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Mycorrhization of strawberry plantlets potentiates the synthesis of phytochemicals during *ex vitro* acclimatization

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ABSTRACT. *Ex vitro* strawberry plantlets from micropropagation and coinoculated with arbuscular mycorrhizal fungi (AMF) and biochar can provide beneficial health effects. In the present study, we evaluated the effects of different proportions of biochar in the presence and absence of AMF on the production of secondary metabolites in the leaves and roots of strawberry plantlets during *ex vitro* acclimatization. Additionally, the enzymatic activity of the substrate enriched with AMF and biochar was analyzed. The experiment consisted of the control (absence of the mycorrhizal community) and four biochar proportions (0, 3, 6, and 9% of the volume of the container) coinoculated with AMF. Plantlets produced on substrates enriched with AMF showed higher levels of polyphenols, flavonoids, phenolic acids, and tannins in the tissues analyzed than control plantlets. The combination of AMF and 9% biochar increased the content of total flavonoids in the leaves of strawberry plantlets and increased the activity of phosphatase. The substrate with up to 6% biochar and mycorrhizae showed increased β-glucosidase activity. In conclusion, mycorrhizae are excellent tools to improve the phytochemical quality of strawberry plantlets acclimatized *ex vitro*. The association between host plants, mycorrhizal symbionts, and bioactivators of these fungi potentiates properties beneficial to health, which can be exploited efficiently in sustainable agriculture.

Keywords: *Fragaria* x *ananassa* Duch.; arbuscular mycorrhiza; biochar; biomolecules; enzymatic activity.

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Introduction

Strawberry (*Fragaria* x *ananassa* Duch., Rosaceae) is a fruit that is appreciated for its excellent flavor, fragrance, and chemical qualities, and has important biological properties, such as antioxidant potential (Chaves, Calvete, & Reginatto, 2017), anti-inflammatory action (Duarte et al., 2018), and antihypertensive and anticancer activities (Giampieri et al., 2015), which are all related to the presence of secondary metabolites, mainly anthocyanins.

Micropropagation is one of the methods of *in vitro* cultivation and is considered a fast form of multiplication that generates plants that are genetically homogeneous and produce disease-free material (Cavallaro, Tringali, & Patanè, 2011). However, micropropagation is a complex procedure that requires the development of appropriate techniques in guaranteeing the success of the process, justifying the high investment in micropropagation. One of the stages of *in vitro* plant production that generates high mortality is acclimatization (Kapoor, Sharma, & Bhatnagar, 2008). After the transfer of plant materials from *in vitro* to *ex vitro* conditions, micropropagated plantlets are susceptible to various stresses owing to environmental changes; thus, the plantlets need to change from heterotrophic to autotrophic feeding. Plantlets grown *in vitro* have poorly developed cuticles, non-functional stomata, and a weak root system (Palei, Das, & Rout, 2015), which can reduce their survival or result in the formation of weak plantlets. This problem can be overcome through special conditions during *ex vitro* cultivation. The coinoculation of the growth substrate with arbuscular mycorrhizal fungi (AMF) and biochar can guarantee success at this stage. This is justified

because AMF form symbiotic relationship with plants and increase their chances of survival, ensuring great acquisition of water and nutrients, and thus promoting plantlets with a more vigorous root system.

Additionally, studies have shown four mechanisms by which biochar can increase AMF activity in growth media and/or plant roots: i) addition of biochar to the soil/substrate results in the availability of nutrients and/or other changes in physical-chemical parameters, which have effects on plants and mycorrhizae; ii) an increase in the bioactivator results in changes that are beneficial or harmful to other soil microbes, such as mycorrhiza helper bacteria or phosphate solubilizing bacteria; iii) changes in signaling dynamics between plants and mycorrhizae; and iv) refuge for colonizing fungi and bacteria (Warnock, Lehmann, Kuyper, & Rillig, 2007).

Many studies have shown that AMF improves the acclimatization of micropropagated horticultural crops, such as artichoke (*Cynara cardunculus* L.) (Campanelli, Ruta, Tagarelli, Morone-Fortunato, & De Mastro, 2014), banana (*Musa* spp.) (Villarreal et al., 2016), and strawberry (Borkowska, 2002).

The symbiosis between AMF and plants results in the promotion of growth and accumulation of secondary metabolites, such as alkaloids, flavonoids, and terpenoids. Therefore, mycorrhizal technology can be used to improve plant secondary metabolites, which can be effective as antioxidants, antibacterials, and antifungals, and in the conservation of endangered plant species (Raghuwanshi & Sinha, 2014). Some studies have also revealed that AMF promote drastic changes in primary and secondary plant metabolisms (Ceccarelli et al., 2010). Thus, under stressful conditions, vegetables tend to increase the production of antioxidant compounds and enzymes such as free radical scavengers, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase, peroxidases, and polyphenoloxidase (Gutteridge & Halliwell, 2010).

Another mechanism in plants that is induced by AMF is the reduction of oxidative stress (Mirjani, Salimi, Matinizadeh, Razavi, & Shahbazi, 2019). Oxidative stress can be prevented by the elimination of hydrogen peroxide in cells, mediated by the activity of CAT, SOD, and APX (Mirjani et al., 2019). Phenolic compounds are antioxidant biomolecules, the main role of which is the elimination of reactive oxygen species (Chaves et al., 2017). Therefore, coinoculation with mycorrhizae is an attempt to combat and minimize oxidative damage. Studies on strawberry plantlets from *in vitro* cultivation are scarce, and these studies are essential as they could effectively contribute to the improvement of plantlet production processes and subsequently the pharmaceutical and cosmetic industries (Ferrari et al., 2020).

Therefore, in this study, we hypothesized that the use of AMF with biochar in the cultivation substrate, in an isolated or combined form, not only interferes with the growth of plantlets but also enhances the contents of secondary metabolites in the leaves and roots of strawberry plantlets during the acclimatization phase. Additionally, it is important to check biochemical processes in plants, such as the production of phenolic compounds and tannins, antioxidant activity, and enzymatic activity in the substrates, to evaluate the action of AMF (Ferrari et al., 2020).

Given the above, the objective of this study was to evaluate whether different biochar concentrations and inoculation with AMF affect the production of secondary metabolites in the leaves and roots of strawberry plantlets during *ex vitro* acclimatization.

Material and methods

Plant material and *in vitro* cultivation

Strawberry meristems of the 'Albion' cultivar, classified as neutral days (ND) regarding flowering, were collected from stolons of plants from the Llahuén/Chilean Patagonia nursery (33º50'15.41" S, 70º40'03.06" W). The explants (stem apexes) were grown in a basic culture medium containing hormones. Multiplication and rooting were performed in the basic MS medium (Murashige & Skoog, 1962), containing 2 mg L^{-1} of benzinoaminopurine (BAP) + 0.5 mg L^{-1} of gibberellic acid for multiplication and 0.005 mg L^{-1} BAP + 6 g agar for rooting. The crops were kept in the dark until the beginning of sprouting and thereafter subjected to controlled light and temperature (16h light per day between 24 and 25ºC). The steps for obtaining the seedlings are shown in Figure 1.

Strawberry acclimatization

After *in vitro* cultivation, the plants were removed from the flasks (Figure 1E), washed in water to remove the residues from the culture medium next to the roots, and placed in plastic trays with paper moistened with water (Figure 1F). They were then transferred to 72-cell polystyrene trays (100 cm^3 $cell^{-1}$) and filled with sterile commercial S10B® substrate (120ºC for 20 min.).

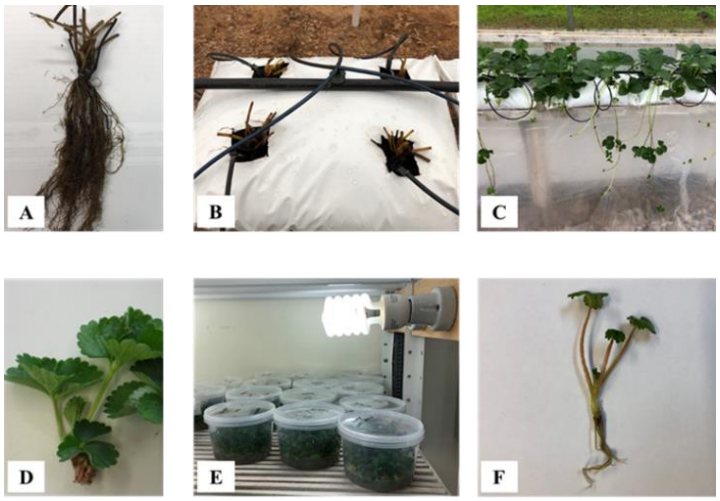

Figure 1. Strawberry plantlets of the 'Albion' cultivar. A) Mother plants; B) Cultivation of mother plant; C) Production of stolons; D) Stolons; E) *In vitro* cultivation; and F) Micropropagated plantlets.

In the experiment, biochar and mycorrhizae were used. Biochar, supplied by *São Paulo Pesquisa e Tecnologia Ltda*., was obtained using a continuous operation reactor at 400ºC with rice husk as the raw material. The AMF was obtained from the trap-cultivation of agricultural soil collected at the reference site for strawberry cultivation in the municipality of Flores da Cunha (29º01'50" S, 51º11'30" W), Rio Grande do Sul State, Brazil (Chiomento et al., 2019a) and is composed of six fungal species, according to Glomeromycota classification by Redecker et al. (2013): *Claroideoglomus claroideum* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler, *Claroideoglomus etunicatum* (W.N. Becker & Gerd.) C. Walker & A. Schüßler, *Funneliformis* aff. *geosporum*, *Glomus* aff. *versiforme*, *Glomus* sp. (*caesaris* like), and *Glomus* sp2.

Experimental design

The experiment consisted of eight treatments outlined in a bifactorial scheme, with four biochar proportions (0, 3, 6, and 9% of the volume of the container) in the presence and absence (control) of inoculation with the AMF community. The experiment was designed in randomized blocks with four replicates.

The biochar was mixed with the cultivation substrate, and in the treatments that received the AMF, 5 g of inoculant was added to the planting pit. To assess the physical and chemical properties of the S10B® substrate, a 500 g sample of the different biochar proportions was used (Table 1).

Table 1. Physical and chemical characterization of the substrate S10B® in the absence and presence of biochar.

Physical properties[2]						
Substrates[1]	D	TP	AS	RAW	BW	RW
	($kg\ m^{-3}$)			($m^3\ m^{-3}$)		
S10B+0%B	241	0.861	0.459	0.155	0.024	0.223
S10B+3%B	243	0.856	0.384	0.219	0.016	0.237
S10B+6%B	242	0.860	0.334	0.278	0.019	0.229
S10B+9%B	237	0.859	0.380	0.234	0.021	0.224
Chemical properties[3]						
Substrates[1]	N	P_2O_5	K_2O	OC	pH	EC	CEC
	% ($m\ m^{-1}$)					$mS\ cm^{-1}$	$mmol_c\ kg^{-1}$
S10B+0%B	1.01	0.83	0.17	21.28	4.6	1.12	473.34
S10B+3%B	0.90	0.82	0.17	18.31	4.7	0.92	451.73
S10B+6%B	1.04	0.70	0.17	21.74	4.7	1.03	488.67
S10B+9%B	0.86	0.66	0.17	18.05	4.8	0.97	471.81

[1]S10B+0%B: substrate S10B + 0% biochar; S10B+3%B: substrate S10B + 3% biochar; S10B+6%B: substrate S10B + 6% biochar; S10B+9%B: substrate S10B + 9% biochar. [2]D: density; TP: total porosity; AS: aeration space; RAW: readily available water; BW buffer water; RW: remaining water. [3]N: nitrogen; P_2O_5: phosphorus pentoxide; K_2O: potassium oxide; OC: organic carbon; pH: potential of hydrogen; EC: electric conductivity; CEC: cation exchange capacity.

Experiment location and procedures

The research was developed at the University of Passo Fundo (28º15'46" S, 52º24'24" W), Rio Grande do Sul State, Brazil, during the period from July (winter) 2017 to September (spring) 2018.

The experiment was carried out in a greenhouse (90 m^2), with a semicircular roof installed in the northeast-southeast direction. The galvanized steel structure was covered with low-density polyethylene film that is anti-UV additive and 150 microns thick, and the sides were covered with an anti-aphid screen.

Sprinkler irrigation was used in the experiment, in a mechanized system, with a flow rate of 1.8 L $min.^{-1}$ per unit. The sprinklers were activated seven times a day, with a total wetting time of 14 min. The water blade supplied to the plantlets was 7.8 mm day^{-1}. The average temperature recorded during the experiment, monitored through a meteorological mini-station, was 20.53ºC. The evaluations were started 2 months after transplant in September 2018.

Mycorrhizal colonization

To verify the infective capacity of the AMF community, root portions of mycorrhized plantlets were prepared according to the method described by Phillips and Hayman (1970) and their mycorrhizal colonization percentages (MC, %) were determined according to Trouvelot, Kouch, and Gianinazzi-Pearson (1986) using the equation:

$$MC\ (\%) = \frac{\text{total number of fragments with mycorrhizal roots}}{\text{total number of fragments}} \times 100 \quad (1)$$

Total polyphenol and flavonoid contents and antioxidant activity

The preparation of leaf and root extracts of strawberry daughter plants was performed according to the methods described by Chaves et al. (2017) and Cvetković et al. (2017). The extracts were obtained from 20 g of dried and ground plant material. A plant material portion (5 g) was subjected to the extraction process with 100 mL of ultrapurified water (plant material:water ratio 1:20 m/v) in the presence of ultrasound. Extraction was performed for 75 min in an ultrasonic washer (SoniClean 2, Sanders®). The extracts obtained were filtered (20 µm), frozen (-70ºC), and lyophilized (LS3000 freeze dryer, Terroni®). Prior to phytochemical analysis, 10 mg of lyophilized extracts of leaves and roots were resuspended with 5 mL of ultrapurified water (plant material:water ratio 2:1 m/v) and kept for 10 min. in an ultrasonic washer and then centrifuged for 5 min. at 3,000 revolutions per minute (rpm).

The total polyphenol content in extracts of leaves (TPL) and roots (TPR) was determined according to the Folin-Ciocalteu method, according to Singleton, Orthofer, and Lamuela-Raventos (1999), using spectrophotometry (PerkinElmer Lambda 20 spectrophotometer, Perkin Elmer®). The total polyphenol content was expressed in grams of gallic acid equivalent per 100 g of dry extract (g GAE 100 g^{-1} DE).

The total flavonoid content in extracts of leaves (TFL) and roots (TFR) was spectrophotometrically obtained according to the method described by Miliauskas, Venskutonis, and Van Beek (2004). The total flavonoid content was expressed in grams of rutin per 100 g of dry extract (g rutin 100 g^{-1} DE).

The antioxidant activity of leaf and root extracts was determined based on their ferric reducing activity, according to the method described by Zhu, Hackman, Ensunsa, Holt, and Keen (2002), by using spectrophotometry. The antioxidant capacity was expressed in grams of gallic acid equivalent per 100 g of dry extract (g GAE 100 g^{-1} DE).

Phenolic acid and tannin contents

The extracts were also evaluated for the identification/quantification of phenolic acids and tannins by using high-performance liquid chromatography attached to a diode array detector (HPLC-DAD).

For phenolic acid analysis of the plant extracts, the chemical references (CR) used were: caffeic acid (> 98%, Sigma Aldrich), chlorogenic acid (⩾ 95%, Sigma Aldrich), coumaric acid (> 98%, Sigma Aldrich), ferulic acid (99%, Sigma Aldrich), and vanillic acid (> 97%, Sigma Aldrich). Regarding the tannin analysis of the extracts, the CR used were catechin (99%, Sigma Aldrich) and epicatechin (99%, Sigma Aldrich).

Prior to the analysis, 20 mg of lyophilized leaf and root extracts were resuspended in 10 mL of ultrapurified water (plant material:water ratio 2:1 m/v), kept for 10 min. in an ultrasonic washer, and filtered through a 0.45 µm nylon membrane.

HPLC analysis was performed using a Flexar LC Perkin Elmer high-performance liquid chromatograph (Burnsville, MN, USA), equipped with a Flexar LC binary pump, Flexar PDA detector at 280 nm, and autosampler. Chromatographic data were analyzed using Chromera Workstation® software. A C18 ACE (250 × 4.6 mm) reverse phase column was used. To prepare the mobile phase, ultrapurified water was used. It was obtained using the Directi-Q System – Millipore®/Millipore Corporation (USA), acetonitrile and methanol

with HPLC grade and phosphoric acid with PA grade. The injection volume was 20 µL of each extract. The CR detection wavelength was 280 nm. These methods were previously validated according to the International Conference on Harmonization (ICH, 2005) and Brasil (2017).

Enzymatic activity of substrate enriched with AMF and biochar

We analyzed the enzymatic activity of the substrate in all treatments tested. Thus, the activities of phosphatase (Tabatabai & Bremmer, 1969) and β-glucosidase (Tabatabai, 1982) were determined.

Statistical analyses

Data were subjected to analysis of variance and regression analysis. When there was statistical significance only for the qualitative factor (mycorrhization), the means of the treatments were compared using Tukey test, at 5% error probability, using the Costat® software.

Results

Mycorrhizal colonization

During the *ex vitro* acclimatization of strawberry plantlets of the 'Albion' cultivar, the effect of mycorrhizal colonization was not found in the root system in relation to the different biochar proportions ($p \geq 0.05$). However, AMF infection was observed in the plantlets, regardless of the biochar that was mixed with the cultivation substrate. The average infectious capacity of the AMF community was 18.50% (Table 2). The fungal structures observed inside the roots were hyphae (mostly) and arbuscules.

Table 2. Effect of mycorrhization on the root system of strawberry plantlets of the 'Albion' cultivar.

Analysis of variance		
Causes of variation	Medium square	
	Degrees of freedom	Mycorrhizal colonization (%)
Blocks	3	137.5^{ns}
Biochar	3	20.83^{ns}
AMF	1	2,812.5*
Biochar x AMF	3	20.83^{ns}
Residue	21	30.35
Total	31	
Comparing means		
Mycorrhization		Mycorrhizal colonization (%)
- AMF		0.00±0.00 b
+ AMF		18.50±8.85 a
Coefficient of variation (%)		18.77

*significant at 5% probability level ($0.01 \leq p < 0.05$). ^{ns}Not significant ($p \geq 0.05$). Data are presented as mean ± standard deviation. Means followed by the same letter in the column do not differ significantly by Tukey's test ($p \leq 0.05$).

Total polyphenol and flavonoid content and antioxidant activity

The total polyphenol and flavonoid content and the antioxidant activity in leaves and roots of strawberry plantlets of the 'Albion' cultivar were analyzed, regardless of the biochar proportions and the absence or presence of AMF. The contents obtained were as follows: a) total polyphenols (g GAE 100 g^{-1} DE) = 12.36 (leaves) and 23.40 (roots), b) total flavonoids (g rutin 100 g^{-1} DE) = 5.16 (leaves) and 2.52 (roots), and c) antioxidant activity (g GAE 100 g^{-1} DE) = 7.28 (leaves) and 7.51 (roots).

AMF had an effect on the TPL, TPR, and TFR regardless of the biochar proportion. The TFL was influenced by the joint action of AMF and biochar.

The TPL of plantlets inoculated with AMF was 10% higher than that of non-mycorrhizal plantlets (Figure 2A). The TFL variable is represented by the positive regression as a function of biochar proportions: the levels of TFL increased in a linear manner with the increase in biochar proportions, regardless of whether the plantlets were inoculated with AMF or not (Figure 2B). In plantlets developed on a substrate without biochar (0%), the TFL levels did not differ in the presence or absence of mycorrhizae. However, plantlets in the medium with 9% biochar showed 6.22 and 7.26 g rutin 100 g^{-1} DE, when developed on substrates in the absence and presence of mycorrhiza, respectively. Thus, these differences in TFL accumulation were 17% higher in mycorrhizal plantlets (Figure 2B).

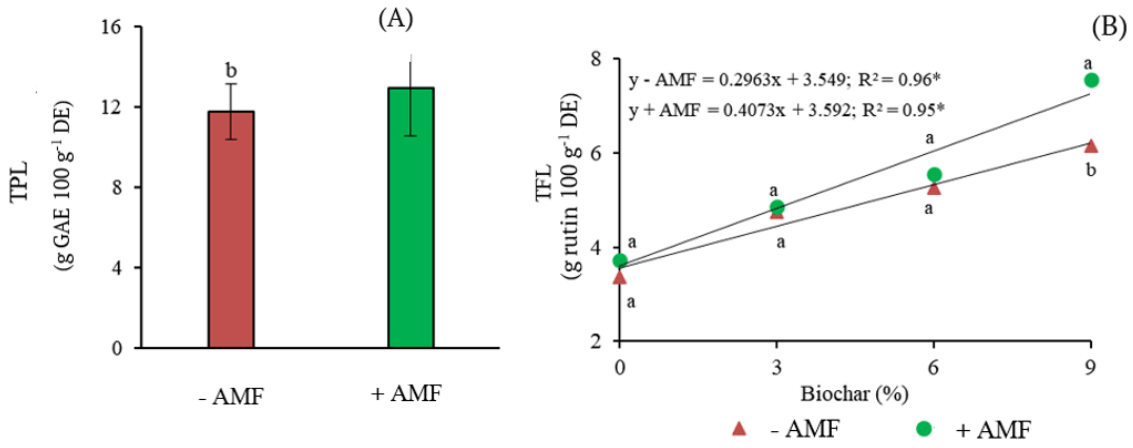

Figure 2. Phytochemical composition of leaves of strawberry plantlets of the 'Albion' cultivar, produced with AMF and biochar. (A) Total polyphenols in leaves (TPL). Data presented as mean ± standard deviation. Different letters above columns indicate significant differences by the Tukey test ($p \leq 0.05$). (B) Total flavonoids in leaves (TFL). Means followed by the same lowercase letter for mycorrhization do not differ statistically by Tukey test ($p \leq 0.05$). *significant at 5% error probability level ($0.01 \leq p < 0.05$).

The phytochemical composition of the root system of strawberry plantlets was changed only by mycorrhization (Figure 3). Plantlets produced with AMF showed 27 and 31% higher TPR (Figure 3A) and TFR (Figure 3B), respectively, than those without mycorrhization.

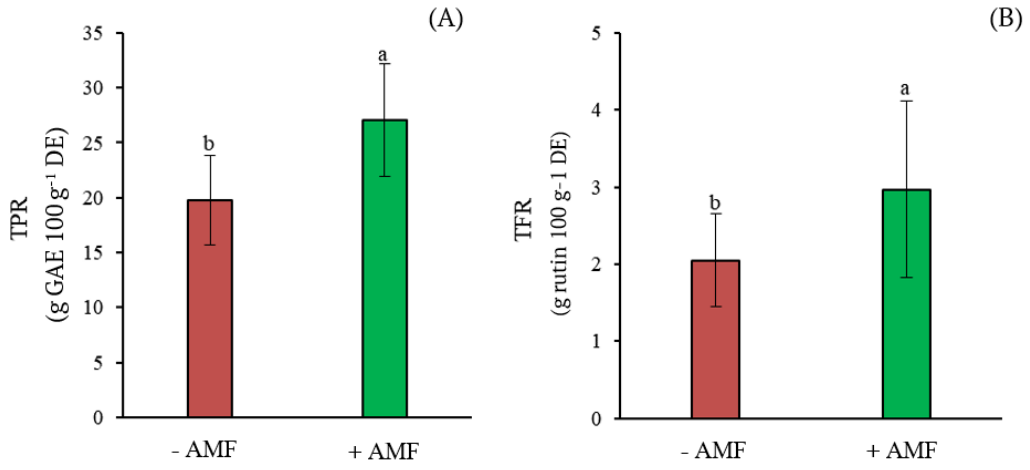

Figure 3. Effect of mycorrhization on the phytochemical composition of roots of strawberry plantlets of the 'Albion' cultivar. (A) Total polyphenols in roots (TPR). (B) Total flavonoids in roots (TFR). Data presented as mean ± standard deviation. Different letters above columns indicate significant statistical differences by the Tukey test ($p \leq 0.05$).

Phenolic acid and tannin content

Among the five phenolic acids studied (caffeic, chlorogenic, coumaric, ferulic, and vanillic acids), only coumaric acid was not detected in the analyzed plantlets. In addition, only caffeic acid was detected in the leaves and vanillic acid in the roots of strawberry plantlets.

Similar to total polyphenol and flavonoid content and antioxidant activity, the levels of phenolic acids and tannins in leaves and roots of strawberry plantlets of the 'Albion' cultivar, were analyzed, regardless of biochar proportions and the absence or presence of AMF. The result indicated a variation between phytochemicals (g substance 100 g^{-1} DE): in the leaves, the highest chlorogenic acid (4.000) and the lowest caffeic acid and epicatechin (0.010) contents were observed, while in the roots, the highest chlorogenic acid (5.384) and the lowest ferulic acid (0.010) contents were observed.

The use of biochar alone and the interaction of AMF with biochar did not alter the composition of phenolic acids and tannins in plantlets. However, an effect of mycorrhization was observed, regardless of biochar proportions, on the contents of phenolic acids (Figure 4) and tannins (Figure 5).

In case of phenolic acids, a positive response was observed in plantlets inoculated with mycorrhizae compared to those that were not inoculated with mycorrhizae. Thus, the level of caffeic acid in the leaves of

the plantlets was 25% more (Figure 4A), whereas in the roots of the plantlets, there was an increase of 42% in ferulic acid (Figure 4B) and 21% in vanillic acid (Figure 4C).

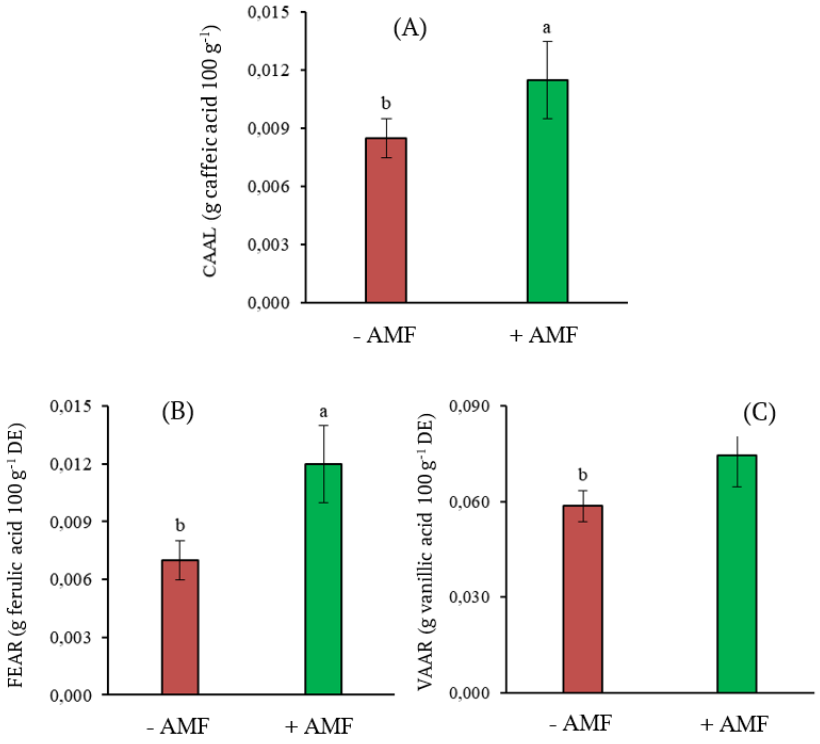

Figure 4. Effect of mycorrhization on the phenolic acid composition of strawberry plantlets of the 'Albion' cultivar. (A) Caffeic acid in leaves (CAAL). (B) Ferulic acid in roots (FEAR). (C) Vanillic acid in roots (VAAR). Data presented as mean ± standard deviation. Different letters above columns indicate significant statistical differences by the Tukey test ($p \leq 0.05$).

In case of tannins, a positive effect was observed in plantlets inoculated with mycorrhizae compared to those that were not inoculated with mycorrhizae. Thus, the catechin content of leaves (Figure 5A) resulted in an increase of 62% compared to those without mycorrhization. In contrast, the roots of plantlets grown with AMF showed a 30% increase in catechin content compared to without mycorrhization (Figure 5B).

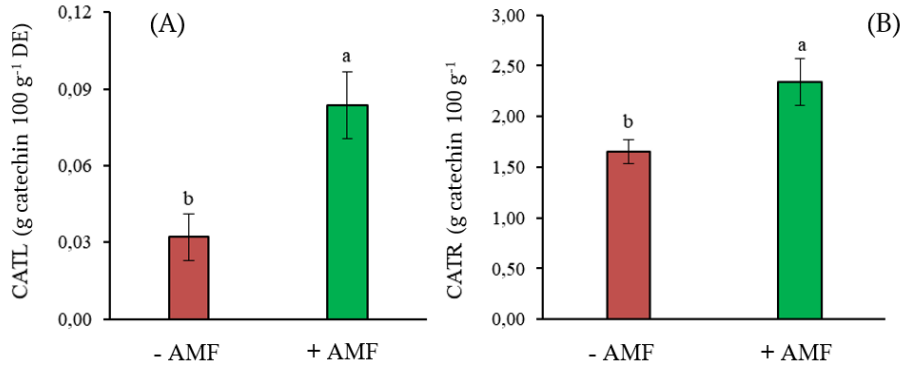

Figure 5. Effect of mycorrhization on the tannin composition of strawberry plantlets of the 'Albion' cultivar. (A) Catechin in leaves (CATL). (B) Catechin in roots (CATR). Data presented as mean ± standard deviation. Different letters above columns indicate significant statistical differences by the Tukey test ($p \leq 0.05$).

Enzymatic activity of substrate enriched with AMF and biochar

Analysis of the activity of phosphatase and β-glucosidase enzymes showed that there was an influence of the absence or presence of mycorrhizae in relation to the different proportions of biochars mixed with the substrate. Similar to the TFL, there was a positive linear increase in enzyme activities as greater proportions of biochar were mixed in the culture medium (Figure 6).

The substrate enriched with 9% biochar and coinoculated with AMF showed greater activity of enzyme phosphatase (3598.66 μg p-nitrophenol g^{-1} substrate h^{-1}) than the substrate containing the same biochar

proportion without mycorrhizae (3247.67 µg p-nitrophenol g^{-1} substrate h^{-1}); the enzyme activity differed statistically between the treatments (Figure 6A).

The greatest difference in β-glucosidase enzyme activity occurred in the absence of biochar (0%); the values of this enzyme in the absence and presence of AMF were 660 µg p-nitrophenol g^{-1} substrate h^{-1} and 902 µg p-nitrophenol g^{-1} substrate h^{-1}, respectively (Figure 6B). Thus, there was a 60% increase in the action of β-glucosidase on substrates containing AMF. This difference diminished as there was an increase in the biochar proportions. Therefore, with the addition of 9% biochar to the substrate, β-glucosidase activity was similar in the absence and presence of mycorrhizae, with only 13% difference between the treatments (Figure 6B).

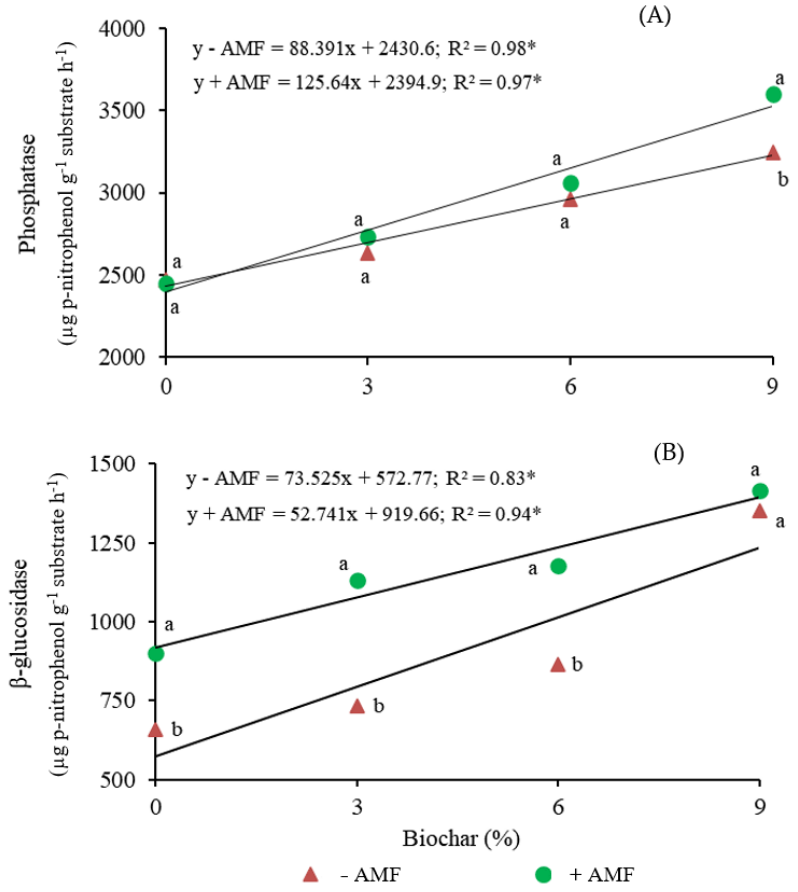

Figure 6. Phosphatase (A) and β-glucosidase (B) activity on substrate enriched with AMF and biochar. Means followed by the same lowercase letter for mycorrhization do not differ statistically by Tukey test ($p \leq 0.05$). *significant at 5% error probability level ($0.01 \leq p < 0.05$).

Discussion

AMF have the capacity to live symbiotically with numerous plants of economic interest, mainly cereals and horticultural crops. In our study, significant increases in phytochemical contents in mycorrhizal strawberry plantlets were observed (total polyphenols and caffeic acid in the leaves; total flavonoids, total polyphenols, ferulic acid, and vanillic acid in roots) compared to that in plantlets not coinoculated with AMF, during *ex vitro* acclimatization.

The concentration of total polyphenols increased (+10%) in the plantlets that received AMF compared to those in the control (without mycorrhization) (Figure 2A). Based on the average of all the biochar proportions, in the roots, the increase was 27% for total polyphenols and 31% for total flavonoids. Thus, the present study demonstrates the effect of AMF on the secondary metabolism of plants, resulting in the biosynthesis of phytochemicals with properties beneficial to health, such as flavonoids and tannins. The biosynthesis of secondary metabolites in mycorrhizal plants involves the activity of enzymes that lead to the production of flavonoids, terpenoids, and polyphenol precursors (López-Ráez, Flors, Garcia, & Pozo, 2010). Such molecules can be modulated by plant hormones, possibly involved in defense responses to mycorrhization (Adolfsson et al., 2017). The high concentration of secondary metabolites in leaves and roots can be attributed to the activation of a host defense response through mycorrhizal colonization (Lingua et al., 2013). Mycorrhizal

colonization of the root system of plantlets was 18.50 ± 8.85% (Table 2); this value can be considered low, but it was enough to promote an increase in phytochemicals in the leaves and roots of plantlets.

The positive effect of mycorrhizae on the phytochemical properties of strawberry fruits has been documented in several cultivars, such as 'Selva' (Lingua et al., 2013), 'Fortuna', 'Sabrina', 'Splendor' (Cecatto, Ruiz, Calvete, Martínez, & Palencia, 2016), and 'Albion' (Chiomento et al., 2019b). Unlike those studies, our study focused on the leaves and roots. Another important aspect to be highlighted in this study was the use of a mycorrhizal community from soil adapted to strawberry cultivation, unlike other studies that used monospecific inoculants or only the genus *Claroideoglomus* or *Glomus*. Research using native populations of AMF generally results in the study's success. This is due to fungus-host compatibility and increased mutualistic effects of two or more symbionts instead of just one (Koron, Sonjak, & Regvar, 2014). It is noteworthy that other studies have already analyzed the phytochemical profile of leaves and roots of strawberry (Besbes, Habegger, & Schwab, 2019), but without using mycorrhizal biotechnology.

Biochar had no effect on the phytochemical properties of plantlets. Few studies have reported the benefit of biochar in increasing secondary metabolites in plants (Umaru, Samling, & Umaru, 2018). Researchers generally target plant growth and crop productivity (Liu et al., 2017). However, in this study, we confirmed the hypothesis that AMF are activated by biochar, both included in the strawberry plantlet growth medium during *ex vitro* acclimatization, owing to the high content of TFL (Figure 2B) and the high activity of phosphatase and β-glucosidase enzymes present in the substrate (Figure 6). The greatest action of phosphatase was found on the substrate with 9% biochar and can be attributed to the low content of P_2O_5 (0.66 m m^{-1}) in the mixture (Table 1). In the medium with 9% biochar, differences in TFL accumulation were found owing to the action of mycorrhizae, with 17% superiority in plantlets (Figure 2B).

The role of biochar occurs indirectly, as there is evidence that its biotechnology improves microbial activity (as is the case with AMF) in the rhizosphere of plant growth media (De Tender et al., 2016), resulting in great enzymatic action of the growth media (Gómez, Denef, Stewart, Zheng, & Cotrufo, 2014). Enzymes respond to the application of biochar in several ways depending on the type and amount of biochar and the properties of the soil/substrate (Zhu, Chen, Zhu, & Xing, 2017). Some components of biochar, such as volatile organic compounds, may be responsible for altering the action of enzymes in a plant growth medium (Paz-Ferreiro, Fu, Mendez, & Gasco, 2014). Enzymatic activity provides information on anthropogenic, agronomic, chemical, and climatic changes in the soil/substrate and can be considered a good indicator of the quality of an agroecosystem (Guerra, Sanjúan, & López, 2018).

From another perspective, soil and/or substrate enzymes are directly involved in nutrient cycling and act as indicators of microbial activity in that medium. Enzymes, produced and released by roots, and microorganisms promote the availability of nutrients in the rhizosphere. Enzymes participate in the hydrolysis of carbon and organic forms of nutrients such as nitrogen (N), phosphorus (P), and sulfur (S). The positive regression found between phosphatase and increasing doses of biochar (Figure 6A) suggests that phosphorus absorption by plantlets occurs, especially in the rhizosphere of those colonized by AMF (Gianfreda, 2015). Thus, the beneficial effect of AMF on substrate enzymes and on metabolic compounds in strawberry leaves and roots may indicate the development of organic and ecological products for agriculture (Zayova, Nikolova, Dimitrova, & Petrova, 2016). However, we recorded a decrease in the activity of β-glucosidase enzyme as the percentage of biochar to the mixture increased, possibly owing to the low N value (0.88 m.m^{-1}) in the substrate (Table 1). It has been reported that this enzyme has a relationship with nitrogen content and is involved in the decomposition of cellulose (Guerra et al., 2018).

The search for studies similar to ours that analyzed secondary metabolites in strawberry leaves and roots indicated that no study had previously investigated the benefits of AMF for the production of these compounds in acclimatized strawberry plantlets *ex vitro*. Our study may be the first to investigate AMF-biochar interface during *ex vitro* acclimatization of strawberry plantlets. However, the relevance of this study is to show that the *in vitro* environment and the *ex vitro* acclimatization of plantlets can alter their primary metabolism and, consequently, generate a spectrum of biomolecules from secondary metabolism, which may differ from those originating from the mother plant. This makes it possible to investigate changes in the phytochemical composition of certain plant species. Secondary metabolites in plants are produced in response to biotic and abiotic stresses. In fact, they are involved in plant defense mechanisms. In tissue culture, when explants are excised during the preparation for culture, it stimulates phenolic exudation.

The increase in the production of active phytochemical constituents is a well-established technology for genetic manipulation through biotechnology, but it presents some challenges, which include identifying the

metabolic pathways by which the compounds are biosynthesized and the genes involved in the main enzymatic or regulatory steps (Raghuwanshi & Sinha, 2014). It is already well established that AMF, when forming symbiotic associations with plants, increases their chances of survival, guaranteeing greater acquisition of water and nutrients and thus promoting plantlets with a more vigorous root system.

Thus, the production of horticultural crops with a high level of phytochemicals meets consumer demands owing to their beneficial health effects (Rouphael et al., 2015). Although the leaves and roots of strawberry plants are sources of phytochemicals, they are the main by-products of the harvest, and tons of them are wasted annually. Although this research obtained answers only for the production of plantlets *ex vitro*, the data suggest the possibility of using these strawberry organs for medicinal purposes (for example, leaves can be used in the form of teas). The phenolic compounds in berry leaves, for example, are known for their antioxidant, anti-inflammatory, and antimicrobial properties (Ferlemi & Lamari, 2016). In addition, secondary metabolites are important for plant defense and contribute to improving plant health (Björkman et al., 2011). Therefore, the use of biotechnological tools that result in the higher accumulation of phytochemicals in plant organs, as is the case with AMF, plays an important role in contributing beneficially to human health and plant protection.

Conclusion

Strawberry fruits are a source of bioactive compounds, which are also synthesized by the process of mycorrhization in strawberry plantlets of the 'Albion' cultivar, during acclimatization *ex vitro*. Thus, the isolated effect of AMF is verified by substances such as total polyphenols in the leaves and total flavonoids in the roots. In addition, biochar acts as an activator of mycorrhizae, potentiating the flavonoid content in leaves and the action of enzymes present in the substrate. In the leaves of mycorrhized plantlets, the presence of caffeic acid was verified, and high levels of ferulic and vanillic acid were found in the roots compared to those that were not coinoculated with mycorrhizae. Thus, in conclusion, mycorrhizae are excellent tools to improve the phytochemical properties of plantlets acclimatized *ex vitro*. This study opens new lines of research to investigate the long-term effect of early AMF inoculation, *ex vitro* acclimatization phase, and its increase into adulthood in the production of these phytochemicals, with benefits in the pharmaceutical and cosmetic industries.

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