Plant growth, steviol glycosides and nutrient uptake as affected by arbuscular mycorrhizal fungi and phosphorous fertilization in *Stevia rebaudiana* Bert.

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Abstract

The increasing demand for products based from *Stevia rebaudiana* Bertoni (both leaves and purified steviol glycosides) makes of interest the research on sustainable production systems, in order to guarantee secure availability and high quality of agricultural raw materials. Arbuscular mycorrhizal fungi (AMF) symbiosis represents an interesting tool for increasing crop production and quality, especially thanks to improved nutrient absorption, particularly phosphorus (P). In the present study, *Stevia rebaudiana* Bert. plants were exposed to different levels of P fertilization (0, 25 and 50 mg P_2O_5 kg^{-1} soil) with or without *Rhizoglomus irregulare* inoculation, in order to evaluate root colonization, plant growth and productive parameters, steviol glycosides (SVglys) yield, as well as nitrogen (N) and P concentrations and uptake. A nutrient balance was also carried out and the nutrient use efficiency was evaluated. Stevia roots were highly colonized by *Rhizoglomus irregulare*, especially in the absence of P fertilization. During the whole vegetative growth, the AMF symbiosis, in association with the P supply, benefitted stevia growth, especially with regard to leaf dry biomass production and SVglys yield. Arbuscular mycorrhizal fungi symbiosis was able to modify the growth habit of stevia plants, with increased branching and a reduced plant height. At the end of the vegetative growth, mycorrhizal plants reached the highest leaf dry yield, together with the highest SVglys production. The application of 25 mg P_2O_5 kg^{-1} soil in association with AMF symbiosis seemed to be the most effective treatment in improving stevia SVglys yield and P uptake together with P nutrient use efficiency.

Keywords: Biofertilizers, Diterpene glycosides yield, Nutrient use efficiency, *Rhizoglomus irregulare.*

1. Introduction

Stevia rebaudiana Bert. (hereafter stevia), a perennial semi-shrub of *Asteraceae* family, has been long used as a sweetener and herbal remedy by the Guaranì people (Ramesh et al. 2006; Madan et al., 2010). This steviol glycoside-rich plant now represents an economic opportunity, especially after the approval of the use of steviol glycosides (SVglys) as a food additive in many countries (Angelini et al., 2016), and the very recent recognition, in Europe, of stevia leaves as "traditional food" in tea, herbal and fruit infusions (Novel Food catalogue, European Commission, 2017).

More than 30 SVglys have been detected in stevia leaf extracts (Wölwer-Rieck, 2012), of which the most abundant are stevioside and rebaudioside A, followed by rebaudiosides B-E, dulcoside A and steviolbioside (De et al., 2013; Pal et al., 2015; Tavarini et al., 2015). The sweetness of SVglys ranges between 250-300 times that of sucrose (Crammer and Ikan, 1987). In addition to the sweet taste, a recent review assessed the health-promoting properties of steviol glycosides and other active principles of stevia (Marcinek and Krejpcio, 2016).

Stevia leaves present a unique composition in terms of the presence of several biologically important secondary metabolites, such as labdanes, flavonoids, phenolic acids, sterols, triterpenoids, chlorophylls, organic acids, mono-disaccharides, and inorganic salts (Gardana et al., 2010; Tavarini et al, 2015; Tavarini and Angelini, 2013). The global market for stevia has grown considerably (Mintel, 2014): consumers increasingly tend to opt for products with a natural origin, and their concerns derived from the use of synthetic sweeteners (Soffritti et al., 2006, 2016; Chiozzotto et al., 2011; Seuez et al., 2014; Kuk and Brown, 2016). As the agricultural production of stevia is still problematic and insufficient to meet such a growing global demand, its cultivation could represent a great opportunity for farmers.

Identifying the main pre-harvest factors that affect the phytochemical profile of stevia is key to improving its productivity and the amounts of beneficial active compounds in its leaves. One of the

main challenges in stevia production is the use of arbuscular mycorrhizal fungi (AMF) which are ubiquitous beneficial root symbionts which promote plant growth and affect the production of health-promoting secondary metabolites (Sbrana et al., 2014; Pedone-Bonfim et al., 2015). Sharma et al. (2015) highlighted the lack of information on the use of AMF as biofertilizers in stevia, as useful tools for modern agriculture that can reduce chemical inputs as well as the impact on the environment.

Arbuscular mycorrhizal symbioses may increase mineral nutrients and water uptake, and photosynthetic rate (Goslin et al., 2006). The improvement in P absorption in mycorrhizal plants is widely recognized, together with AMF activity as bioenhancers, biostimulants and biocontrol agents (Smith and Read, 2008; Smith et al., 2011; Rouphael et al., 2015; Bücking and Kafle, 2015; Corrêa et al., 2014). However, studies on the effects of mycorrhizal symbiosis on the quantitative and qualitative production of stevia are limited (Portugal et al., 2006; Mandal et al., 2015) and do not consider the interaction between AMF and P fertilization on the biosynthesis of the different steviol glycosides.

The aim of this study was to evaluate the effects of AMF inoculation, P fertilization levels (P) and their reciprocal interaction (AMFxP) on stevia root colonization, the main biometric and productive parameters, and SVglys yield, throughout the vegetative growth. In addition, nitrogen and phosphorus concentrations in three different plant organs (leaves, stems and roots) and the nutrient uptake and partitioning within the plant were analyzed at harvest.

2. Material and Methods

2.1. *Chemicals*

The HPLC-grade solvents, acetonitrile, formic acid and water were purchased from J. T. Baker (Phillipsburg, NJ, USA). Common Stevia Glycosides Standards Kit (steviolbioside, dulcoside A,

rebaudioside B, stevioside, rebaudioside A and rebaudioside C) were purchased from Chromadex (LGC Standards S.r.L., Milan, Italy). All solvents and water were thoroughly degassed prior analyses.

2.2. *Plant Material and Experimental Conditions*

A pot trial was carried out at the Experimental Centre of the Department of Agriculture, Food and Environment (DAFE) (Central Italy, Pisa, 43° 40' N; 10° 19' E), during the 2015 growing season. The stevia plants were obtained from a high rich-rebaudioside C genotype, belonging to the DAFE germplasm collection, through stem cuttings to ensure the production of uniform plant material. In December 2014, apical portions were cut from the mother plants grown in greenhouse conditions, and transferred on sterile peat-based growing media, into plug trays, for elongation and rooting. The derived plantlets were maintained under controlled conditions in the greenhouse until the beginning of the trial. At the beginning of May 2015, uniform sized (5-7 cm height) plants were selected and transplanted to 3 L pots with 2 kg of autoclaved soil in each pot. The substrate used was a mixture of 9/10 sandy loam soil (sand 75%; silt 22%; clay 3%; organic matter 15 g kg^{-1}; pH 8.1; total nitrogen 0.6 g kg^{-1}; available phosphorus 11.9 mg kg^{-1}; exchangeable potassium 107.1 mg kg^{-1}) and 1/10 peat-based growing media (VALCOFERT S.r.l, Empoli, Italy). The substrate obtained was mixed and autoclaved twice (121°C for 1 h), with a 24 h gap between one cycle and the next, in order to kill naturally occurring AMF propagules.

The trial was conducted in open-air conditions, from May to the first ten days of September. A weather station located near the experimental site was used to record any changes in minimum, maximum and mean air temperatures and total rainfall. Mean maximum and minimum temperatures in the growing season were 28.8°C and 15.8°C, respectively, with 283.8 mm of total rainfall.

The plants were exposed to six treatments, consisting in three phosphorus doses (0, 25 and 50 mg P_2O_5 kg^{-1} of soil) with (M) and without (NM) mycorrhizal inoculum: NM+0P (without AMF

inoculum and without P fertilization); NM+25P (without AMF inoculum with 25 mg P_2O_5 kg^{-1} of soil); NM+50P (without AMF inoculum with 50 mg P_2O_5 kg^{-1} of soil); M+0P (mycorrhizal without P); M+25P (mycorrhizal with 25 mg P_2O_5 kg^{-1} of soil); M+50P (mycorrhizal with 50 mg P_2O_5 kg^{-1} of soil).

According to Tavarini et al. (2015), a randomized block design with two treatment factors (phosphorous, mycorrhizal inoculum) occurring in a factorial structure was used. Three harvests were carried out at 69 (16 July 2015), 89 (5 August 2015) and 123 (8 September 2015) days after transplanting (DAT) sampling 3 replicates for each treatment. Phosphorus, as triple superphosphate, was added at the beginning of the trial. One month after the beginning of the trial, nitrogen fertilization was supplied to each plant (0.25 g N pot^{-1}, as ammonium nitrate). The plants were well-watered through the experiment (75-80% of field capacity) thanks to a drip irrigation system.

On the last sampling date, the plants were at the beginning of the reproductive stage, when the SVglys leaf concentration reaches the maximum (Sumida, 1980; Xiang, 1983; Ramesh et al., 2006). At each sampling date, plant height, branch number, total fresh above- and below-ground biomass were measured. The plants were then air-dried in a ventilated oven from 30°C to 40°C until constant weight, for dry weight determination of the leaves, stems and roots. Root to shoot ratio was also measured as: root dry mass/(leaf + stem) dry mass. The various dry plant parts were ground to a fine powder, by a laboratory mill (IKA universal grinder M20), and used for subsequent analyses.

2.3. *AMF inoculum and mycorrhizal colonization*

Mycorrhizal treatments were set up using inoculum consisting of mycorrhizal roots and soil containing spores and extraradical mycelium of the AMF species *Rhizoglomus irregulare* (N.C. Schenck & G.S. Sm.) Sieverd., G.A. Silva & Oehl (syn. *Rhizophagus irregularis* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüssler, formerly known as *Glomus intraradices*), isolate IMA6. The inoculum was obtained

131 from *Medicago sativa* L. and *Zea mays* L. pot cultures in a mixture of sandy loam soil and calcinated
132 clay (OILDRI, Chicago, IL, USA) (1:1 v/v), kept at the Microbiology laboratory, Department of
133 Agriculture, Food and Environment (DAFE), University of Pisa, Italy. After excision of the shoots
134 from the host plants, the substrate was air dried at room temperature, roots were ground, carefully
135 mixed with the soil, and stored until use.

136 All pots received 50 mL of a filtrate, obtained by sieving the mycorrhizal inoculum through a
137 50 μm pore diameter sieve and then through Whatman paper no. 1 (Whatman International Ltd,
138 Maidstone, Kent, UK), to ensure a common microflora to all treatments. In the mycorrhizal treatment,
139 stevia plants were inoculated with 130 mL of inoculum, while NM plants received the same volume of
140 a mock inoculum, prepared by steam-sterilization of the whole inoculum. The inoculation occurred just
141 before transplanting.

142 One month after the transplant, root samples from three pots per treatment were collected for
143 the determination of mycorrhizal colonization. The method was based on clearing and then staining
144 with 0.05% Trypan blue in lactic acid root samples (Phillips and Hayman, 1970). The percentage of
145 colonized AMF root length was assessed on representative root samples from each plant, using the
146 gridline intersect method (Giovannetti and Mosse, 1980).

147

148 2.4. *Evaluation of soil physical-chemical characteristics*

149 Soil physical characterization was carried out according to the Soil Survey Laboratory Methods
150 Manual (USDA-NRCS, 1996) before plant transplanting. Bulk density (BD) was determined by a
151 separate undisturbed soil core (3 cm diameter) collected from a depth of 0-15 cm from each pot. This
152 was calculated by dividing the mass of the oven-dried sample at 105°C for 48 h by the volume of the
153 probe (USDA-NRCS, 1996).

Soil organic matter (SOM) was evaluated by multiplying soil organic carbon × 1.724 (Nelson and Sommers, 1982). Soil pH, total N and available P were assessed by McLean (1982), Bremner and Mulvaney (1982), and Olsen and Sommers (1982), respectively. $CaCO_3$ was evaluated according to Derimains (1962), while for cation exchange capacity (C.E.C.), the method of Mehlich (1948) was followed. Electrical conductivity (E.C.) was determined in a 1:5 (m:v) substrate:water suspension after 30 min of stirring with GLP-31 Crison conductimeter using a 52.93 electrode and corrected to 20°C.

2.5. *Evaluation of nitrogen and phosphorus content and uptake*

The nitrogen and phosphorus concentrations in the plant organs (leaf, stem and root) were determined according to Jones et al. (1991). Nutrient uptake (N and P uptake) of the stems, leaves and roots were determined by multiplying their nutrient concentrations by their corresponding dry yield biomass per plant (g plant^{-1}).

2.6. *Nutrient balance and nutrient use efficiency*

At the end of the experimental period, the nutrient balance was evaluated as the differences between the total inputs (N and P entering into the pot by mineral fertilization), and the total outputs (N and P leaving the soil, through the uptakes by above-ground biomass) (Di Bene et al., 2011). For P balance, the P0 thesis was not considered since no P input was added to the crop.

Nutrient use efficiency (NUE) was also calculated for all treatments, except for the P0 thesis, according to D'Haene et al. (2007) as follows:

NUE (%)=(total output/total input) × 100 (Eq. 1).

2.7. *Preparation of extract*

For each treatment and sampling date, 0.1 g of powder leaves were dissolved in 10 mL of 5 mM ammonium formate in water/acetonitrile (5:95, v/v) at pH 3.0, in order to obtain a concentration of 1 g L^{-1}, and sonicated for 30 min at 60°C. The obtained extracts were then filtered using 0.45 μm nylon filters.

2.8. *Steviol glycoside content*

Steviol glycoside determination was performed according to Zimmermann et al. (2012). A Hydrophilic Liquid Interaction Chromatography column (Luna HILIC 200A, 5 μm, 250 mm × 4.6 mm; Phenomenex, Italy), was used in conjunction with the corresponding guard column (4 x 3.0 mm), in a HPLC system (Jasco PU980) coupled with a UV-visible wavelength detector. Operating HPLC conditions and chromatogram acquisition are based on the procedure described by Tavarini et al. (2015). Steviol glycoside quantification was performed using authentic standards, through calibration curves (0.05-0.5 g L^{-1}), obtained from standard mixtures containing steviolbioside, dulcoside A, rebaudioside B, stevioside, rebaudioside A, and rebaudioside C.

2.9. *Statistical Analysis*

Data were subjected to analysis of variance (ANOVA) using CoStat version 6.2 (CoHort Software, Monterey, CA, USA). Two-way completely randomized ANOVA was carried out to estimate the variance components of phosphorus (P), arbuscular mycorrhizal fungi (AMF), and their interaction (AMFxP). Means were separated on the basis of the least significant difference (LSD) only when the ANOVA *F* test showed significance at the 0.05 or 0.001 probability level.

3. Results and discussion

3.1. Mycorrhizal colonization

Rhizoglomus irregulare successfully established mutualistic symbiosis with stevia roots, with a high percentage (from 76% to 88%) of mycorrhizal root length (Table 1). This thus confirms that stevia is highly susceptible to AMF colonization (Portugal, 2006; Mandal et al., 2013, 2015). No colonization was observed in NM plants. With increased P additions to the soil, a significant decrease in the percentage of AMF colonized roots was found, in line with previous data on stevia (Mandal et al. 2013) and other plant species, such as cucumber (*Cucumis sativus* L.) (Bruce et al., 1994), pea (*Pisum sativum* L.) (Balzergue et al., 2011), and *Mentha crispa* L. (Urcoviche et al., 2015). Several environmental factors affect AMF symbiosis. Nutrient availability can influence the symbiotic interaction and, consequently, the total AMF root biomass (Menge et al., 1978; Thomson et al., 1986; Breuillin et al., 2010; Smith et al., 2011; Balzergue et al., 2013; Bonneau et al., 2013). This phenomenon is correlated to strigolactone biosynthesis, a new class of plant hormones involved in the pre-symbiotic stage as signal molecules facilitating the contact between AMF and host plant roots (López-Ráez and Pozo, 2013). It has been shown that strigolactone biosynthesis is negatively correlated with phosphate levels, which increase under deficient phosphate conditions, thus promoting fungal development and establishment of symbiosis (Yoneyama et al. 2007; López-Ráez et al. 2008; López-Ráez et al. 2011).

3.2. *Soil physical-chemical parameters*

The substrate had a sandy-loam texture (sand 71.2%; silt 23.8%; clay 5.0%) with a bulk density of 1.55 g cm^{-3}. The chemical characteristics, evaluated both before plant transplanting and at the end of the experiment, are reported in Table 2. The substrate's characteristics were favourable for stevia growth, since this species requires well-drained soil, which is rich in organic matter and nutrients. During the experiment, SOM concentration (%) decreased significantly from the initial substrate to M+25P and

M+50P substrates, with an average reduction of 13.6% between the initial SOM concentration and the SOM measured after cultivation of M-plants treated with 25 and 50P. A similar trend was also found for the total N concentration, with the lowest values in the M+25P substrate (Table 2). These observations are consistent with those reported by Hodge et al. (2001), who suggested that AMF can influence SOM mineralization by accelerating its decomposition and N acquisition. The lowest soil pH value was thus found in the initial substrate, with the maximum SOM concentration.

A clear effect of AMF inoculation and P application was found for the P levels into the soil after stevia cultivation. In particular, the AMF treatment significantly reduced the P soil concentration at the end of the experimental period, indicating that AMF provided the plant with a greater supply of P. The lowest soil P concentrations were recorded in the M+0P substrate, followed by the M+25P substrate (Table 2). These observations were confirmed by the plant P uptake (Figure 1). In fact, a higher P absorption from the substrate was observed in M+0P and M+25P-treated plants compared with NM counterpart (Figure 1).

3.3. *Biometric characteristics*

The branch number increased in M plants at the highest P fertilization levels, although this increase was significant only at 89 DAT ($p < 0.01$ for AMFxP) (Table 3).

As a general trend, both plant height and branch number of stevia plants, were significantly influenced by the mycorrhizal symbiosis, but not by the P fertilization level (Table 3). Significant differences between NM and M plants were recorded, for both traits, at all sampling dates. The NM plants were characterized by a larger stem height and a lower branch number, compared with M plants.

The obtained results showed that, in stevia, AMF symbiosis influenced the above-ground architecture, irrespectively of the addition of P to the soil, by stimulating shoot branching. Little is known about the impact of AMF symbionts on plant growth. In a study designed to assess the effect of

AMF on the growth parameters of nine plant species, Touati et al. (2014) highlighted that all mycorrhizal plants were significantly higher and more branched, compared with non-inoculated plants. Similarly, in a study on stevia biofertilization, Vadafar et al. (2014), found that inoculated plants reached a greater stem height, compared with controls. Since, in stevia, the biosynthesis of gibberellins and SVglys share common steps (Richman et al., 1999; Brandle and Telmer, 2007; Ceunen and Geuns, 2013; Guleria and Yadav, 2013), Mandal et al. (2015) suggested that AMF may induce a shift in the metabolite flow towards SVglys synthesis, thus explaining the increased shoot branching detected in mycorrhizal plants. Similarly, low levels of gibberellins produced phenotypes with increased branching in gibberellin-deficient mutants of *Arabidopsis* (Silverstone et al., 1997), rice (*Oryza sativa* L.) (Lo et al., 2008) and pea (Murfet and Reid, 1993). However, although gibberellins were reported to affect internode elongation (Davies, 2010), their role in shoot branching has yet to be fully unraveled (Rameau et al., 2014). On the other hand, strigolactones may play a role in the modulation of shoot branching and the control of plant above-ground architecture (Gomez-Roldan et al. 2008; Kapulnik et al. 2011).

3.4. *Biomass production and root:shoot ratio*

Regarding the biomass production, the AMFxP interaction significantly influenced leaf dry yield only at 69 DAT, with the lowest dry leaf production observed for NM+50P plants (Table 4). At 123 DAT, both leaf dry yield and total dry above-ground biomass were affected by mycorrhizal symbiosis, with a higher leaf dry yield (+17.6%) and total dry above-ground biomass (+9%) in M plants compared with NM ones (Table 4). On the other hand, at 89 DAT, a negative effect of P supply was observed in the total dry above-ground biomass with the lowest values at the maximum P level (Table 4). Taken together, these results demonstrated that, in stevia at the end of the vegetative growth, mycorrhizal symbiosis led to improved leaf dry and total dry above-ground production. These results confirm

previous reports. Portugal et al. (2006) found that, in stevia, *Glomus intraradices* enhanced total dry biomass and leaf dry yield. Mandal et al. (2013) and Vafadar et al. (2014) underlined the effectiveness of AMF inoculation in increasing the shoot dry weight of stevia.

In order to assess the above- and below-ground biomass allocation in stevia plants affected by AMF symbiosis and P fertilization, the root to shoot ratio (R/S) was evaluated. The patterns of biomass allocation were inconsistent, as mycorrhizal symbiosis affected R/S only at 0P and 50P, at 69 DAT; and at 0P, at 89 DAT, with the highest ratio in NM plants. Conversely, at 89 DAT, R/S showed at 25Pthe lowest values in NM plants (Table 4), confirming previous findings (Veresoglou et al., 2012). However, the present study highlighted that P fertilization was the major factor influencing the R/S ratio in stevia, at 123 DAT sampling. Finally, R/S was higher at the beginning of the vegetative growth, in comparison with the last harvest, suggesting an effect of plant growth stage and seasonality on R/S in stevia (Ledig and Perry, 1966; Haolin et al., 2008).

3.5. *Mineral concentration and uptake*

Nitrogen and phosphorous concentration and uptake were recorded in the different plant organs at the end of the experiment (123 DAT). Nitrogen and phosphorous concentrations were significantly affected by AMFxP interaction, apart from leaf N concentration, which was dependent on both the AMF and P level (Table 5). Regarding this latter parameter, NM plants were characterized by a significantly higher N concentration in the leaves, than M plants, and, at the same time, the plants treated with 25P showed the lowest value (Table 5). In stems and roots, the highest N concentrations were recorded in NM plants treated with the maximum P dose. A similar trend was observed for P concentrations, with the highest values in leaves, and roots of NM plants treated with the 50P dose. NM plants, together with M plants receiving 25P treatment, also showed the highest stem P concentration

296 (Table 5). These findings highlight that, at the maximum P treatment (50 mg P_2O_5 kg^{-1} soil), all the
297 organs (leaves, stems and roots) of NM plants were characterised by the highest P concentrations.

298 Interestingly, with lower P doses (25 and 0 mg P_2O_5 kg^{-1} soil), the M plants showed
299 significantly higher P concentrations in their organs than NM plants, as result of a more effective P
300 absorption due to mycorrhizal symbiosis. Similarly, Hoseini et al. (2015) found that stevia plants
301 inoculated with *Glomus mossae* were characterised by a higher P concentration than non-inoculated
302 plants. In terms of the P concentration in roots, Earanna (2007) did not find significant differences
303 between non-inoculated and *Glomus fasciculatum* inoculated stevia plants.

304 The results obtained in the present study would seem to indicate that mycorrhizal inoculation in
305 stevia was very beneficial without or with a low P fertilization level, in terms of P concentration in the
306 various plant organs. However, this effect disappeared at the highest fertilization rate, which means
307 that, at this level of fertilization, mycorrhizal symbiosis became irrelevant for stevia plants (Figure 1
308 B). In fact, total P uptake by stems, leaves and roots was significantly higher in M than in NM plants at
309 0P and 25P ($p < 0.001$). In contrast, no significant variation was recorded for NM and M plants at the
310 highest P level (Figure 1 B).

311 The findings observed in the present study confirm Mandal et al. (2015), who found that P
312 uptake by M stevia plants was 216% higher compared with that taken up by non-inoculated plants.
313 Such data are consistent with the well-known role of AMF in plant P nutrition at low soil P availability
314 (Smith and Read, 2008). The lack of differences in P uptake in M and NM stevia plants at a high P
315 availability may be ascribed to the predominance of direct root P uptake on the AMF-mediated P
316 pathway (Smith et al., 2011). The high P absorption by M plants is mainly due to the activity of the
317 extraradical mycelium which provides an additional pathway for P assimilation (Smith et al., 2011).

318 The N uptakes by stevia plants are shown in Figure 1A. When no P fertilization was added to
319 the soil, M plants were characterised by a significantly higher N uptake than NM plants ($p < 0.01$). On

320 the other hand, NM stevia plants showed a higher N uptake, compared with M plants, at the highest P
321 dose, while no significant variation was observed between NM and M plants at 25P.

322 However, little is known about the influence of AMF on N uptake (Bücking and Kafle, 2015;
323 Corrêa et al., 2015). A low N availability can be limiting for both the plant and AMF, resulting in the
324 retention of N by the fungus, thereby reducing N availability for the plant (Johansen, 1999; Nouri et al.,
325 2014; Corrêa et al., 2014).

326 In the present study, AMF produced inconsistent results on N uptake by stevia plants,
327 depending on P fertilization levels, thus confirming previous studies showing positive (Saia et al.,
328 2014; Mensah et al., 2015), neutral (Hawkins and George, 1999) and negative (George et al., 1995)
329 effects of AMF on N plant nutrition. The average amount of N and P extracted by the stevia plants and
330 required to produce 1 kg of dry leaf biomass was 11.91 g and 1.55 g of N and P, respectively for NM
331 plants. The nutrient requirements of M plants were 10.49 g N and 2.10 g P to produce 1 kg of dry leaf.
332 These data suggest that M plants need a lower amount of nitrogen to produce the same quantity of dry
333 leaf biomass, than NM plants, while the opposite was observed for P.

334

335 3.6. *Nutrient balance and nutrient use efficiency*

336 A positive N balance (input > output) was found for all treatments at the end of the experiments (Table
337 6). On the other hand, a negative P balance was observed for M+25P-treated plants. Despite the AMF
338 colonization, N use efficiency, calculated according to Eq.(1), was similar for both NM and M plants
339 (Table 6). Conversely, the P use efficiency was maximum in M+25P plants. In fact, M+25P plants
340 were characterised by similar outputs to those registered for M+50P plants, however their input was
341 50% lower with respect to M+50P.

342

343 3.7. *Steviol glycoside yield*

As expected, the SVglys yield increased during the vegetative growth, from the first to the last sampling. At 69 and 123 DAT, the SVglys yield was significantly ($p < 0.05$) affected by the AMFxP mutual interaction (Table 7). In the first sampling, differences between inoculated and non-inoculated plants were recorded for 0 and 50P, with the lowest values for NM plants. However, at the 25P level, no differences were observed between NM and M stevia plants. In the last sampling, the highest yields were recorded for M plants receiving P fertilization (25 and 50 mg P_2O_5 kg^{-1} soil). These findings indicate that a double P dose applied to M plants did not lead to an increase in SVglys yield. This suggests that 25P is likely the best P level in order to achieve the maximum SVglys yield at the end of the vegetative growth. At 89 DAT, the SVglys yield was significantly influenced only by mycorrhizal inoculation, with higher yields in M plants in comparison with NM plants (Table 7).

Definitively, these findings indicate that M plants receiving P fertilization are able to achieved the maximum SVglys yield, as previously observed by Mandal et al. (2013). This behaviour is probably due to the improved P uptake in M plants, which, in turn, has been shown to be responsible, not only for an enhanced nutritional status of the plant, but also for stimulation of secondary metabolite biosynthesis, as observed in several fruits and vegetables (Sbrana et al., 2014).

4. Conclusions

This study underlines that, in stevia, AMF, in association with P supply, can be beneficial for stevia growth, especially with regard to leaf dry biomass production and SVglys yield. AMF symbiosis was able to modify plant architecture, with an increase in branching and a reduction in plant height. At the end of the vegetative growth, mycorrhizal plants reached the highest leaf dry yield, together with the highest SVglys production. At the same time, the application of 25 mg P_2O_5 kg^{-1} soil in M plants improved P uptake, P nutrient use efficiency as well as SVglys yield. Based on these data, AMF can be

368 considered a valid biofertilizer for stevia cultivation, thereby leading to more sustainable agricultural
369 systems.
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References

Angelini, L.G., Martini, A., Passera, B., Tavarini, S., 2016. Cultivation of Stevia rebaudiana Bertoni and Associated Challenges. In: Sweeteners, Reference Series in Phytochemistry, Springer International Publishing Switzerland 2016 J.-M. Mérillon, K.G. Ramawat (eds.). http://dx.doi.org/10.1007/978-3-319-26478-3_8-1

Balzergue, C., Chabaud, M., Barker, D.G., Bécard, G., Rochange, S.F., 2013. High phosphate reduces host ability to develop arbuscular mycorrhizal symbiosis without affecting root calcium spiking responses to the fungus. Front. Plant Sci. 4, 426. https://doi.org/10.3389/fpls.2013.00426

Balzergue, C., Puech-Pagès, V., Bécard, G., Rochange, S.F., 2011. The regulation of arbuscular mycorrhizal symbiosis by phosphate in pea involves early and systemic signalling events. J. Exp. Bot. 62, 1049-1060. https://doi.org/10.1093/jxb/erq335

Bonneau, L., Huguet, S., Wipf, D., Pauly, N., Truong, H.N., 2013. Combined phosphate and nitrogen limitation generates a nutrient stress transcriptome favorable for arbuscular mycorrhizal symbiosis in *Medicago truncatula*. New Phytol. 199, 188-202. http://dx.doi.org/10.1111/nph.12234

Brandle, J.E., Telmer, P.G., 2007. Steviol glycoside biosynthesis. Phytochemistry 68, 1855-1863. http://dx.doi.org/10.1016/j.phytochem.2007.02.010

Bremner, J.M., Mulvaney, C.S., 1982. Nitrogen-total. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2: Chemical and Micro biological Properties. American Society of Agronomy, Inc., Madison, WI, pp. 595-624.

Breuillin, F., Schramm, J., Hajirezaei, M., Ahkami, A., Favre, P., Druege, U., Hause, B., Bucher, M., Kretzschmar, T., Bossolini, E., Kuhlemeier, C., Martinoia, E., Franken, P., Scholz, U., Reinhardt, D., 2010. Phosphate systemically inhibits development of arbuscular mycorrhiza in Petunia hybrida and represses genes involved in mycorrhizal functioning. Plant J. 64, 1002-1017. http://dx.doi.org/10.1111/j.1365-313X.2010.04385.x

Bruce, A., Smith, S.E., Tester, M., 1994. The development of mycorrhizal infection in cucumber: effects of P supply on root growth, formation of entry points and growth of infection units. New Phytol. 127, 507-514.

Bücking, H., Kafle, A., 2015. Role of arbuscular mycorrhizal fungi in the nitrogen uptake of plants: current knowledge and research gaps. Agronomy 5, 587-612. http://dx.doi.org/10.3390/agronomy5040587

Ceunen, S., Geuns, J.M., 2013. Steviol glycosides: chemical diversity, metabolism, and function. J. Nat. Prod. 76, 1201-1228. http://dx.doi.org/10.1021/np400203b

Chiozzotto, D., Soffritti, M., Falcioni, L., Tibaldi, E., Manservisi, F., Manservigi, M., Manserivi, M., Bua, L., Belpoggi, F., 2011. Results of life span carcinogenicity bioassay on Sprague-Dawley rats exposed to aspartame since foetal life. Eur. J. Oncol. 16, 81-97.

Corrêa, A., Cruz, C., Ferrol, N., 2015. Nitrogen and carbon/nitrogen dynamics in arbuscular mycorrhiza: the great unknown. Mycorrhiza 25, 499-515. http://dx.doi.org/10.1007/s00572-015-0627-6

Corrêa, A., Cruz, C., Pérez-Tienda, J., Ferrol, N., 2014. Shedding light onto nutrient responses of arbuscular mycorrhizal plants: nutrient interactions may lead to unpredicted outcomes of the symbiosis. Plant Sci. 221-222, 29-41. http://dx.doi.org/10.1016/j.plantsci.2014.01.009

Crammer, B., Ikan, R., 1986. Sweet glycosides from the stevia plant. Chem. Br. 22, 915-917.

D'Haene, K., Magyar, M., De Neve, S., Palmai, O., Nagy, T., Németh, T., Hofman, G., 2007. Nitrogen and phosphorus of Hungarian farms. Eur. J. Agron. 26, 224-234. http://dx.doi.org/10.1016/j.eja.2006.10.005

Davies, P.J., 2010. The plant hormones: their nature, occurrence, and functions, in: Davies, P.J. (Eds.), Plant Hormones. Springer Netherlands, pp. 1-15. http://dx.doi.org/10.1007/978-1-4020-2686-7_1

De, S., Mondal, S., Banerjee, S., 2013. Enhancement of stevioside recovery by diafiltration, in: Stevioside: technology, applications and health. John Wiley & Sons, Oxford. http://dx.doi.org/10.1002/9781118350720.ch9.

Di Bene, C., Tavarini, S., Mazzoncini, M., Angelini, L.G., 2011. Changes in soil chemical parameters and organic matter balance after 13 years of ramie [*Boehmeria nivea* (L.) Gaud.] cultivation in the Mediterranean region. Europ. J. Agronomy 35, 154-163.

Dreimanis, A., 1962. Quantitalive gasometric determination of calcite and dolomite by using Chittick apparatus. J.Sediment Petrol. 32, 520-529.

Earanna, N., 2007. Response of *Stevia rebaudiana* to biofertilizers. Karnataka Journal of Agricultural Sciences 20, 616-617.

Gardana, C., Scaglianti, M., Simonetti, P. 2010. Evaluation of steviol and its glycosides in *Stevia rebaudiana* leaves and commercial sweetener by ultra-high-performance liquid chromatography-mass spectrometry. J Chromatogr A, 1217, 1463-1470. http://dx.doi.org/10.1016/j.chroma.2009.12.036

George, E., Marschner, H., Jakobsen, I., 1995. Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Crit. Rev. Biotechnol. 15, 257-270

Giovannetti M., Mosse B., 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol. 84:489-500.

Gomez-Roldan, V., Fermas, S., Brewer P.B., Puech-Pagès V., Dun E.A., Pillot J.P., Letisse F., Matusova R., Danoun S., Portais J.C., Bouwmeester H., Bécard G., Beveridge C.A., Rameau C., Rochange S.F., 2008. Strigolactone inhibition of shoot branching. Nature 455, 189-194. http://dx.doi.org/10.1038/nature07271

Gosling, P., Hodge, A., Goodlass, G., Bending, G.D., 2006. Arbuscular mycorrhizal fungi and organic farming. Agric Ecosyst Environ. 113, 117-135. http://dx.doi.org/10.1016/j.agee.2005.09.009

Guleria, P., Yadav, S.K., 2013. Agrobacterium mediated transient gene silencing (AMTS) in *Stevia rebaudiana*: insights into steviol glycoside biosynthesis pathway. PloS ONE 8, e74731. http://dx.doi.org/10.1371/journal.pone.0074731

Haolin, G., Yuhu,i W., Fengyu, W., Bingrui, J., 2008. Dynamics of root-shoot ratio and environmental effective factors of recovering *Leymus chinensis* steppe vegetation in Inner Mongolia, China. Acta Ecol. Sinica 28, 4629-4634. http://dx.doi.org/10.1016/S1872-2032(09)60002-0

Hawkins, H.J., George, E., 1999. Effect of plant nitrogen status on the contribution of arbuscular mycorrhizal hyphae to plant nitrogen uptake. Physiol. Plant. 105, 694-700.

Hodge, A., Campbell, C.D., Fitter, A.H., 2001. An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413,297-299. http://dx.doi.org/10.1038/35095041

Hoseini, R.Z., Mohammadi, E., Kalatejari, S., 2015. Effect of bio-fertilizer on growth, development and nutrient content (leaf and soil) of *Stevia rebaudiana* Bertoni. J. Crop Prot. 4, 691-704.

Johansen, A., 1999. Depletion of soil mineral N by roots of *Cucumis sativus* L. colonized or not by arbuscular mycorrhizal fungi. Plant Soil. 209, 119-127.

Jones Jr, J.B., 1991. Kjeldahl method for nitrogen determination. Kjeldahl method for nitrogen determination.

Kapulnik, Y., Delaux, P.M., Resnick, N., Mayzlish-Gati, E., Wininger, S., Bhattacharya, C., SejalonDelmas, N., Combier, J.P., Bécard, G., Belausov, E., Beeckman, T., Dor, E., Hershenhorn, J., Koltai, H., 2011. Strigolactones affect lateral root formation and root-hair elongation in Arabidopsis. Planta 233, 209-216. http://dx.doi.org/10.1007/s00425-010-1310-y

Kuk, J.L., Brown, R.E., 2016. Aspartame intake is associated with greater glucose intolerance in individuals with obesity. Appl. Physiol. Nutr. Metab. 41, 795-798. http://dx.doi.org/10.1139/apnm-2015-0675

Ledig, F.T., Perry, T.O., 1966. Physiological genetics of the shoot-root ratio. In: Proceedings of the Society of American Foresters, Society of American Foresters, Washington DC, 39-43.

Lo, S.F., Yang, S.Y., Chen, K.T., Hsing, Y.I., Zeevaart, J.A., Chen, L.J., 2008. A novel class of gibberellin 2-oxidases control semidwarfism, tillering, and root development in rice. Plant Cell 20, 2603-2618. http://dx.doi.org/10.1105/tpc.108.060913

López-Ráez, J.A., Charnikhova, T., Gómez-Roldán, V., Matusova, R., Kohlen, W., De Vos, R., Verstappen, F., Puech-Pages, V., Bécard, G., Mulder, P., Bouwmeester, H., 2008. Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytol. 178, 863-874. http://dx.doi.org/10.1111/j.1469-8137.2008.02406.x.

López-Ráez, J.A., Pozo, M.J., 2013. Chemical signalling in the arbuscular mycorrhizal symbiosis: biotechnological applications. In: Arcora, R. (Ed), Springer Berlin Heidelberg. Symbiotic Endophytes pp. 215-232. http://dx.doi.org/10.1007/978-3-642-39317-4_11

López-Ráez, J.A., Pozo, M.J., García-Garrido, J.M., 2011. Strigolactones: a cry for help in the rhizosphere. Bot. 89, 513-522. http://dx.doi.org/10.1139/b11-046

Madan, S., Ahmad, S., Singh, G.N., Kohli, K., Kumar, Y., Singh, R., Garg, M., 2010. *Stevia rebaudiana* (Bert.) Bertoni - a review. Indian J. Nat. Prod. Resour. 1, 267-286. http://nopr.niscair.res.in/handle/123456789/10287

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. http://dx.doi.org/10.1016/j.apsoil.2013.07.003

Mandal, S., Upadhyay, S., Singh, V.P., Kapoor, R., 2015. Enhanced production of steviol glycosides in mycorrhizal plants: a concerted effect of arbuscular mycorrhizal symbiosis on transcription of biosynthetic genes. Plant Physiol. Biochem. 89, 100-106. http://dx.doi.org/10.1016/j.plaphy.2015.02.010

Marcinek, K., Krejpcio, Z., 2016. *Stevia rebaudiana* Bertoni: health promoting properties and therapeutic applications. J. Verbrauch. Lebensm. 11, 3-8. http://dx.doi.org/10.1007/s00003-015-0968-2

McLean, E.O., 1982. Soil pH and lime requirement. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties. American Society of Agronomy, Inc., Madison, WI, pp. 199-224.

Mehlich, A., 1948. Determination of Cation-and Anion-exchange properties of soil. Soil Sci. 66, 429-446.

Menge, J.A., Steirle, D., Bagyaraj, D.J., Johnson, E.L.V., Leonard, R.T., 1978. Phosphorus concentrations in plants responsible for inhibition of mycorrhizal infection. New Phytol. 80 575-578. http://dx.doi.org/10.1111/j.1469-8137.1978.tb01589.x

Mensah, J.A., Koch, A.M., Antunes, P.M., Hart, M.M., Kiers, E.T., Bücking, H., 2015. High functional diversity within arbuscular mycorrhizal fungal species is associated with differences in phosphate and nitrogen uptake and fungal phosphate metabolism. Mycorrhiza 25, 533-546. http://dx.doi.org/10.1007/s00572-015-0631-x

Mintel, 2014. Stevia set to steal intense sweetener market share by 2017, reports Mintel and Leatherhead Food Research, 13 January 2014.

Murfet, I., Reid, J.B., 1993. Developmental mutants. In: CaseyR, DaviesDR, eds. Peas: genetics, molecular biology and biotechnology. Wallingford, UK: CAB International.

561 Nelson, P.W., Sommers, C.E., 1982. Total C, organic C and organic matter. In: Page, A.L., Miller,
562 R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis, Part 2: Chemical and Microbiological
563 Properties. American Society of Agronomy, Inc., Madison, WI, pp. 539-579.
564
565 Nouri, E., Breuillin-Sessoms, F., Feller, U., Reinhardt, D., 2014. Phosphorus and nitrogen regulate
566 arbuscular mycorrhizal symbiosis in *Petunia hybrida*. PLoS ONE 9, e90841.
567 http://dx.doi.org/10.1371/journal.pone.0090841
568
569 NRCS, U., 1996. Soil survey laboratory methods manual. Soil survey investigations report 2, Version
570 3.0. USDA, Natural Resources Conservation Service, Washington, DC.
571
572 Olsen, S.R., Sommers, L.E., 1982. Phosphorus. In: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.),
573 Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties. American Society
574 of Agronomy, Inc., Madison, WI, pp. 403-430.
575
576 Pal, P.K., Mahajan, M., Prasad, R., Pathania, V., Singh, B., Ahuja, P.S., 2015. Harvesting regimes to
577 optimize yield and quality in annual and perennial *Stevia rebaudiana* under sub-temperate
578 conditions. Ind. Crops. Prod. 65, 556-564. http://dx.doi.org/10.1016/j.indcrop.2014.09.060
579
580 Pedone-Bonfim, M.V.L., da Silva, F.S.B., Maia, L.C., 2015. Production of secondary metabolites by
581 mycorrhizal plants with medicinal or nutritional potential. Acta Physiol. Plant. 37, 1-12.
582 http://dx.doi.org/10.1007/s11738-015-1781-3
583
584 Phillips, J. M., Hayman, D. S., 1970. Improved procedures for clearing roots and staining parasitic and
585 vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of the
586 British mycological Society 55, 158IN16-161IN18.
587
588 Portugal, E.P., Mercuri Quitério, G.C., Honório, S.L., 2006. Seleção de fungos micorrízicos
589 arbusculares para estévia, *Stevia Rebaudiana* (bert.) Bertoni. Edilberto. MultiCiencia: Revista
590 interdisciplinar dos Centros e Nucleos da Unicamp 7, 2-20.
591

592 Rameau, C., Bertheloot, J., Leduc, N., Andrieu, B., Foucher, F., Sakr, S., 2014. Multiple pathways
593 regulate shoot branching. Front. Plant Sci. 5, 741. https://doi.org/10.3389/fpls.2014.00741
594
595 Ramesh, K., Singh, V., Megeji, N.W., 2006. Cultivation of stevia [*Stevia rebaudiana*(Bert.) Bertoni]: A
596 comprehensive review. Adv. Agron. 89, 137-77. http://dx.doi.org/10.1016/S0065-
597 2113(05)89003-0
598
599 Richman, A.S., Gijzen, M., Starratt, A.N., Yang, Z., Brandle, J.E., 1999. Diterpene synthesis in *Stevia*
600 *rebaudiana*: recruitment and up-regulation of key enzymes from the gibberellin biosynthetic
601 pathway. Plant J. 19, 411-421.
602
603 Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., De Pascale, S.,
604 Bonini, P., Colla, G., 2015. Arbuscular mycorrhizal fungi act as biostimulants in horticultural
605 crops. Sci. Hortic. 196, 91-108. http://dx.doi.org/10.1016/j.scienta.2015.09.002
606
607 Saia, S., Benitéz, E., Garcia-Garrido, J.M., Settanni, L., Amato, G., Giambalvo, D., 2014. The effect of
608 arbuscular mycorrhizal fungi on total plant nitrogen uptake and nitrogen recovery from soil
609 organic material. J. Agric. Sci. 152, 370-378. http://dx.doi.org/10.1017/S002185961300004X
610
611 Sbrana, C., Avio, L., Giovannetti, M., 2014. Beneficial mycorrhizal symbionts affecting the production
612 of health-promoting phytochemicals. Electrophoresis, 35,1535-1546.
613 http://dx.doi.org/10.1002/elps.201300568
614
615 Sharma, S., Walia, S., Singh, B., Kumar, R., 2015. Comprehensive review on agro technologies of low-
616 calorie natural sweetener stevia (*Stevia rebaudiana* Bertoni): a boon to diabetic patients. J. Sci.
617 Food Agric. 96, 1867-1879. http://dx.doi.org/10.1002/jsfa.7500
618
619 Silverstone, A.L., Mak, P.Y., Martinez, E.C., Sun, T.P., 1997. The new RGA locus encodes a negative
620 regulator of gibberellin response in *Arabidopsis thaliana*. Genetics 146, 1087-1099.
621
622 Smith, S.E., Jakobsen, I., Grønlund, M., Smith, F.A., 2011. Roles of arbuscular mycorrhizas in plant
623 phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular

mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiol. 156, 1050-1057. http://dx.doi.org/10.1104/pp.111.174581

Smith, S.E., Read, D.J., 2008. Mycorrhizal symbiosis, 3rd edn. Academic press/Elsevier Ltd, London. ISBN 978-0-12-370526-6.

Soffritti, M., Belpoggi, F., Esposti, D.D., Lambertini, L., Tibaldi, E., Rigano, A., 2006. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed to Sprague-Dawley rats. Environ. Health Perspect. 114, 379-385. http://www.jstor.org/stable/3436681

Soffritti, M., Padovani, M., Tibaldi, E., Falcioni, L., Manservisi, F., Lauriola, M., Bua, L., Manservigi, M., Belpoggi, F. 2016. Sucralose administered in feed, beginning prenatally through lifespan, induces hematopoietic neoplasias in male swiss mice. Int. J. Occup. Env. Health. 22, 7-17. http://dx.doi.org/10.1080/10773525.2015.1106075

Suez, J., Korem, T., Zeevi, D., Zilberman-Schapira, G., Thaiss, C.A., Maza, O., Gilad, S., Weinberger, A., Kuperman, Y., Harmelin, A., Kolodkin-Gal, I., Shapiro, H., Halpern, Z., Segal, E., Elinav E., Kuperman, Y., 2014. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature 514, 181-186. http://dx.doi.org/10.1038/nature13793

Sumida, T.,1980. Studies on *Stevia rebaudiana* Bertoni as a new possible crop for sweetening resource in Japan. Kenkyu hokoku, Journal of the Central Agricultural Experiment Station-Japan, Nogyo Shikenjo, Konosu.

Tavarini, S., Angelini, L.G., 2013. *Stevia rebaudiana* Bertoni as a source of bioactive compounds: the effect of harvest time, experimental site and crop age on steviol glycoside content and antioxidant properties. J. Sci. Food Agric. 93, 2121-2129. http://dx.doi.org/10.1002/jsfa.6016

Tavarini, S., Sgherri, C., Ranieri, A.M., Angelini, L.G., 2015. Effect of nitrogen fertilization and harvest time on steviol glycosides, flavonoid composition, and antioxidant properties in *Stevi*a

rebaudiana Bertoni. J. Agric. Food Chem. 63, 7041-7050. http://dx.doi.org/10.1021/acs.jafc.5b02147

Thomson, B.D., Robson, A.D., Abbott, L.K., 1986. Effects of phosphorus on the formation of mycorrhizas by Gigaspora calospora and Glomus fasciculatum in relation to root carbohydrates. New Phytol. 103 751-765. http://dx.doi.org/10.1111/j.1469-8137.1986.tb00850

Touati, J., Chliyeh, M., Touhami, A.O., Benkirane, R., Douira, A., 2014. Effect of the arbuscular mycorrhizal fungi on the growth and root development of selected plant species suggested for slope revegetation. Int. J. Pure App. Biosci. 2, 163-177.

Urcoviche, R.C., Gazim, Z.C., Dragunski, D.C., Barcellos, F.G., Alberton, O., 2015. Plant growth and essential oil content of *Mentha crispa* inoculated with arbuscular mycorrhizal fungi under different levels of phosphorus. Ind. Crops. Prod. 67, 103-107. http://dx.doi.org/10.1016/j.indcrop.2015.01.016.

Vafadar, F., Amooaghaie, R., Otroshy, M., 2014. Effects of plant-growth-promoting rhizobacteria and arbuscular mycorrhizal fungus on plant growth, stevioside, NPK, and chlorophyll content of *Stevia rebaudiana*. J. Plant. Interact. 9, 128-136. http://dx.doi.org/10.1080/17429145.2013.779035

Veresoglou, S.D., Chen, B., Rillig, M.C., 2012. Arbuscular mycorrhiza and soil nitrogen cycling. Soil Biol Biochem. 46, 53-62. http://dx.doi.org/10.1016/j.soilbio.2011.11.018

Wölwer-Rieck, U., 2012. The leaves of *Stevia rebaudiana* (Bertoni), their constituents and the analyses thereof: a review. J. Agric. Food Chem. 60, 886-895. http://dx.doi.org/10.1021/jf2044907

Xiang, Z.P., 1983. Stevia (partial English translation). General Bureau of State Farms, Heilongjiang, China.

Yoneyama, K., Xie, X., Kusumoto, D., Sekimoto, H., Sugimoto, Y., Takeuchi, Y., Yoneyama, K., 2007. Nitrogen deficiency as well as phosphorus deficiency in sorghum promotes the

688 production and exudation of 5-deoxystrigol, the host recognition signal for arbuscular
689 mycorrhizal fungi and root parasites. Planta, 227, 125-132. http://dx.doi.org/10.1007/s00425-
690 007-0600-5
691
692 Zimmermann, B.F., Wölwer-Rieck, U., Papagiannopoulos, M., 2012. Separation of steviol glycosides
693 by hydrophilic liquid interaction chromatography. Food Anal. Methods 5, 266-271.
694 http://dx.doi.org/10.1007/s12161-011-9229-x.

Table 1. Root colonization of *Stevia rebaudiana* Bert. plants by the arbuscular mycorrhizal fungal species *Rhizoglomus irregulare* under three phosphorus fertilization levels. The roots were analysed one month after transplanting.

	Root colonization (%)		
	0P	25P	50P
NM	nd	nd	nd
M	87.6 ± 6.0 a	76.1 ± 6.4 b	75.8 ± 8.9 b

nd = not detectable

Results are the means (n=3) ± SD. Data were arc sin transformed before statistical analysis. Mean values followed by different letters were significantly different at the $p < 0.05$ probability level according to the LSD test.

Table 2. Mean chemical characteristics of three replicates (± standard deviation) of the initial substrate and at the end of the trial.

	Initial substrate	NM+0P	NM+25P	NM+50P	M+0P	M+25P	M+50P
SOM [a] (%)	4.63 ± 0.01 ab	4.54 ± 0.01 b	4.75 ± 0.20 a	4.34 ± 0.06 c	4.24 ± 0.04 c	4.00 ± 0.05 d	4.04 ± 0.13 d
Total N (g kg^{-1})	0.99 ± 0.01 b	1.05 ± 0.01 a	0.97 ± 0.03 bc	0.94 ± 0.02 cd	0.93 ± 0.01 d	0.87 ± 0.02 e	0.92 ± 0.04 d
Available P (mg kg^{-1})	15.21 ± 0.03 c	14.31 ± 0.04 d	16.58 ± 0.02 b	20.85 ± 0.01 a	9.57 ± 0.02 g	11.66 ± 0.01 f	14.27 ± 0.01 e
$CaCO_3$ (g kg^{-1})	2.08 ± 0.18 a	2.06 ± 0.23 a	2.22 ± 0.45 a	2.06 ± 0.23 a	2.22 ± 0.23 a	2.06 ± 0.23 a	2.22 ± 0.23 a
pH	8.07 ± 0.01 d	8.29 ± 0.01 b	8.27 ± 0.01 c	8.27 ± 0.01 c	8.32 ± 0.01 a	8.32 ± 0.01 a	8.32 ± 0.01 a
C.E.C. [b] (meq $100g^{-1}$)	18.50 ± 0.41 a	18.48 ± 0.32 ab	18.62 ± 0.37 a	18.21 ± 0.48 ab	18.80 ± 0.51 a	17.11 ± 0.23 c	17.84 ± 0.31 b
E.C. [c] (µs cm^{-1})	368.10 ± 1.20 f	372.08 ± 1.12 e	427.31 ± 1.18 c	408.42 ± 1.10 d	427.27 ± 1.43 c	444.18 ± 1.91 a	430.20 ± 1.47 b

[a] Soil organic matter
[b] Cation exchange capacity
[c] Electrical conductivity
Mean values followed by different letters were significantly different at the $p < 0.05$ probability level according to the LSD test.

Table 3. Effect of phosphorus fertilization and mycorrhizal inoculation on plant height and branch number of *Stevia rebaudiana* Bert. at 69, 89 and 123 days after transplanting (DAT).

		Plant height (cm)		Branch number	
		NM	M	NM	M
69 DAT	0P	45.50 ± 2.76	39.27 ± 3.02	2.50 ± 0.30	3.40 ± 0.27
	25P	40.63 ± 3.90	38.81 ± 3.96	2.71 ± 0.74	3.00 ± 0.69
	50P	46.26 ± 5.47	38.72 ± 3.52	2.30 ± 0.76	4.20 ± 0.74
	Mean	44.13 A	39.03 B	2.50 B	3.25 A
	Source of variation: AMFxP = ns; AMF = *; P = ns			Source of variation: AMFxP = ns; AMF = **; P = ns	
89 DAT	0P	56.76 ± 3.21	48.27 ± 2.14	2.42 ± 0.11 c	3.53 ± 0.22 b
	25P	52.86 ± 4.10	48.02 ± 3.44	2.60 ± 0.21 c	3.28 ± 0.31 b
	50P	56.87 ± 3.58	45.49 ± 3.15	2.30 ± 0.29 c	4.18 ± 0.35 a
	Mean	55.50 A	47.26 B		
	Source of variation: AMFxP = ns; AMF = ***; P = ns			Source of variation: AMFxP = **; AMF = ***; P = ns	
123 DAT	0P	75.20 ± 3.92	62.88 ± 3.19	2.33 ± 0.41	3.67 ± 0.67
	25P	72.78 ± 3.62	61.58 ± 3.91	2.40 ± 0.32	3.60 ± 0.46
	50P	70.88 ± 3.43	57.82 ± 3.07	2.50 ± 0.58	4.17 ± 0.67
	Mean	72.95 A	60.76 B	2.41 B	3.81 A
	Source of variation: AMFxP = ns; AMF = ***; P = ns			Source of variation: AMFxP = ns; AMF = ***; P = ns	

Results are the means (n=3) ± SD. A two-way ANOVA was used to evaluate the effect of the interaction between mycorrhizal inoculation (AMF) and phosphorus fertilization (P) (AMFxP). Lower-case letters indicate AMFxP interaction, upper-case letters indicate effect of mycorrhizal inoculation (AMF). Significance is indicated as follows: ns, not significant; *, significant at $p < 0.05$ level; **, significant at $p < 0.01$ level; ***, significant at $p < 0.001$ level.

Table 4. Effect of phosphorus fertilization and mycorrhizal inoculation on leaf dry yield, total dry above-ground biomass, and root to shoot ratio of *Stevia rebaudiana* Bert. at 69, 89 and 123 days after transplanting (DAT).

		Leaf dry yield (g plant^{-1})		Total dry above-ground biomass (g plant^{-1})			Root to shoot ratio		
		NM	M	NM	M	Mean	NM	M	Mean
69 DAT	0P	5.72 ± 0.162 a	5.91 ± 0.418 a	8.52 ± 0.130	8.91 ± 0.542		0.89 ± 0.006 a	0.79 ± 0.041 bc	
	25P	5.95 ± 0.478 a	5.35 ± 0.522 a	8.86 ± 0.921	8.22 ± 0.870		0.66 ± 0.005 d	0.72 ± 0.018 cd	
	50P	4.58 ± 0.153 b	5.68 ± 0.489 a	7.24 ± 0.329	8.45 ± 0.358		0.87 ± 0.066 ab	0.76 ± 0.082 c	
	Source of variation: AMFxP = *; AMF = ns; P = *			Source of variation: AMFxP = ns; AMF = ns; P = ns			Source of variation: AMFxP = *; AMF = ***; P = ***		
89 DAT	0P	7.58 ± 0.878	6.61 ± 0.977	12.25 ± 1.602	11.80 ± 0.112	12.02 A	0.74 ± 0.010 a	0.62 ± 0.008 b	
	25P	7.57 ± 0.990	6.32 ± 0.078	12.52 ± 1.550	10.58 ± 0.500	11.55 A	0.60 ± 0.069 b	0.69 ± 0.047 a	
	50P	6.53 ± 0.223	6.63 ± 0.142	10.40 ± 0.149	10.58 ± 0.151	10.49 B	0.70 ± 0.018 a	0.70 ± 0.021 a	
	Source of variation: AMFxP = ns; AMF = ns; P = ns			Source of variation: AMFxP = ns; AMF = ns; P = *			Source of variation: AMFxP = ***; AMF = ns; P = *		
123 DAT	0P	9.05 ± 0.572	10.58 ± 0.547	19.38 ± 0.241	20.66 ± 0.240		0.72 ± 0.003	0.68 ± 0.001	0.70 AB
	25P	9.23 ± 0.536	10.94 ± 0.238	18.71 ± 1.202	21.70 ± 1.303		0.66 ± 0.068	0.66 ± 0.038	0.66 B
	50P	9.72 ± 1.280	11.41 ± 0.219	20.53 ± 1.988	21.56 ± 0.091		0.74 ± 0.051	0.73 ± 0.033	0.73 A
	Mean	9.33 B	10.97 A	19.54 B	21.31 A				
	Source of variation: AMFxP = ns; AMF = ***; P = ns			Source of variation: AMFxP = ns; AMF = **; P = ns			Source of variation: AMFxP = ns; AMF = ns; P = *		

Results are the means (n=3) ± SD. A two-way ANOVA was used to evaluate the effect of the interaction between mycorrhizal inoculation (AMF) and phosphorus fertilization (P) (AMFxP). Lower-case letters indicate AMFxP interaction, upper-case letters indicate effect of mycorrhizal inoculation (AMF) and phosphorus level (P). Significance is indicated as follows: ns, not significant; *, significant at $p < 0.05$ level; **, significant at $p < 0.01$ level; ***, significant at $p < 0.001$ level.

Table 5. Effect of phosphorus fertilization and mycorrhizal inoculation on nitrogen and phosphorus concentration in the leaves, stems and roots of *Stevia rebaudiana* Bert. plants at 123 days after transplanting (DAT).

		N concentration (g $100g^{-1}$)			P concentration (g $100g^{-1}$)	
		NM	M	Mean	NM	M
Leaf	0P	0.87 ± 0.026	0.79 ± 0.001	0.83 A	0.05 ± 0.005 e	0.11 ± 0.001 b
	25P	0.80 ± 0.003	0.76 ± 0.011	0.78 B	0.07 ± 0.001 d	0.10 ± 0.011c
	50P	0.87 ± 0.009	0.80 ± 0.029	0.84 A	0.12 ± 0.003 a	0.11 ± 0.005 b
	Mean	0.85 A	0.78 B			
	Source of variation: AMFxP = ns; AMF = *; P = ***				Source of variation: AMFxP = ***; AMF = ***; P = ***	
Stem	0P	0.30 ± 0.010 ab	0.29 ± 0.026 bc		0.04 ± 0.001 c	0.11 ± 0.005 b
	25P	0.30 ± 0.027 ab	0.30 ± 0.013 ab		0.04 ± 0.002 c	0.12 ± 0.006 a
	50P	0.33 ± 0.007 a	0.26 ± 0.032 c		0.12 ± 0.001 a	0.11 ± 0.005 b
	Source of variation: AMFxP = *; AMF = *; P = ns				Source of variation: AMFxP = ***; AMF = ***; P = ***	
Root	0P	0.36 ± 0.004 b	0.38 ± 0.005 ab		0.03 ± 0.001e	0.09 ± 0.001c
	25P	0.38± 0.011 ab	0.22 ± 0.005 d		0.05 ± 0.005 d	0.10 ± 0.004 b
	50P	0.39± 0.011 a	0.32± 0.031 c		0.11 ± 0.006 a	0.10 ± 0.001 b
	Source of variation: AMFxP = ***; AMF = ***; P = ***				Source of variation: AMFxP = ***; AMF = ***; P = ***	

Results are the means (n=3) ± SD. A two-way ANOVA was used to evaluate the effect of the interaction between mycorrhizal inoculation (AMF) and phosphorus fertilization (P) (AMFxP). Lower-case letters indicate AMFxP interaction, upper-case letters indicate effect of mycorrhizal inoculation (AMF) and phosphorus level (P). Significance is indicated as follows: ns, not significant; *, significant at $p < 0.05$ level; ***, significant at $p < 0.001$ level.

Table 6. Mean nutrient balance of *Stevia rebaudiana* Bert. cultivation, and nutrient use efficiency (NUE) during 123 days of cultivation.

		NM+0P	NM+25P	NM+50P	M+0P	M+25P	M+50P
N (g pot^{-1})	Input:						
	F	0.250	0.250	0.250	0.250	0.250	0.250
	Output:						
	U	0.110	0.103	0.121	0.112	0.115	0.117
	Balance [a]: input − output	0.140	0.147	0.129	0.138	0.135	0.133
	NUE [b] (%)	44	41	48	45	46	47
P (g pot^{-1})	Input:						
	F	-	0.022	0.043	-	0.022	0.043
	Output:						
	U	-	0.010	0.025	-	0.024	0.023
	Balance: input − output	-	0.011	0.018	-	−0.003	0.021
	NUE (%)	-	48	58	-	112	53

[a] Nutrient Balance was calculated as the difference inputs-outputs
[b] Nutrient Use Efficiency (NUE) was evaluated according to Eq.(1).

Table 7. Effect of phosphorus fertilization and mycorrhizal inoculation on steviol glycoside yield of *Stevia rebaudiana* Bert. measured at 69, 89 and 123 days after transplanting (DAT).

		Steviol glycosides yield (g plant^{-1})	
		NM	M
69 DAT	0P	0.60 ± 0.02 b	0.71 ± 0.05 a
	25P	0.75 ± 0.06 a	0.70 ± 0.07 a
	50P	0.59 ± 0.02 b	0.70 ± 0.06 a
Source of variation: AMFxP = *; AMF = *; P = *			
89 DAT	0P	0.86 ± 0.08	0.98 ± 0.08
	25P	0.89 ± 0.08	0.91 ± 0.09
	50P	0.82 ± 0.03	1.04 ± 0.02
	Mean	0.86 B	0.98 A
Source of variation: AMFxP = ns; AMF = **; P = ns			
123 DAT	0P	1.39 ± 0.09 bc	1.30 ± 0.07 c
	25P	1.28 ± 0.07 c	1.59 ± 0.04 a
	50P	1.43 ± 0.08 b	1.66 ± 0.03 a
Source of variation: AMFxP = ***; AMF = ***; P = ***			

Results are the means (n=3) ± SD. A two-way ANOVA was used to evaluate the effect of the interaction between mycorrhizal inoculation (AMF) and phosphorus fertilization (P) (AMFxP). Lower-case letters indicate AMFxP interaction, upper-case letters indicate effect of mycorrhizal inoculation (AMF). Significance is indicated as follows: ns, not significant; *, significant at $p < 0.05$ level; **, significant at $p < 0.01$ level; ***, significant at $p < 0.001$ level.

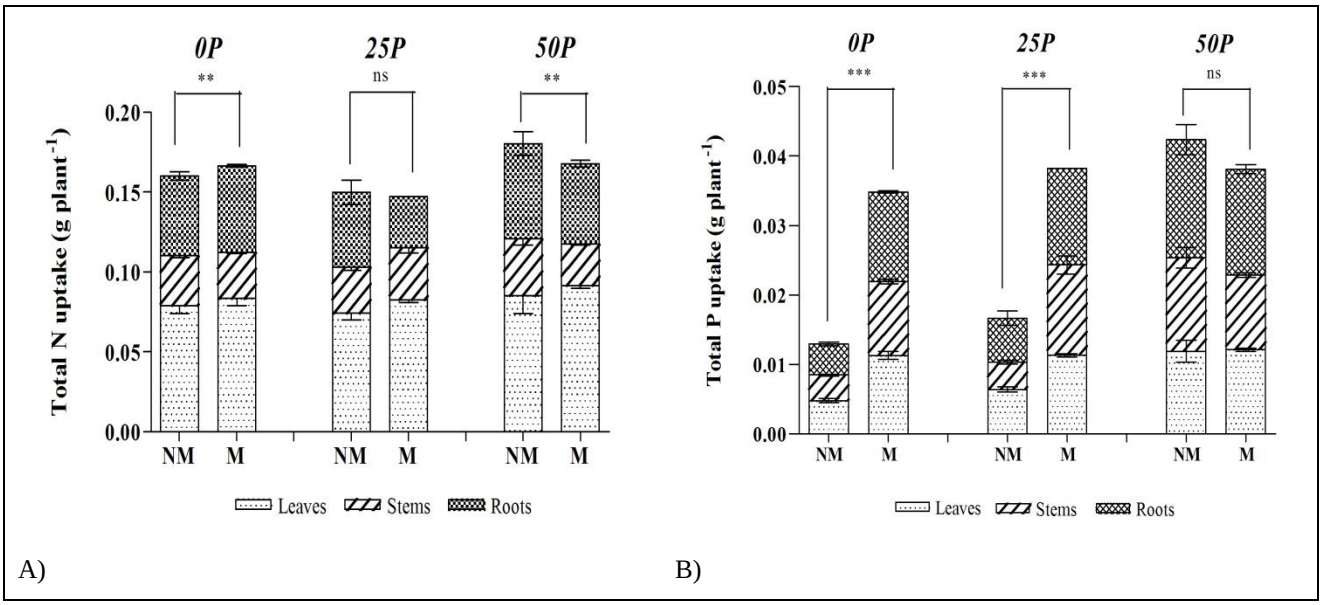

Figure 1. Effect of phosphorus fertilization and mycorrhizal inoculation on total nitrogen (A) and phosphorus (B) uptake (g plant^{-1}) of *Stevia rebaudiana* Bert. at 123 days after transplanting (DAT). Results are the means (n=3) ± SD. Significance is indicated as follows: ns, not significant; **, significant at $p < 0.01$ level; ***, significant at $p < 0.001$ level, according to *t*-test.