Bertoni**

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ABSTRACT

This work investigated the effect of nitrogen fertilization and harvest time on flavonoid composition and antioxidant properties of *Stevia rebaudiana* leaves. At the same time, changes in stevioside (Stev) and rebaudioside A (RebA) contents were recorded. A pot trial under open air conditions was set up, testing five N rates and three harvest times. The results showed that, by using an adequate N rate and choosing an appropriate harvest time, it was possible to significantly increase and optimize the bioactive compound levels. In particular, higher RebA, RebA/Stev ratio, total phenols and flavonoids, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside levels, and antioxidant capacity, were recorded by supplying 150 kg N ha^{-1}. Reduced or increased N availability in comparison with N150 had no consistent effect on Stevia phytochemicals content. Significant correlations were also found between stevioside and some of the flavonoids, indicating a possible role of flavonoids in the stevioside metabolic pathway, which deserves more investigations.

KEY WORDS

Stevia rebaudiana Bertoni; nitrogen, flavonoid composition, steviol glycosides, antioxidant activity.

INTRODUCTION

In the past few years, the demand for both dietetic products and natural food ingredients is continuously increasing. Much attention has been focused on dietary natural antioxidants[1], capable to inhibit reactive oxygen species (ROS), which are involved in determining several human pathologies, such as cancer, arteriosclerosis, diabetes, cardio-vascular diseases and degenerative illness connected to the aging process. At the same time, the increasing health consciousness and concern over the consumption of sugar and the problems related to the safety of some artificial non-nutritive sweeteners (NNS), have stimulated the interest toward natural sweetners.[2] Stevioside (Stev) and other steviol glycosides extracted from the leaves of the plant *Stevia rebaudiana* Bertoni were the first natural high-potency sweeteners to be approved for consumption in the United States, the European Union[3], as well as in Australia and new Zealand. *Stevia rebaudiana* Bertoni is a perennial shrub belonging to the *Asteraceae* family, which is native of the Amambay region, in the northeastern Paraguay. It represents an interesting species for the development of new ingredients characterized by a low caloric contribution and a high antioxidant and phytochemical properties.[4] Steviol glycosides (SG), more than 30 *ent*-kaurene-type diterpene glycosides, including Stev, rebaudiosides A-G, rubusoside, dulcoside A, account for about 4-20 % of the dry weight of the leaves.[4,5] Native people from South America have been using Stevia extract as sweetener and traditional medicine for several hundred years. The great advantages of SG are especially for those who suffer from several kinds of pathologies, such as obesity, diabetes mellitus, heart diseases and dental caries.[6-9] Furthermore, Stevia leaves show many pharmacological properties since they contain important constituents, such as minerals, vitamins, phenolic compounds, flavonoids, alkaloids, water soluble chlorophylls, xanthophylls and hydroxycynnamic acids, with potential beneficial effects on human health.[4, 9-12] Particularly important for their high antioxidant capacity in the Stevia leaf extract are flavonoids belonging to the subgroups of flavonols and flavones.[9] These unique properties make Stevia a good candidate for pharmaceutical, food, and beverage industries. The promotion and maintenance of high

56 standards of product quality in terms of secondary metabolites content, may be the key for a possible
57 commercial expansion of Stevia production all over the world. To achieve this goal, specific studies
58 closely related to the geographical areas are needed, e.g. the accurate evaluation of crop responses to
59 both growth conditions and management, and the identification of their best combinations.

60 It is well known how the biosynthesis of secondary metabolites in medicinal plants is strongly
61 influenced by several pre-harvest factors, such as environmental conditions, agricultural
62 management, harvest time, water and nutrient requirements. Among these different factors, nitrogen
63 fertilization, and thus plant N status, plays a key role in defining the concentrations of secondary
64 compounds. Generally, the plants fertilized with high nitrogen levels tend to increase their
65 photosynthesis, enhancing consequently their biomass.[15] However, previous works showed several
66 conflicting results regarding the effects of N fertilization on the plant antioxidant concentrations and
67 capacities.[16] To best of our knowledge, till now no study has investigated the effect of nitrogen
68 fertilization on flavonoid composition as well as on antioxidant activity of *S. rebaudiana*. Moreover,
69 information on the effects of N on SG content is scanty. In the 1970s, Japanese researchers
70 investigated the effects of N, as well as of P and K, on SG levels[17-20]. These authors found that Stev
71 content was not affected by the presence or absence of nitrogen fertilizer[17] and no effect of P and K
72 on this SG was shown [18]. Similarly, rebaudioside A was not affected by supply of different
73 concentrations of N, P and K. [19,20] Thus, according to the importance of phytochemicals and
74 antioxidant power for functional aspect of Stevia, the aim of this study was to evaluate the effects of
75 N supply on SG content, total phenols and flavonoid composition, as well as on total antioxidant
76 capacity in Stevia leaves. At the same time, the effects of harvest time, as well as of N supply x
77 harvest time interaction, were also investigated, with the aim to define the best conditions to maximize
78 the levels of beneficial bioactive compounds in Stevia leaves.

79

80 **MATERIALS AND METHODS**

81 **Chemicals.** Ethanol, methanol and ferrous sulfate were purchased from Carlo Erba SpA
82 (Milan, Italy). Water and acetonitrile were obtained from JT Baker (Phillipsburg, NJ, USA). Pure
83 Stev (99.9% purity) and pure Reb A (97.4% purity) were purchased from Extrasynthese (Genay,
84 France). DPPH (2,2-diphenyl-1-picrylhydrazyl), gallic acid monohydrate (3,4,5-trihydroxybenzoic
85 acid), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), Trizma acetate, Folin–Ciocalteu reagent, sodium
86 carbonate and ferric chloride were obtained from Sigma-Aldrich Chemical Co. (Milan, Italy). The
87 standards luteolin-7-*O*-glucoside, rutin, myricetin, apigenin-7-*O*-glucoside, quercitrin, kaempferol
88 were purchased from Extrasynthèse (Genay, France). All reagents and solvents used were analytical
89 or high-performance liquid chromatography (HPLC) grade. All solvents and water were accurately
90 degassed before being used in the analyses.
91 **Plant material and experimental conditions.** A pot trial was carried out at the Experimental
92 Centre of Department of Agriculture, Food and Environment (DAFE) of the University of Pisa,
93 located in San Piero a Grado, Pisa, Italy (43°40'N; 10°19'E, 5 m above sea level), during the 2010
94 growing season. A selected clone (RG) of *Stevia rebaudiana*, belonging to the DAFE germplasm
95 collection, was used. The plants were obtained by stem cuttings taken from 3-years old parental plants
96 grown in the open field at the DAFE experimental centre. The stem cuttings, 4-5 cm long, were placed
97 into 20 L pots to allow the maximum development of the roots, and then filled with sandy-loam and
98 low fertility soil (68.5% sand; 25.9% silt; 5.6% clay; pH 7.74; organic matter 0.83%; total N 0.32 g
99 kg^{-1}; available P 11.49 mg kg^{-1}; exchangeable K 44.84 g kg^{-1}; 1.65 g cm^{-3} bulk density). The trial was
100 conducted in open air from May to September. The area has a Mediterranean climate, with rainfall
101 mainly concentrated in the autumn and spring (mean 948 mm $year^{-1}$). Mean maximum and minimum
102 temperatures in the growing season were 26.9°C and 13.1°C, respectively.
103 Stevia plants were grown under different rates of nitrogen: N0 (without N fertilization); N50
104 (50 kg N ha^{-1} equal to 0.4 g N per pot as ammonium nitrate); N150 (150 kg N ha^{-1}, equal to 1.2 g N
105 per pot as ammonium nitrate), N300 (300 kg N ha^{-1}, equal to 2.4 g N per pot as ammonium nitrate)

106 and Norg (150 kg N ha^{-1}, equal to 1.2 g N per pot as organic nitrogen from Nutex N7 based on wool
107 wastes, poultry manure, blood and pomace with 7% organic N and 35% organic carbon).

108 The pots were arranged in a completely randomized block design with five replications (1 pot
109 per replicate) for each treatment. Starting from the middle part of May, during the vegetative growth,
110 the nitrogen was split in four applications every 30 days. In addition, a mix of macro (Mg) and
111 microelements (Bo, Cu, Fe, Mn, Mo, Zn) was supplied, at the dose of 0.1 g L^{-1} per pot. A constant
112 source of phosphorus and potassium was distributed to all treatments at the rate of 100 kg ha^{-1} of P
113 and K, respectively (equal to 0.80 g P per pot as triple superphosphate, and 0.80 g K per pot as
114 potassium sulphate), before transplanting the plants. Water (20 m^3 ha^{-1}) was supplied to all pots to
115 facilitate transplanting recovery. During the trial, the plants were maintained under optimal water
116 supply through a drip irrigation system in order to maintain soil moisture to 75-80% of field capacity.
117 No pests and diseases have been observed during the trial.

118 **Crop sampling.** Three different samplings of the leaves were carried out in order to evaluate
119 the dynamic of accumulation of bioactive compounds in Stevia leaves. Leaf samplings were
120 accomplished at the beginning and at the end of July, during the vegetative growth (H1 = July, 9th,
121 H2 = July, 21st), and at the beginning of September, when the plants started flowering (H3 =
122 September 10th). After each sampling, the leaves were air-dried in a ventilated oven at 40°C until
123 constant weight (8% residual water content) and ground to fine powder using a laboratory mill.
124 Samples were used to determine SG content, total phenols, flavonoid composition and antioxidant
125 capacity.

126 **Preparation of extracts.** Samples (0.5 g powder) were randomly chosen from each plant
127 treatment and placed in 250 mL Erlenmeyer flasks. Extracts were sonicated for 30 min at 60°C with
128 50 mL 70% (v/v) ethanol. Before analysis, extracts were passed through a 0.45 μm nylon filter
129 attached to a syringe to remove any suspended material.

130 **Steviol glycoside content.** The procedure was performed following the method described by
131 Hearn and Subedi[21] and Kolb et al.[22] Briefly, 20 µL of filtered extract were injected into a HPLC
132 system (Jasco PU980) coupled with a UV-visible wavelength detector. A LiChrospher NH2 column,
133 5µm, 250 mm x 4.6 mm (Alltech Italia), in conjunction with LiChrospher Amino All-Guard and All-
134 Guard Cartridge Holder (Alltech Italia) was used. The following HPLC operating conditions were
135 used: an isocratic mobile phase, acetonitrile/water (80/20), pH 5 adjusted with acetic acid, a flow rate
136 of 1.0 mL/min and a run time of 20 min. Detection was at 210 nm at ambient temperature.
137 Chromatograms were acquired online and data were collected via a Jasco interface (Hercules 2000
138 Interface Chromatography). The identity of SG was confirmed by chromatography on HPLC with
139 authentic standards and quantification was performed using a standard curve in the range 0.25-1 g/L
140 of standard mixtures containing Stev and Reb A.

141 **Total phenolic content.** Determination of total phenolic compounds was performed on
142 ethanolic extracts by the Folin–Ciocalteu method according to Dewanto et al.[23] This procedure
143 involves the reduction of Folin–Ciocalteu reagent by phenolic compounds, with concomitant
144 formation of a blue complex determined at 765 nm by UV–visible spectrophotometer (Varian Cary
145 1E, Palo Alto, CA U.S.A.). Calculations were performed using a calibration curve prepared with
146 gallic acid as standard and results were expressed as mg gallic acid equivalents (GAE)/g DW.

147 **Ferric-reducing antioxidant power (FRAP) assay.** The FRAP method, based on the
148 reduction of the ferric tripyridyltriazine complex (Fe^{3+} - TPTZ) by antioxidants, was carried out
149 according to Benzie and Strain[24] with slight modifications. Briefly, 2 mL of freshly prepared FRAP
150 reagent, containing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution (in 40 mM HCl) and 20
151 mM ferric chloride in 10:1:1 (v/v/v) ratio, was added to a known aliquot of Stevia leaf ethanolic
152 extract. After incubation at 37° C for 10 min absorbance at 593 nm was recorded. Ferrous sulphate
153 was used as a standard (0-1500 µM). Total antioxidant capacity was expressed as µmol Fe^{2+} g^{-1} DW.

154 **DPPH radical-scavenging assay**. The free radical-scavenging activity of Stevia leaf extracts was
155 evaluated by the DPPH free radical method according to Tadhani et al.[25] Briefly, 200 μL of ethanolic
156 extract was placed in a test tube and the volume was made up to 1mL with methanol. Then 3mL of
157 freshly prepared DPPH solution (200 μmol L^{-1} in methanol) was added and the tube contents were
158 mixed vigorously. The tube was subsequently kept in a water bath at 37°C for 20 min, after which
159 the absorbance of the sample was measured at 517nm using a UV–visible spectrophotometer (Varian
160 Cary 1E, Palo Alto, CA U.S.A.). The decrease in absorbance at 517nm was measured against a blank
161 of pure methanol to estimate the radical scavenging capacity of the samples. Radical-scavenging
162 activity was calculated as the inhibition of the free radical by the sample using the following formula:
163 $\% \text{ inhibition } (\% \text{ I}) = [(A0 - At) / A0] \times 100$
164 where A0 is the absorbance of the control DPPH solution at 0min and At is the absorbance in the
165 presence of the extract after t =20min. The extract concentration providing 50% of radicals
166 scavenging activity (IC_{50}) was calculated from the graph of inhibition percentage against extract
167 concentration. The results were also expressed as ascorbic acid equivalent.
168 **Flavonoid determination.** Qualitative and quantitative analyses were performed by RP-
169 HPLC. 20 μL of extract were injected into a Waters model 515 HPLC system (Waters, Milford, MA)
170 fitted with a 4.6 mm x 250 mm Prodigy ODS column (Phenomenex, Bologna, Italy). Detection was
171 carried out at 360 nm, using a Waters 2487 dual λ UV-visible detector. Mobile phase A contained
172 water acidified with formic acid (pH 2.7), and mobile phase B contained methanol. A linear gradient
173 of 10-90% mobile phase B was run for 26 min at 1 mL/min. The identity of the free flavonoids was
174 confirmed by co-chromatography with authentic standards. Quantification was performed using
175 standard curves in the range of 10-500 ng of a standard mixture containing luteolin-7-*O*-glucoside,
176 rutin, myricetin, apigenin-7-*O*-glucoside, quercitrin, quercetin, luteolin and kaempferol.
177 Chromatogram analysis was performed by the software Millennium 32 (Waters).

178 **Statistical Analysis**. All data were subjected to analysis of variance (ANOVA) using CoStat
179 Version 6.2 (CoHort Software, Monterey, CA U.S.A.). Two-way completely randomised ANOVA
180 was carried out to estimate the variance components of N supply (N), harvest time (H) and their
181 interaction (NxH). Means were separated on the basis of least significant difference (LSD) only when
182 the ANOVA *F* test showed significance at 0.05 or 0.01 probability level. Finally, linear regression
183 analyses using GraphPad PRISM Version 4.0 (GraphPad Software, Inc., La Jolla, CA U.S.A.) were
184 performed in order to evaluate the relationships between antioxidant capacity, total phenols, total
185 flavonoids and SG. For inhibition percentage data, obtained by the DPPH assay, an arcsine
186 transformation was applied before statistical analysis was performed.
187
188 **RESULTS**
189 Nitrogen fertilization (N), harvest time (H) and their mutual interaction (NxH) significantly
190 affected the Stev and Reb A contents, although the individual compounds showed different behavior
191 (Table 1). The results indicated that the Stev content in the leaf changed with the stage of plant
192 development. Results showed that the Stev content was higher in the vegetative stage (H1), and
193 decreased until the beginning of flowering time (H3). In fact, the maximum Stev content was reached
194 in the leaves collected at the beginning of July (H1) during maximum vegetative development, and it
195 decreased thereafter (Table 1). Regarding to N, the maximum Stev content was observed in plants
196 grown without N (N0), followed by Norg. Taking into consideration NxH effect, the highest Stev
197 contents were recorded in plants grown at N0 and Norg and harvested in H1.
198 In contrast to Stev, the Reb A content increased significantly from the beginning to the end of
199 July, reaching its highest value in the second harvest (Table 1). Considering mean values, also N rate
200 significantly affected Reb A content, with the maximum values in leaves of Stevia plants grown with
201 150 and 300 kg N ha^{-1}. In contrast, considering NxH interaction, at H2 the Norg-treated plants showed

202 the lowest level of this compound whereas no significant differences were observed among the
203 mineral N doses (Table 1).

204 Due to the changes in the two main SG, the RebA/Stev ratio significantly varied depending
205 on N rates, harvest time and NxH interaction. The maximum ratio was recorded in plants harvested
206 at H2 and H3, and in plants grown with 150 and 300 kg N ha^{-1}. In particular, the leaves of plants
207 grown with 150 kg N ha^{-1} and harvested in H2 showed the highest ratio, while the lowest ratio was
208 found in plants grown without N and harvested at H1.

209 Similarly to SG, total phenols and total flavonoids were significantly affected by N rate,
210 harvest time and NxH interaction (Figures 1 A and B). As concern total phenols, the highest values
211 were recovered at H1 in leaves of plants grown with 50 and 150 kg N ha^{-1} (105.50 and 110.41 mg g^{-}
212 ^{1} DW, respectively). At the same harvest time, the N 150-treated plants showed the highest level of
213 total flavonoids (104.03 mg g^{-1} DW). As general trend, by averaging over N-treatments, both phenols
214 and flavonoids were significantly higher in H1 (83.4 and 68.9 mg g^{-1} DW, for phenols and flavonoids,
215 respectively) than H2 (60.8 and 34.7 mg g^{-1} DW, for phenols and flavonoids, respectively) and H3
216 (69.9 and 46.9 mg g^{-1} DW, for phenols and flavonoids, respectively). Considering the N effect, as an
217 average over harvest times, the N50-treated plants showed the highest phenolic content (86.7 mg g^{-1}
218 DW), followed by N150 and N300 treatments (76.5 and 73.9 mg g^{-1} DW, respectively). Otherwise,
219 the N150-treated plants were characterized by the highest flavonoids (58.0 mg g^{-1} DW), followed by
220 N0-treated plants (54.5 mg g^{-1} DW).

221 Among the flavonoids, rutin, myricetin, apigenin-7-*O*-glucoside, quercitrin, kaempferol and
222 luteolin-7-*O*-glucoside have been identified (Table 2). All the flavonoids were significantly affected
223 by the N rate, harvest time and NxH interaction, with the exception of myricetin for which H was not
224 significant. The luteolin-7-*O*-glucoside was the main flavonoid in Stevia leaf extracts, ranging from
225 43% to 74% of the total flavonoids (Table 2), depending on nitrogen rate and harvest time. In
226 particular, it was recovered at higher amounts in H1 compared to the other harvests, reaching the

227 highest content in plant grown with 150 kg N ha^{-1} (74%). In contrast, in H2, luteolin-7-*O*-glucoside
228 content was 2-4 times lower than the one recorded in H1. In H3, the amount of this compound
229 increased in each N treatment in comparison with H2, with the exception of N150 treatment, whereas,
230 under N0 and Norg treatments, it approached again values recorded in H1 (Table 2).

231 Among the identified flavonoids, kaempferol appeared to be present in minor amount,
232 representing the 0.1-2% (Table 2). Myricetin and quercitrin showed values around 2-3 mg g^{-1} DW,
233 representing the 3-9% of the flavonoid total amount. Rutin and apigenin-7-*O*-glucoside values were
234 about 4-5 and 7-10 mg g^{-1} DW, respectively. Apigenin-7-*O*-glucoside showed a value 2-fold higher
235 than rutin, representing the 14% of the total (Table 2). As observed for luteolin-7-*O*-glucoside, both
236 rutin and apigenin-7-*O*-glucoside showed the highest value at H1 under N150 treatment, contributing
237 to determine also the highest value of total flavonoid in these conditions (Figure 1, Table 2). In
238 contrast to what observed for luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, rutin and quercitrin
239 showed no increase from H2 to H3, and the lowest values were recorded in the last harvest.

240 At present, none of the antioxidant capacity assays truly reflect the "total antioxidant capacity"
241 of a particular plant extract, since this measure needs to reflect both lipophilic and hydrophilic
242 capacity, and to differentiate between hydrogen atom transfer (radical quenching) and electron
243 transfer (radical reduction). Consequently, to comprehensively evaluate a sample's ROS scavenging
244 capacity, more than one assays measuring individual ROS scavenging capacity are needed. For this
245 reason, both FRAP and DPPH assay have been performed (Figures 2 A and B). N, H and NxH
246 interaction significantly affected also the antioxidant activity, measured both with FRAP and DPPH
247 assays. According both methods, the higher antioxidant capacities, and consequently the higher
248 presence of bioactive molecules, have been observed in H1 (589 mmol $FeSO_4$ g-^{1} DW and 83 mmol
249 ASA g^{1} DW, for FRAP and DPPH, respectively) and H3 (475 mmol $FeSO_4$ g^{1} DW and 80 mmol
250 ASA g^{1} DW, for FRAP and DPPH, respectively) in comparison with H2 (354 mmol $FeSO_4$ g^{-1} DW
251 and 78 mmol ASA g^{-1} DW, for FRAP and DPPH, respectively). The only exception to this trend was

observed for Norg treatment, where the values recorded in H1 did not differ significantly to those obtained in H2 whereas at H3 they reached the highest values (Figure 2 A, B). As concerns the effect of N rate, the on average highest values, measured by FRAP method, were recorded under N50 and Norg treatments (545 and 536 mmol $FeSO_4$ g^{-1} DW, respectively), whereas, considering the NxH interaction, the highest FRAP values were recorded for N50 and N150 treated plants when harvested at H1 (Figure 2A).

The DPPH radical scavenging has been used widely to test the antioxidant activities of plant extracts and foods. Figure 4 showed the IC_{50} value (the antioxidant concentration necessary to decrease the initial concentration of DPPH by 50%), calculated, for each N dose, pooling data from the three harvest times, and for ascorbic acid, luteolin and BHT, used as positive controls (Figure 3). In DPPH assay, the lower is the IC_{50} value, the higher is its ability to scavenge radicals, particularly peroxy radicals, which are the propagators of the autoxidation of lipid molecules and thereby break the free radical chain reaction.[26] Our results showed that the Stevia leaf extracts were potent radical scavengers. In fact, notwithstanding their IC_{50} values were higher than those of ascorbic acid and luteolin, they were lower than BHT (Figure 3).

In this work, significant high correlations between secondary metabolites of Stevia leaves and antioxidant capacities were found by both antioxidant measuring methods (Table 3). In particular, positive correlations were found among antioxidant activity and total phenols and flavonoids for N50, N150 and N300 treatments (Table 3). In addition, it was demonstrated that the antioxidant activity, measured by both assays, was negatively correlated with the amount of Reb A for N0 treatment, and positively correlated with the Stev content under N150 treatment. In the plants grown under Norg treatment, total flavonoid, Stev and Reb A contents did not correlate with the antioxidant activities. In addition, interesting positive statistic correlations have been found in N150-treated plants between Stev and total flavonoids, rutin, myricetin, apigenin-7-*O*-glucoside, quercitrin, and luteolin-7-*O*-glucoside (Table 4). In contrast, no significant correlation was observed in N150-treated plants either

277 between Reb A and total flavonoids or between Reb A and individual flavonoids (Table 4). Moreover,
278 Reb A was negatively correlated at N0 and N50 with total flavonoid contents, apigenin-7-*O*-
279 glucoside, and luteolin-7-*O*-glucoside (Table 4). Noteworthy, regarding the two latter N doses Stev
280 did not in general correlate either with either total or individual flavonoids.

281

282 **DISCUSSION**

283 The achieved results showed that most of the phytochemical contents, as well as the
284 antioxidant capacity, were significantly affected by N rate, harvest time and the NxH interaction,
285 underlining the importance of these pre-harvest factors in the biosynthesis of Stevia secondary
286 metabolites and, consequently, in determining the Stevia leaf extract quality. Considering the mean
287 N values, our findings point out that 150 kg N ha^{-1} appeared to be the optimal dose giving an
288 improvement of the quality, since, for this N rate, the highest contents of Reb A, Reb A/Stev ratio,
289 total phenol and flavonoid, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside and antioxidant activity
290 have been recorded.

291 Our study demonstrates that, using an adequate N rate and choosing an appropriate harvest
292 time, it is possible to increase significantly the content of Reb A and, consequently the ratio between
293 RebA/Stev, considered to be a good qualitative measure of sweetness.[27] The maximum Stev content
294 was recorded in N deficiency conditions (N0), while, in presence of higher N supply, the Stev
295 decreased and Reb A significantly increased (N150 and N300). Since Stev is the substrate for the
296 synthesis of Reb A[28], our findings indicated that N supply could modulate the composition of SG,
297 promoting the transformation of Stev in Reb A. At the same time, the harvest time was found to be
298 an extremely important factor in affecting the SG content and, consequently, Reb A/Stev ratio, that
299 increased in the Stevia leaves from July to September.

300 These findings are extremely important since the RebA represents the preferred SG for
301 sweetening purposes. Due to the commercialization of Reb A, intensive selection and cultivation

302 practices have yielded cultivars with markedly altered SG compositions in their leaves.[29-30] One
303 parameter to evaluate cultivars/genotypes for the commercial extraction of RebA is the ratio of its
304 concentration against that of Stev within the leaves.[31] Some studies have been carried out in order to
305 investigate this ratio in function of different parameters, but no evidence are reported about the effect
306 of N status on RebA/Stev ratio. For example, it was reported that, within the same cultivar, this ratio
307 appeared to be relatively stable during vegetative growth, but a 35% increase occurred in the lower
308 leaves during reproductive development.[31] Similarly to the individual SG pools, this pattern was also
309 influenced by photoperiod and genotype.[32,33] Consequently, our study gives new information for
310 better understanding the complex interactions underlie the accumulation of SG within the Stevia
311 leaves. The SG accumulation has emerged as a dynamic process, strongly influenced not only by the
312 genetic variability[31], but also by the nutrient conditions and their temporal availability.

313 Similarly to that observed for SG, total phenols and flavonoids, as well as the antioxidant
314 activity and flavonoid composition, considerably changed in response to different N rates. Such
315 variability may be due to the regulation of individual enzymes involved in the biosynthesis of these
316 compounds, such as phenylalanine ammonia-lyase (PAL, EC 4.3.1.5).[34,35] Our findings showed an
317 increase in total phenols and flavonoids from N0 to N150 treatments. At high supply rates, N may
318 inhibit the synthesis of phenols and flavonoids via enhancing the channeling of L-phenylalanine
319 towards proteins.[36] Since polyphenols contribute mostly to the antioxidant activity of plant extract [37],
320 also the antioxidant activity of Stevia may be reduced at high N supply.

321 There are conflicting reports regarding the effects of N fertilization on the plant antioxidant
322 concentrations and capacities[16] and, generally, it was found that high nitrogen supply has negative
323 effect on the biosynthesis of soluble phenolics, condensed tannins and flavonoids in plant tissues.[38,39]
324 The lack of plant nutrients may cause alterations in phenolic content and composition for several
325 crops, although most studies conclude that its effect is difficult to prove.[40] Besides the effects of other
326 potential co-variables (e.g. weather conditions, UV radiation, pest incidence), the variable effect of

327 nutrient shortage on phenolic content might also be due to the complexity of plant response to nutrient
328 availability. According to the growth/differentiation balance theory, plants always optimize the
329 resources available by exploiting them in growth or differentiation processes (primary or secondary
330 metabolism) even taking into account the particular needs of each crop stage.

331 In the case of Stevia, our findings demonstrate that the bioactive compounds and their related
332 antioxidant activity increased by optimizing the N supply and decreased with increasing or decreasing
333 N rates compared to the optimal N dose (150 kg N ha^{-1}). The lower values recorded in Norg, compared
334 with N150, could be related to the slow N release pattern of organic N sources, attributable to the
335 dependence of organic manure on microbial decomposition and subsequent mineralization of N, a
336 process largely affected by climate as well as by manure quality, such as C:N ratio and polyphenolic
337 content.[41-43]

338 Regarding to flavonoid composition, the flavonoids detected in Stevia leaves, under the
339 different N rates, belong to the subgroups of flavonols and flavones, as already observed in previous
340 studies. [9,11,44] All flavonoids, except myricetin, have already been described.[9,11,44,45] Myricetin has a
341 unique chemical structure and, recently, the health benefits of this flavonoid have been demonstrated,
342 in particular, its therapeutic potential in diabetes mellitus has been investigated.[46] This finding adds
343 to Stevia extracts particular importance, confirming beneficial role of Stevia and its metabolites on
344 health promoting properties. Particular attention should be paid to its insulinotropic activity, that
345 could make Stevia's roles one of most promising.[47]

346 The most abundant flavonoid appears to be luteolin-7-*O*-glucoside, which was strongly
347 affected by the harvest time, with a tow fold higher value in the first sampling than in the other two
348 harvests. Furthermore, the choice of the optimal harvest time can contribute to enhance the
349 phytochemical quality of Stevia leaf extracts. The great differences in the bioactive compounds and
350 antioxidant activity of Stevia leaves harvested at different times, may be a consequence of some
351 physiological changes. Samples collected at H1 (beginning of July) generally showed higher phenolic

352 and flavonoid content, antioxidant activity and Stev levels, compared to samples collected in H2 and**,**
353 with the exception of a few cases, in H3. Environmental factors such as photoperiod, water
354 availability, temperature, pest and disease incidence, as well as physiological status of the plant have
355 a direct impact on biochemical pathways, thus affecting the metabolism of secondary products.[48] Our
356 results points out that time of harvest, being related with the physiological stage of the plant, is an
357 important factor in influencing the secondary metabolites of Stevia leaf extracts. Stevia is an obligate
358 short-day plant with a critical day length of about 12-13 h.[32,33] Under long-day condition, the
359 vegetative growth phase of short-day plant is retained for a long time by prohibiting precocious
360 flowering. It was reported that the long-day conditions significantly increased leaf biomass and Stev
361 content in Stevia leaves.[49] Similarly, it was reported that, in leaves of sweet potato plants, photoperiod
362 regimes influenced the accumulation of phenolic acids, anthocyanins and other flavonoids by
363 increasing the total contents of these compounds under long day conditions, suggesting a role for
364 these compounds in the protection against enhanced light exposure.[48]

365 Antioxidant capacity depends on the type of antioxidant which prevails in the extract and,
366 thus, on the kind of plant extract, which can be essentially distinguished on the base of its
367 hydrophobic/hydrophilic nature. The difference found here by applying the two assays (FRAP and
368 DPPH) on Stevia extracts (Figure 2) lie in the chemistry of the reaction involved, namely the
369 biomolecule acting as reductant (type and number of functional groups involved) as well as the
370 detection radical and the solvent used.[50] However, both with FRAP and DPPH assay, the high
371 correlations found between secondary metabolites of Stevia leaves and antioxidant capacities confirm
372 that the high antioxidant capacity characterizing Stevia leaves is mainly attributable to the presence
373 of both phenolic compounds, such as flavonoids, and SG. In particular, the high and positive
374 correlations found between Stev and FRAP and between Stev and DPPH in the N150-treated plants
375 (Table 3) confirm that SG are potent ROS scavengers and that Stev is a stronger scavenger than Reb
376 A for superoxide radicals, as reported by Geuns et al. [51]

377 Interestingly, in leaves of plants grown under N150 treatment, Stev positively correlated with
378 both the content of total and individual flavonoids, with the exception of kaempferol (Table 4). In
379 contrast, for N150 dose, Reb A did not significantly correlate either with total or individual
380 flavonoids, while significant and negative correlations have been found between this compound and
381 total flavonoid content and luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, in N0- and N50-
382 trated plants (Table 4).

383 Further investigations are needed to elucidate a possible interrelation of flavonoids with the
384 metabolic pathway of Stev synthesis. In fact, key functions of flavonoids as developmental regulators-
385 signaling molecules in plants and humans have been reported.[52] This could shed a light on a possible
386 additional role of flavonoids, besides those well-known of being potent antioxidants.

387 All together, the results obtained in this study provide useful information on Stevia response
388 to different N regimes and harvest date, underling the possibility to increase, from a phytochemical
389 point of view, the Stevia quality. Moreover, this work succeeded in improving the knowledge about
390 the changing in SG, flavonoid composition and antioxidant capacity of Stevia in response to different
391 N rates and harvesting dates. In fact, our results indicate that the manipulation of N fertilization might
392 be an effective tool to increase the accumulation of bioactive compounds in Stevia. In fact, higher
393 RebA/Stev ratio, total phenolic content, total flavonoids, luteolin-7-*O*-glucoside levels, and
394 antioxidant capacity were observed in Stevia when nitrogen supply was equal to 150 kg ha^{-1}.
395 Moreover, at the highest nitrogen treatment level, Stevia exhibited significantly lower bioactive
396 compounds and antioxidant activity than under N150 treatment. The lower phytochemical content
397 and antioxidant capacity observed in Norg-treated plants in comparison with N150-treated plants may
398 be attributable to a distinct temporal N availability that characterized organic and inorganic N sources.
399 In addition to nitrogen fertilization, harvest time or more precise plant developmental stage was also
400 found to have a significant influence on bioactive compounds and antioxidant activity in Stevia.
401

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Notes

The authors declare no competing financial interest.

408 **ABBREVIATIONS USED**

409 DPPH, 1,1-diphenyl-2-picrylhydrazyl; GAE, gallic acid equivalents; H1, harvest at July 9th; H2,
410 harvest at July 21st; H3, September 10th; N0, no nitrogen; N150, 150 kg N ha^{-1} as mineral nitrogen;
411 N300, 300 kg N ha^{-1} as mineral nitrogen; N50, 50 kg N ha^{-1} as mineral nitrogen; NNS, non-nutritive
412 sweeteners; Norg, 150 kg N ha^{-1} as organic nitrogen; RebA, rebaudioside A; ROS, reactive oxygen
413 species; SG, steviol glycosides; Stev, stevioside.

414

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547

FIGURE CAPTIONS

Fig. 1. Effect of nitrogen rate and harvest time on total phenolic content (A) and total flavonoids (B) in leaves of *Stevia rebaudiana* plants. Data are means of five replicates ± SDs, bars represent standard deviation. Means with the same letters are not significantly different for $P = 0.05$ following two-way ANOVA test with nitrogen rate and harvest time as variability factors.

Asterisks indicate significant differences according to 2-way ANOVA test (***, significant at $P < 0.001$ probability level).

Fig. 2. Effect of nitrogen rate and harvest time on total antioxidant capacity (A) and radical scavenging activity (B) in leaves of *Stevia rebaudiana* plants. Data are means of five replicates ± SDs, bars represent standard deviation. Means with the same letters are not significantly different for $P = 0.05$ following two-way ANOVA test with nitrogen rate and harvest time as variability factors.

Asterisks indicate significant differences according to 2-way ANOVA test (***, significant at $P < 0.001$ probability level).

Fig. 3. DPPH radical scavenging activity (Half-inhibitory concentration, IC_{50}) of different scavengers and *Stevia rebaudiana* leaf extracts. Data are means of three replicates ± SDs, bars represent standard deviation. Means with the same letters are not significantly different for $P = 0.05$ following one-way ANOVA test, with scavenger/leaf extract as variability factor.

Table 1. Effect of nitrogen fertilization (N0, without N fertilisation; N50, 50 kg N ha^{-1}; N150, 150 kg N ha^{-1}; N300, 300 kg N ha^{-1}; Norg, 150 kg N ha^{-1} as organic nitrogen) and harvest time (H1 = July, 9th; H2 = July, 21st; H3 = September 10th) on stevioside and rebaudioside A contents (g $100g^{-1}$ DW) and rebaudioside A/stevioside ratios in leaves of *Stevia rebaudiana*[a].

	N0	N50	N150	N300	Norg	Mean harvest
Stevioside						
H1	6.47 ± 0.73 ab	3.08 ± 0.01 fg	5.60 ± 0.03 cd	4.93 ± 0.29 d	7.08 ± 0.52 a	5.43 A
H2	6.32 ± 0.96 bc	5.08 ± 0.25 d	3.63 ± 0.18 efg	4.18 ± 0.53 e	3.98 ± 0.83 e	4.64 B
H3	4.97 ± 0.07 d	3.18 ± 0.06 fg	3.78 ± 0.11 ef	2.97 ± 0.01 g	3.77 ± 0.01 ef	3.73 C
Mean Nitrogen	5.92 A	3.78 D	4.34 C	4.03 CD	4.94 B	
Source of variation: N = ***; H = ***; NxH = ***						
Rebaudioside A						
H1	1.79 ± 0.05 h	1.69 ± 0.06 h	3.46 ± 0.62 bcde	3.69 ± 0.18 abcd	3.19 ± 0.06 de	2.75 C
H2	3.96 ± 0.35 ab	4.10 ± 0.10 a	3.88 ± 0.64 ab	3.75 ± 0.62 abc	2.35 ± 0.11 g	3.61 A
H3	3.19 ± 0.02 de	3.08 ± 0.08 ef	3.27 ± 0.01 cde	3.17 ± 0.06 e	2.64 ± 0.09 fg	3.07 B
Mean Nitrogen	2.98 B	2.94 B	3.54 A	3.54 A	2.73 B	
Source of variation: N = ***; H = ***; NxH = ***						
Rebaudioside A/Stevioside						
H1	0.28 ± 0.03 j	0.55 ± 0.01 hi	0.62 ± 0.08 gh	0.75 ± 0.06 def	0.45 ± 0.02 i	0.53 B
H2	0.63 ± 0.11 fgh	0.81 ± 0.01 cde	1.07 ± 0.16 a	0.92 ± 0.15 bc	0.60 ± 0.09 gh	0.81 A
H3	0.64 ± 0.01 fgh	0.97 ± 0.01 ab	0.86 ± 0.02 bcd	1.07 ± 0.01 a	0.70 ± 0.02 efg	0.85 A
Mean Nitrogen	0.52 C	0.78 B	0.85 AB	0.91 A	0.58 C	
Source of variation: N = ***; H = ***; NxH = ***						

[a]Results are the means ± SD of five replicates that were each analyzed in triplicate. A two-way ANOVA was used to evaluate the effect of nitrogen fertilization (N), harvest time (H) and the interaction between nitrogen and harvest time (NxH). Significance was as follows: ***, significant at $P < 0.001$ level. Upper-case letter: effect of nitrogen rate (N) and harvest time (H); lower-case letter: NxH interaction.

Table 2. Effect of nitrogen fertilization (N0, without N fertilisation; N50, 50 kg N ha^{-1}; N150, 150 kg N ha^{-1}; N300, 300 kg N ha^{-1}; Norg, 150 kg N ha^{-1} as organic nitrogen) and harvest time (H1 = July, 9th; H2 = July, 21st; H3 = September 10th) on main flavonoids (mg g^{-1} DW) in leaves of *Stevia rebaudiana*[a].

	N0	N50	N150	N300	Norg	Mean harvest
Rutin						
H1	5.58 ± 0.38 bcd	6.45 ± 0.48 ab	7.03 ± 0.40 a	4.12 ± 0.20 fg	5.28 ± 0.02 cde	5.69 A
H2	5.32 ± 0.11 cde	6.63 ± 0.72 ab	4.82 ± 1.46 def	4.80 ± 0.33 def	6.22 ± 1.03 abc	5.55 A
H3	5.01 ± 0.65 def	4.36 ± 0.41 efg	3.32 ± 0.25 g	4.54 ± 0.88 def	6.41 ± 0.53 ab	4.73 B
Mean Nitrogen	5.30 BC	5.81 AB	5.06 CD	4.49 D	5.97A	
Source of variation: N = ***; H = ***; NxH = ***						
Myricetin						
H1	2.19 ± 0.13 def	2.59 ± 0.21 bcde	2.84 ± 0.14 ab	2.15 ± 0.16ef	2.27 ± 0.01 def	2.41 A
H2	2.53 ± 0.00 bcdef	2.65 ± 0.64 bcd	2.22 ± 0.56 def	2.50 ± 0.18 bcdef	2.77 ± 0.36 abc	2.53 A
H3	2.60 ± 0.27 bcde	2.50 ± 0.50 bcdef	2.06 ± 0.07 f	2.31 ± 0.13 cdef	3.24 ± 0.09 a	2.54 A
Mean Nitrogen	2.44 B	2.58 AB	2.37 B	2.32 B	2.76 A	
Source of variation: N = *; H = n.s.; NxH = **						
Apigenin-7-*O*-glucoside						
H1	9.60 ± 0.63 bc	8.91 ± 0.02 bcde	14.68 ± 0.98a	10.04 ± 0.54b	7.25 ± 0.23efg	10.10A
H2	3.97 ± 0.10 h	7.91 ± 0.08 cdefg	9.11 ± 2.90bcde	6.37 ± 0.58g	8.13±1.53bcdefg	7.10C
H3	9.31 ± 1.80 bcd	7.37 ± 0.52 defg	6.42 ± 1.36fg	8.34 ± 0.95bcdef	13.09 ± 0.62a	8.91B
Mean Nitrogen	7.63 B	8.06 B	10.07A	8.25B	9.49A	
Source of variation: N = ***; H = ***; NxH = ***						
Quercitrin						
H1	3.06 ± 0.07a	2.49 ± 0.02d	2.84 ± 0.16bc	2.48 ± 0.08d	3.03 ± 0.19ab	2.78A
H2	2.68 ± 0.05cd	2.09 ± 0.07e	1.77 ± 0.36f	2.15 ± 0.01e	2.64 ± 0.07cd	2.27B
H3	2.79 ± 0.22c	1.23 ± 0.03h	1.51 ± 0.00g	1.65 ± 0.08fg	2.09 ± 0.08e	1.85C
Mean Nitrogen	2.84A	1.94D	2.04CD	2.09C	2.59B	
Source of variation: N = ***; H = ***; NxH = ***						
Kaempferol						
H1	0.16 ± 0.02f	0.11 ± 0.03fgh	0.12 ± 0.04fg	0.03 ± 0.00h	0.04 ± 0.00gh	0.09C
H2	0.47 ± 0.07ab	0.05 ± 0.01gh	0.09 ± 0.01fgh	0.35 ± 0.05cd	0.10 ± 0.00fgh	0.21B
H3	0.43 ± 0.14bc	0.34 ± 0.02de	0.52 ± 0.08a	0.49 ± 0.04ab	0.26 ± 0.06e	0.41A
Mean Nitrogen	0.35A	0.17C	0.24B	0.29B	0.13C	
Source of variation: N = ***; H = ***; NxH = ***						
Luteolin-7-*O*-glucoside						
H1	45.67 ± 3.96b	45.28 ± 2.50b	76.51 ± 0.76a	36.46 ± 0.80c	35.20 ± 0.39c	47.82A
H2	16.04 ± 1.38fg	17.85 ± 1.20fg	19.93 ± 1.44ef	12.15 ± 1.52g	19.16 ± 3.96efg	17.03C
H3	47.30 ± 9.76b	22.18 ± 0.98ef	16.83 ± 5.80fg	26.08 ± 4.01de	30.00 ± 1.80cd	28.48B
Mean Nitrogen	36.34A	28.44B	37.76A	24.90B	28.12B	
Source of variation: N = ***; H = ***; NxH = ***						

[a]Results are the means ± SD of five replicates that were each analyzed in triplicate. A two-way ANOVA was used to evaluate the effect of nitrogen fertilization (N), harvest time (H) and the interaction between nitrogen and harvest time (NxH). Significance was as follows: ***, significant at P <0.001 level; **, significant at P < 0.01 level; *, significant at P < 0.05 level; n.s., not significant. Upper-case letter: effect of nitrogen rate (N) and harvest time (H); lower-case letter: NxH interaction.

Table 3. Correlation coefficients (r^2) among total antioxidant capacity (FRAP, µmol $FeSO_4$ g^{-1} DW and DPPH, , µmol ASA g^{-1} DW) and total phenols (mg GAE g^{-1} DW), total flavonoids (mg g^{-1} DW), stevioside and rebaudioside A (g 100 g^{-1} DW) in leaves of *Stevia rebaudiana* plants grown under different nitrogen rates[a].

Antioxidant assay	Sample	Phenols	Flavonoids	Stevioside	Rebaudioside A
FRAP	N0	0.727 ** y = 0.089x + 21.80	0.807 ** y = 0.101x + 10.59	6.8 10^{-4} n.s.	0.609 ** y = -0.005x + 4.98
	N50	0.641 ** y = 0.098x + 29.45	0.604 ** y = 0.096x – 8.98	0.071 n.s.	0.403 n.s.
	N150	0.583 * y = 0.138x + 15.29	0.913 *** y = 0.206x – 33.89	0.956 *** y = 0.006x + 1.80	0.048 n.s.
	N300	0.885 *** y = 0.059x + 46.60	0.946 *** y = 0.055x + 16.91	0.104 n.s.	0.008 n.s.
	Norg	0.725 ** y = -0.100x + 25.40	0.136 n.s.	0.092 n.s.	0.187 n.s.
DPPH	N0	0.433 n.s.	0.243 n.s.	0.357 n.s.	0.823 ** y = -0.495x + 43.21
	N50	0.923 *** y = 4.905x – 308.3	0.829 *** y = 4.696x – 33.1	0.031 n.s.	0.523 * y = -0.261x + 23.93
	N150	0.500 * y = 4.528x – 278.3	0.867 *** y = 7.369x – 527.7	0.792 ** y = 0.197x – 11.35	0.223 n.s.
	N300	0.661 ** y = 3.643x – 217.4	0.862 *** y = 3.744x – 257.0	0.318 n.s.	0.025 n.s.
	Norg	0.668 * y = 3.429x – 208.9	0.293 n.s.	0.176 n.s.	0.024 n.s.

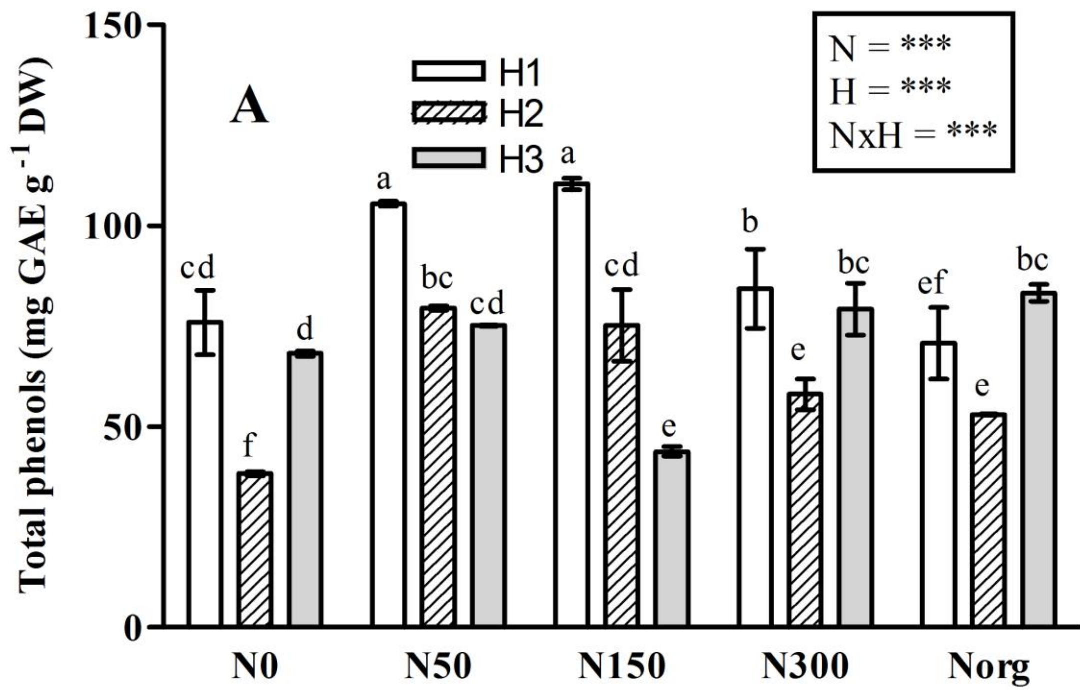
[a]Significance was as follows: ***, significant at $P < 0.001$ level; **, significant at $P < 0.01$ level; *, significant at $P < 0.05$ level; n.s., not significant. N0, without N fertilisation; N50, 50 kg N ha^{-1}; N150, 150 kg N ha^{-1}; N300, 300 kg N ha^{-1}; Norg, 150 kg N ha^{-1} as organic nitrogen.

Table 4. Correlation coefficients (r^2) among steviol glycosides (stevioside and rebaudioside A) (g 100 g^{-1} DW) and total flavonoids (mg g^{-1} DW) and each single flavonoid (Rutin, Myricetin, Apigenin-7-*O*-glucoside, Quercitrin, Kaempferol, Luteolin-7-*O*-glucoside) (mg g^{-1} DW), in leaves of *Stevia rebaudiana* plants grown under different nitrogen rates[a].

Steviol glycosides	Sample	Flavonoids	Rutin	Myricetin	Apigenin-7-*O*-glucoside	Quercitrin	Kaempferol	Luteolin-7-*O*-glucoside
Stevioside	N0	0.078 n.s	0.431 n.s.	0.357 n.s.	0.050 n.s.	0.690 ** y = 2.060x + 0.47	0.155 n.s.	0.052 n.s.
	N50	0.310 n.s	0.271 n.s.	0.582 * y = 11.05x – 24.76	0.044 n.s.	0.023 n.s.	0.378 n.s.	0.426 n.s.
	N150	0.945 *** y = 0.027x + 2.81	0.658 ** y = 0.445x + 2.10	0.648 ** y = 1.863x – 0.09	0.728 ** y = 0.212x + 2.20	0.814 *** y = 1.345x + 1.59	0.139 n.s.	0.979 *** y = 0.032x + 3.11
	N300	0.100 n.s.	0.060 n.s.	0.059 n.s.	0.110 n.s.	0.907 *** y = 2.302x – 0.80	0.781 ** y = -3.725x + 5.13	0.090 n.s.
	Norg	0.138 n.s.	0.742 ** y = -2.127x + 17.65	0.795 ** y = -3.352x + 14.19	0.441 n.s.	0.588 * y = 2.812x – 2.26	0.526 * y = -11.79x + 6.53	0.556 * y = 0.212x – 1.28
Reb A	N0	0.531 * y = -0.038x + 5.06	0.169 n.s.	0.570 * y = 3.360x – 5.23	0.598 * y = -0.263x + 4.99	0.355 n.s.	0.705 ** y = 4.974x + 1.23	0.614 ** y = -0.051x + 4.79
	N50	0.838 *** y = -0.068 + 6.15	2.8 10^{-4} n.s.	0.089 n.s.	0.494 * y = -1.086x + 11.71	0.161 n.s.	0.009 n.s.	0.916 *** y = -0.079x + 5.19
	N150	0.001 n.s.	0.065 n.s.	0.101 n.s.	0.029 n.s.	0.036 n.s.	0.217 n.s.	0.008 n.s.
	N300	0.018 n.s.	0.055 n.s.	0.051 n.s.	0.023 n.s.	0.159 n.s.	0.128 n.s.	0.014 n.s.
	Norg	0.362 n.s.	0.362 n.s.	0.613 * y = -0.614x + 4.49	0.078 n.s.	0.279 n.s.	0.204 n.s.	0.854 *** y = 0.048x + 1.38

[a]Significance was as follows: ***, significant at $P < 0.001$ level; **, significant at $P < 0.01$ level; *, significant at $P < 0.05$ level; n.s., not significant. N0, without N fertilisation; N50, 50 kg N ha^{-1}; N150, 150 kg N ha^{-1}; N300, 300 kg N ha^{-1}; Norg, 150 kg N ha^{-1} as organic nitrogen.

Figure 1



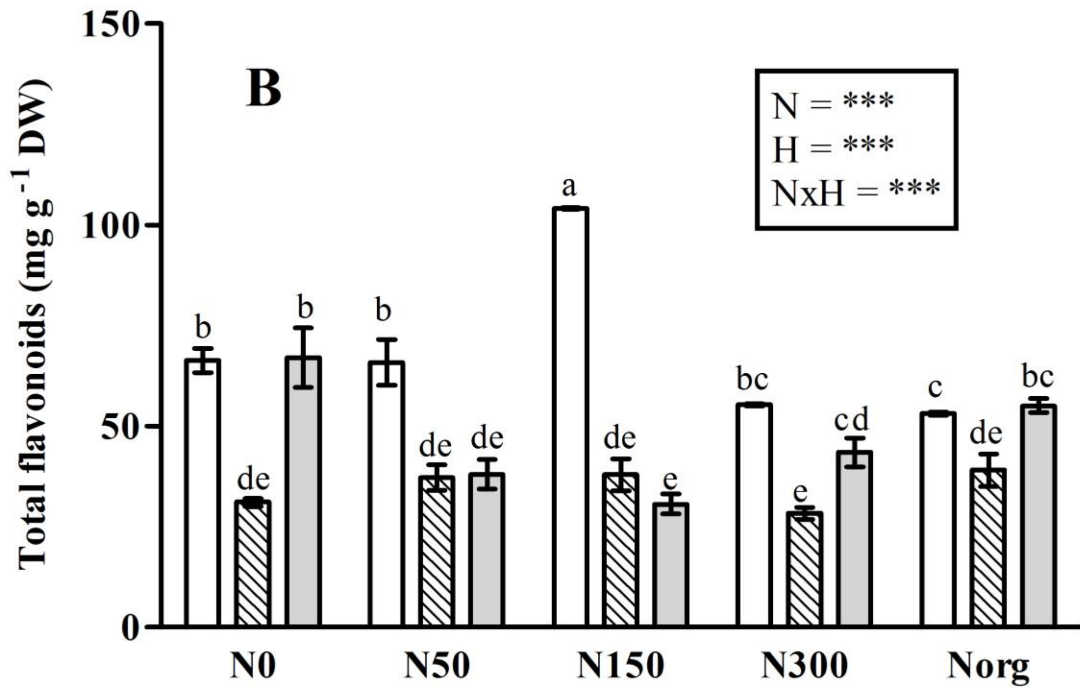


Figure 2

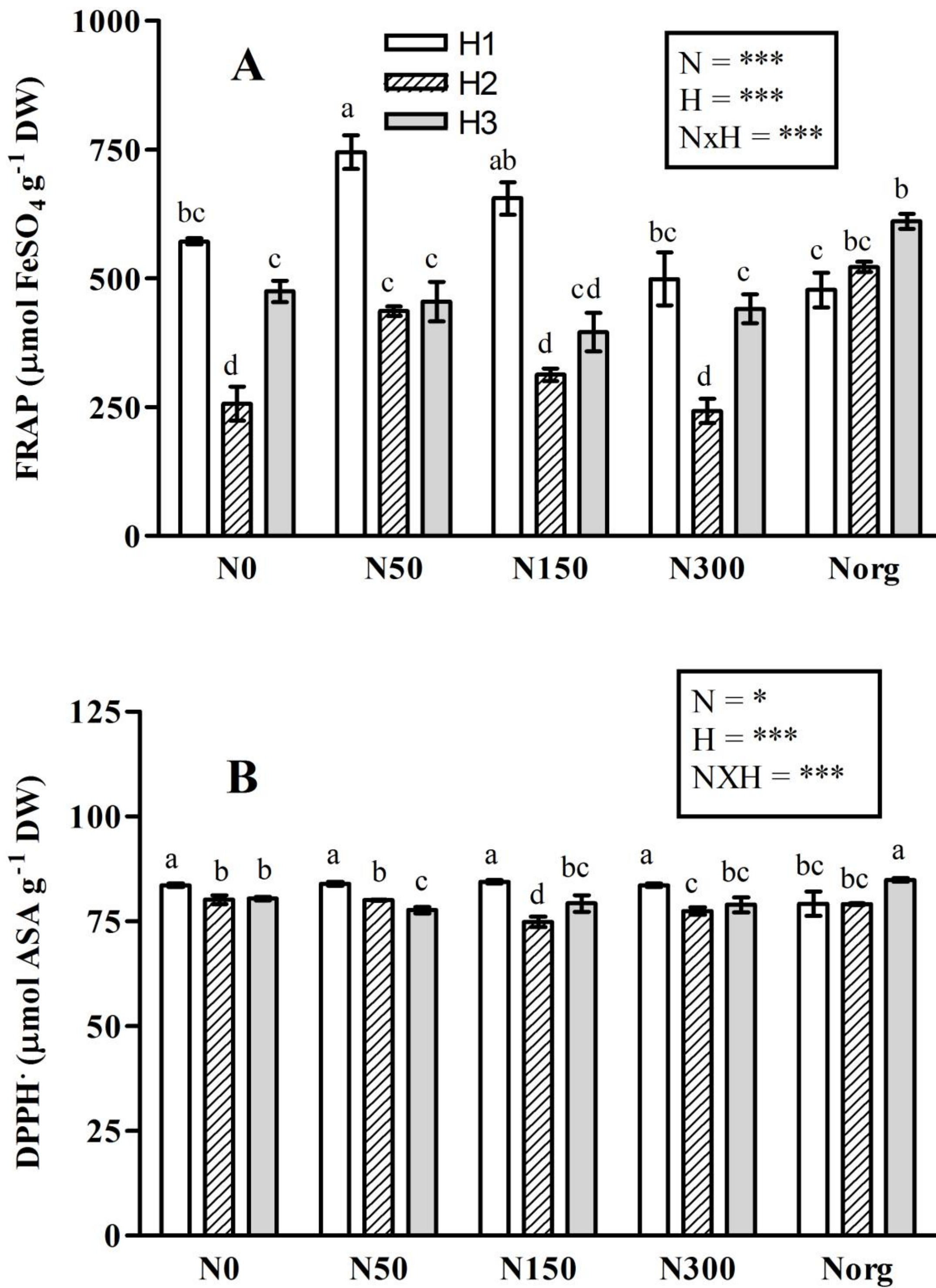
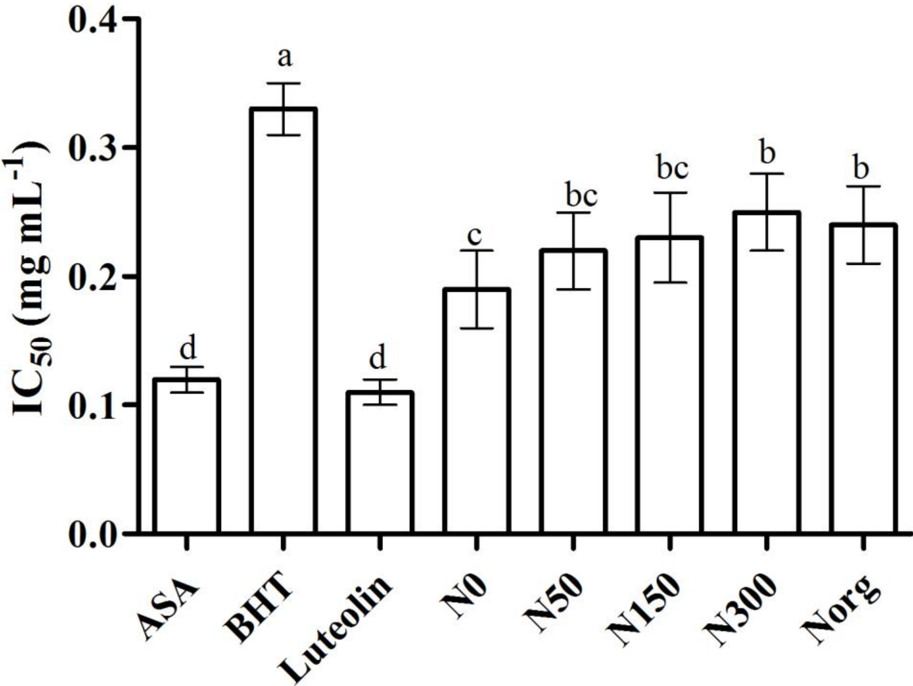


Figure 3



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