

Article

Biostimulatory Action of Arbuscular Mycorrhizal Fungi Enhances Productivity, Functional and Sensory Quality in ‘Piennolo del Vesuvio’ Cherry Tomato Landraces

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Abstract: Arbuscular mycorrhizal fungi (AMF) are a promising tool to improve plant nutrient use efficiency (NUE) and tolerance against abiotic stresses. Moreover, AMF can potentially increase plant productivity and reduce the negative externalities of the agricultural sector. Our study aimed to elucidate whether AMF (containing *Rhizoglyphus irregularis* and *Funneliformis mosseae*) could positively affect not only tomato growth and productivity but also the nutritional and nutraceutical quality of yellow-pigmented type (‘Giagiù’) and red-pigmented type (‘Lucariello’) tomatoes (*Solanum lycopersicum* L.). These cherry tomatoes are landraces of the Protected Designation of Origin (PDO) ‘Pomodoro del Piennolo del Vesuvio’ (PPV), one of the most typical agricultural products of the Campania region (Southern Italy). AMF-rose fruit yield by increasing the number of fruits per plant (+49% and +29% in ‘Giagiù’ and ‘Lucariello’, respectively) but not of the fruit mean mass. AMF increased lycopene (+40%), total ascorbic acid (TAA; +41%), alanine (+162%), gamma-Aminobutyric acid (GABA; +101%) and branched-chain amino acids (BCAAs; +53%) in ‘Lucariello’. In ‘Giagiù’, AMF increased calcium (+63%), zinc (+45%), ASP (+70%), GABA (+53%) and the essential amino acids arginine (+58%) and lysine (+45%), also indicating a genotype-specific response. In both landraces, AMF improved nutrient uptake and biosynthesis of important molecules involved in the control of oxidative stress and cellular pH. In addition to the beneficial effects of human health, the molecules influenced by the AMF treatment are expected to extend the shelf life of tomato fruits, thus further promoting the useful agronomic application of AMF for premium tomatoes marketed fresh or in pendulums (‘piennoli’).

Keywords: *Rhizoglyphus irregularis*; *Funneliformis mosseae*; cherry tomato; essential amino acids; BCAAs; GABA; HPLC; minerals; lycopene

1. Introduction

Growth in population and income, shifts in taste and needs, as well as limited availability of land, have ushered to the perception of crop production as an economic sector different from nature

or the environment, thus downplaying the negative externalities of agriculture [1]. Particularly, intensive use of land associated with overuse of chemical fertilizers has precipitated environmental degradation, pollution and reduction of arable land [2]. Habitat destruction, as a consequence of increasing pressure from agriculture, and wild-life depredation are among the underlying causes of the recent outbreaks of zoonoses like avian influenza, SARS-CoV-1 and the pandemic SARS-CoV-2 (COVID-19) [3,4]. In this view, increasing crop production per unit of land while reducing the use of external inputs, using resource-poor and marginal lands and developing and/or recovering crop varieties with improved yield, palatability and nutritional traits are among the best strategies for sustainable intensification of agricultural systems [5,6]. A promising and environmentally friendly innovation for the realization of such a paradigm could be the use of natural plant biostimulants (PBs) like arbuscular mycorrhizal fungi (AMF). They constitute the most common symbiotic association between plants and microbes. AMF are present in most natural habitats and their action is essential to improve the nutrient use efficiency (NUE), as well as confer resistance and tolerance to stresses [7]. In fact, AMF improve plant uptake and translocation of mineral nutrients by extending their external hyphae from root surfaces to soil areas beyond the mineral resources depletion zone [8]. Inoculation with AMF can boost the adaptability of host plants, including horticultural herbaceous crops [9,10], to changing environment by up-regulating tolerance mechanisms against abiotic stresses like drought, salinity and extreme temperatures [7]. AMF interfere with the plant defense system, increasing not only the activity of antioxidant enzymes like catalase (CAT) and peroxidase (POD), but also the number of antioxidant metabolites like phenolic acids, anthocyanins and flavonoids [11–13], in addition to phytohormones related to defense signaling [10,14]. AMF beneficial effects are mainly exerted through a synergistic tripartite association among host plants, mycorrhizal symbionts and bacterial communities living in the mycorrhizosphere, which carry out nitrogen fixation, P solubilization, in addition to the synthesis of phytohormones, siderophores and antibiotics [15]. The induced changes in secondary metabolism exerted by AMF are also responsible for the increase in phytochemicals in host plants that improve plant growth and resilience but do not always improve commercial quality [8]. Indeed, different plant species respond to AMF inoculation with activation of different secondary metabolic pathways, and most plants undergo activation of these pathways only in the roots and not in above-ground plant organs including edible ones [6]. These drawbacks do not predispose the use of AMF for enhancing the nutritional content and/or the functional quality of crops in a targeted manner. However, it would be insightful to apply these PBs on uniform genetic material presenting limited variation in response to stresses in order to understand the underlying mechanism of action on quality. This is the case with two ‘Pomodoro del Piennolo del Vesuvio’ (PPV) tomato landraces that differ in their capacity of producing lycopene, the main antioxidant metabolite present in tomatoes. PPV are a signature product of Campania (Italy) endangered by genetic erosion [16]. These extensive shelf life PPV landraces undergo rainfed cultivation in a semiarid environment—the lava present on the slopes of the volcano—that enhances the synthesis and accumulation of antioxidant metabolites (e.g., polyphenols and carotenoids), and at the same time the nutraceutical properties of the produce [16]. However, an AMF-triggered increase in protective metabolites could not only enhance the nutraceutical components of the PPVs, and therefore their premium quality, but also function as an instrumental phytochemical tool to attune plant resilience to stress conditions and improve fruit postharvest performance and shelf life [16–18].

Therefore, the aim of the present work was to assess the putative effects of AMF on the yellow-pigmented type (‘Giagiù’) and red-pigmented type (‘Lucariello’) landraces of PPV in terms of quality, growth and marketability; moreover, to provide knowledge on the regulatory mechanisms elicited by these PBs involved in the control of plant metabolism, which constitutes a prerequisite for the standardization of quality in AMF-improved horticultural products.

2. Materials and Methods

2.1. Growth Conditions, Tomato Landraces, Experimental Design and Cultural Practices

A field experiment was carried out in the spring/summer 2019, from 13 April to 29 July, at the experimental station 'Ambrosio' situated at San Giuseppe Vesuviano, Naples, South Italy (Lat: 40.83°, Lon: 14.51°, Alt: 101 a.s.l.). The soil was sandy (89% sand, 7% silt and 4% clay), with an organic matter of 4.0% (*w/w*), pH of 6.6, total N at 0.13%, carbonates at 1.3%, available P at 151 mg kg⁻¹ and exchangeable K at 388 mg kg⁻¹. Figure 1 presents the rainfall distribution patterns during the cultural cycle as well as the minimum, mean and maximum air temperatures.

Two 'Pomodoro del Piennolo del Vesuvio' (PPV) long-shelf-life cherry tomatoes (*Solanum Lycopersicum* L.) landraces were tested in the current experiment: 'Giagiù' and 'Lucariello'. The 'Giagiù' landrace originated from Sant'Anastasia (Naples) is a yellow-pigmented tomato type, while 'Lucariello' originated from Ercolano (Naples) is a red-pigmented tomato type. The two landraces were selected as the most representative long-shelf-life cherry tomatoes cultivated in the Campania region under rainfed conditions differing in their capacity of producing lycopene [16].

Prior to transplanting, organic fertilizer BIOREX manufactured by Italtollina S.p.A (Verona, Italy) was broadcast at a rate of 750 kg ha⁻¹. The composition of the organic fertilizer was as follows: N (2.8%), P₂O₅ (2.5%), K₂O (3.0%), organic matter and carbon (65% and 38%, respectively), C/N (13) and a pH of 6.0. Seedlings of 'Giagiù' and 'Lucariello' cherry tomato landraces were transplanted manually at the three-leaf stage on April 13, at a plant density of 40,000 plants per hectare.

The experiment was arranged according to a Split Plot Design with three replications, AMF application being the main plot and landrace the subplot. Each block consisted of the two landraces and the microbial biostimulant treatment containing two strains of mycorrhizae (+AMF; arbuscular mycorrhizal fungi) and the control (-AMF). Each experimental plot covered 20 m² and contained 80 plants. Mycorrhization was performed by placing 2 g in the planting hole just before transplanting of a commercial microgranular inoculum (Aegis Migrogranule—Italtollina S.p.A) carrying 25 spores g⁻¹ of *Rhizoglyphus irregularis* BEG72 (ex. *Glomus intraradices* BEG72) and 25 spores g⁻¹ of *Funneliformis mosseae* BEG234 (ex. *Glomus mosseae* 234). During the cultural cycle, aphids were controlled by a foliar treatment of Acetameprid (KESTREL, Adama Italia, Grassobbio, Italy), whereas *Tuta absoluta* was controlled by three applications of a commercial formulation containing *Bacillus thuringiensis* var. *kurstaki* (Sequra WG, Sipcam Italia, Rho, Italy). For the containment of the most common fungal and bacterial diseases, a copper-based covering product (Cuproxtat SDI, Nufarm, Melbourne, Australia) was used to avoid any negative interference with the mycorrhization.

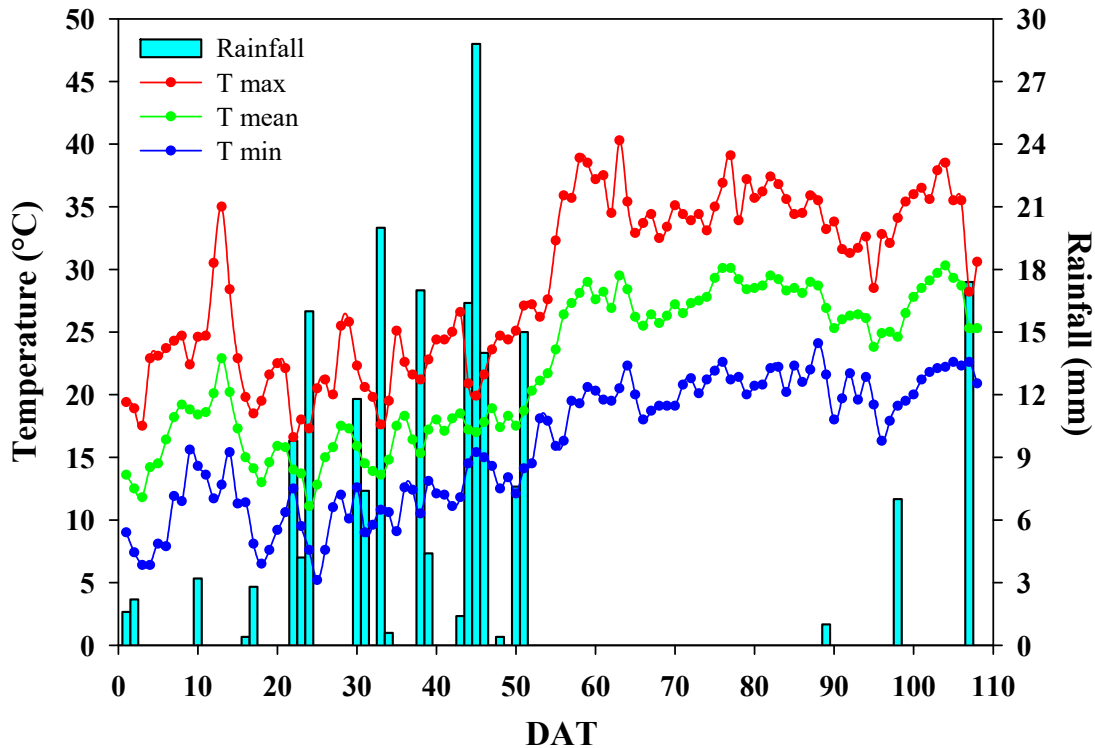


Figure 1. Minimum, mean and maximum air temperature and rainfall during the 2019 growing season.

2.2. Yield Measurements, Fruit Quality Sampling and Arbuscular Mycorrhizal Fungi Root Colonization

Harvesting of fully ripe tomato fruits initiated at 95 days after transplanting (16 July; first truss harvest) and terminated 108 days after transplanting (29 July; third truss harvest). The marketable tomato fruit mean weight and number, together with fresh yield were determined for each experimental replicate plot. Tomato fruits of the second harvest truss were used for quality analyses. A subsample of fresh tomato was used for the determination of total soluble solids and fruit dry matter contents, whereas the remaining subsample was frozen in liquid nitrogen and stored at -80°C for further analyses: lipophilic and hydrophilic antioxidant activities, starch, total ascorbic acid, lycopene, sucrose, macro and microelements, amino acids profile including essential and branched-chain amino acids.

At the end of the experiment (29 July; 108 days after transplanting), tomato roots from 4 plants per replicate (i.e., experimental plot) were rinsed from soil, and subsamples were saved for assessment of AMF root colonization. Root samples were cleared with potassium hydroxide (10%), stained with trypan blue (0.05%) in lactophenol as described by Phillips and Hayman [19], and microscopically assessed for AMF colonization by determining the percentage of colonized root segments using a gridline intercept method [20].

2.3. Total Soluble Solids and Fruit Dry Matter Content

The filtrated tomato juice was used to measure the total soluble solids (TSS) content on a digital refractometer (Atago N1 model, Atago Company Ltd.; Tokyo Japan). A subsample of tomato juice was also used to determine the fruit dry matter content (DM) by desiccation to constant weight in a forced air oven at 70°C for 3 days. The DM was calculated using the following formula: $\text{DM} (\%) = 100 \times \text{fruit dry weight} / \text{fruit fresh weight}$. The dry weight fruit tissue material was stored and used for macro and micro mineral analysis.

2.4. Macro and Micro Mineral Content Analysis

An inductively coupled plasma (ICP) mass spectrometer (ICP-OES Spectroblue, Spectro Ametek, Berwyn, PA, USA) was used to assess the macro (phosphorus, potassium, calcium and magnesium) and micro (sodium, iron, copper, manganese and zinc) mineral content of cherry tomato fruits, based on the method described by Volpe et al. [21]. Briefly, 1 g of oven-dried tomato samples were digested in a microwave digestion system after the addition of nitric acid (65%) and hydrochloric acid (37%). The detected elements were quantified against two different calibration curves, one for nonalkaline elements ranging from 1 to 100 $\mu\text{g L}^{-1}$ and another for alkaline elements ranging from 100 $\mu\text{g L}^{-1}$ to 100 mg L^{-1} . Macro and microelements were expressed as mg g^{-1} dry weight (dw), respectively.

2.5. Antioxidant Activity and Bioactive Compounds Analysis

The antioxidant capacity and lycopene content of lipophilic (LAA) and hydrophilic (HAA) fractions were determined on freeze-dried tomato samples, whereas the total ascorbic acid (TAA) was determined on fresh tomato material and quantified on a Hach DR 2000 spectrophotometer (Hach Co., Loveland, CO, USA), according to [22–25]. Solution absorbances of the two antioxidant activities (LAA and HAA) and the two bioactive compounds (lycopene and TAA) were assessed at 505, 734, 472 and 765 nm, respectively.

2.6. Starch and Sucrose Analysis

Sucrose ($\mu\text{mol g}^{-1}$ dw) was estimated in the supernatant of ethanolic extracts of lyophilized tomato samples by an enzymatic coupled assay based on the spectrophotometric determination of NADH at 340 nm recorded by a Synergy HT spectrophotometer (BioTEK Instruments, Bad Friedrichshall, Germany) [16]. Starch was quantified with the same enzymatic coupled assay in the pellets of the ethanolic extracts after hydrolysis to glucose. Starch was expressed as glucose equivalents [26].

2.7. Amino Acids Analysis

Primary amino acids and proline ($\mu\text{mol g}^{-1}$ dw) were extracted from 15 mg of lyophilized fruit samples in 1 mL ethanol/water (40:60 *v/v*) overnight at 4 °C, and estimated by HPLC after precolumn derivatization with *o*-phthalaldehyde (OPA) according to Carillo et al. [16]. Proline was determined in the same ethanolic extract by using an acid ninhydrin method according to Woodrow et al. [27].

2.8. Statistics

Analysis of variance (ANOVA) was conducted for all traits. Hierarchical cluster analysis was employed to assess the effect of AMF application on the overall performance of the two landraces. In multivariate analysis, all variables included in the model had an equal contribution. Therefore, due to the high number of amino acids examined, two separate hierarchical analyses were conducted. In the first analysis, only the amino acids were included. In the second analysis, all the other variables were included plus total amino acid content. Squared Euclidean distances were estimated on standardized Z values, with a mean of 0 and a standard deviation of 1. Clustering was performed using the 'WARD' method. All analyses were carried out using SPSS (IBM, SPSS ver. 26).

3. Results

3.1. Fungal Concentrations in the Rhizo-Soil and Yield Responses of two Tomato Landraces as Affected by AMF Inoculation

The percentage of mycorrhizal root colonization 108 days after transplanting was significantly higher in the microbial inoculation treatment (avg. 24.2%) than the noninoculated control (4.6%), with no significant differences recorded between the two cherry tomato landraces 'Giagiù' and 'Lucariello'

(data not shown). Concerning the effects of microbial inoculation on crop productivity, our results showed significant differences between the two long-shelf-life cherry tomato landraces, irrespective of AMF treatment with the highest apparent values recorded in the red-pigmented type ('Lucariello') compared to the yellow-pigmented one ('Giagiù') (Table 1). However, the inoculation with arbuscular mycorrhizal fungi caused a significant increase in the fruit yield of both landraces without significant interaction. Interestingly, the higher production observed in cherry tomato landraces inoculated AMF biostimulant was attributed to an increase in fruit number per plant (+38.7%) and not to an increase in fruit mean mass (Table 1). Overall, the microbial-based biostimulant significantly improved the cumulative yield by 30.9%, compared to the noninoculated cherry tomato plants (Table 1).

Table 1. Significance of the main effects and their interactions on total yield, number of fruits and fruit mean weight. Values represent means of three replicates.

Source of Variation	Yield	Number of Fruits	Fruit Mean Weight
	(g m ⁻²)	(no. m ⁻²)	(g fruit ⁻¹)
Mycorrhiza (M)	**	***	*
Landraces (L)	**	ns	**
M x L	ns	ns	ns
Main effect—AMF			
–AMF	728	67.97	10.66
+AMF	953	94.33	10.16
Main effect—Landrace			
'Giagiù'	730	81.75	8.96
'Lucariello'	951	80.55	11.86

ns, *, **, *** Nonsignificant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. AMF: Arbuscular mycorrhizal fungi.

3.2. Mineral Profile of Two Tomato Landraces as Affected by AMF Inoculation

Among the macro minerals analyzed potassium was by far the most abundant in the four tested combinations followed by phosphorus, calcium and magnesium, while iron was the most abundant micro mineral followed by zinc, copper, sodium and finally manganese (Table 2). Neither landrace nor AMF inoculation had a significant effect on K (avg. 32.1 mg g⁻¹ dw), Fe (avg. 43.1 µg g⁻¹ dw) and Mn (avg 7.7 µg g⁻¹ dw) concentrations in tomato fruits (Table 2). The effect of AMF inoculation was significant for P, Ca, Mg, Na, Cu and Zn and the effect of landrace was significant for Ca, Na, Cu and Zn. The concentrations of Ca and Na in tomato fruits were also significantly affected by the interaction of the two tested factors (landrace and microbial-based biostimulant) (Table 2). The highest Ca and Na concentrations in tomato fruits were recorded in 'Giagiù' with (+AMF) and without (–AMF) inoculation, respectively (data not shown). Moreover, when averaged over AMF inoculation, 'Lucariello' was characterized by a higher Cu content compared to 'Giagiù', whereas an opposite trend was observed for Ca, Na and Zn (Table 2). Interestingly, the AMF inoculation averaged over landrace affected positively the mineral status in particular P, Ca, Mg, Cu and Zn concentrations in fruit tissue which were higher by 24.7%, 28.2%, 3.7%, 9.8% and 34.2% than in noninoculated tomato plants (Table 2).

Table 2. Significance of the main effects and the interactions on the macro (phosphorus, potassium, calcium and magnesium) and micro (sodium, iron, copper, manganese and zinc) mineral content. Values represent means of three replicates.

Source of Variation	P (mg g ⁻¹ dw)	K (mg g ⁻¹ dw)	Ca (mg g ⁻¹ dw)	Mg (mg g ⁻¹ dw)	Na (mg g ⁻¹ dw)	Fe (µg g ⁻¹ dw)	Cu (µg g ⁻¹ dw)	Mn (µg g ⁻¹ dw)	Zn (µg g ⁻¹ dw)
Mycorrhiza (M)	*	ns	**	*	***	ns	*	ns	**
Landraces (L)	ns	ns	**	ns	***	ns	*	ns	*
M × L	ns	ns	**	ns	**	ns	ns	ns	ns
Main effect – AMF									
–AMF	2.83	31.86	1.10	1.09	0.02	41.48	12.58	7.55	19.95
+AMF	3.53	32.32	1.41	1.13	0.01	44.66	13.81	7.88	26.78
Main effect – Landrace									
‘Giagiù’	3.24	32.40	1.43	1.13	0.03	42.77	12.52	7.90	26.09
‘Lucariello’	3.12	31.78	1.08	1.09	0.01	43.37	13.87	7.53	20.64

ns, *, **, *** Nonsignificant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively.

3.3. Fruit Soluble Solids, Dry Matter, Sucrose, Starch, Antioxidant Activity and Bioactive Compounds of two Tomato Landraces as Affected by AMF Inoculation

The AMF application significantly affected all traits, whereas the effect of landrace was significant only for LAA, HAA, lycopene and sucrose (Table 3). A significant interaction between microbial-based biostimulant and cherry tomato landrace was observed for sucrose and total ascorbic acid (TAA). Interestingly, inoculation with endophytic fungi enhanced TAA by 14.7% and 29.0% for ‘Giagiù’ and ‘Lucariello’, respectively (Figure 2). Moreover, when averaged over AMF inoculation, ‘Giagiù’ was characterized by higher LAA compared to ‘Lucariello’, whereas an opposite trend was observed for HAA, lycopene and sucrose (Table 3). Finally, the AMF inoculation averaged over landrace affected positively the TSS content, fruit DM percentage, LAA, HAA, starch, TAA and lycopene in fruit tissue which were higher by 9.7%, 9.7%, 12.0%, 8.6%, 31.4%, 28.4% and 46.7% than in noninoculated tomato plants (Table 3).

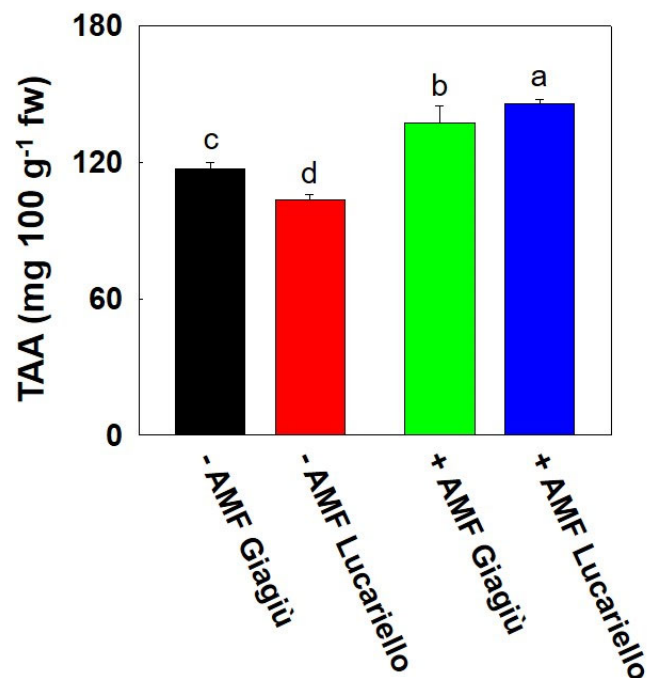


Figure 2. Effects of arbuscular mycorrhizal fungi (AMF) on total ascorbic acid (TAA) of yellow-pigmented type (‘Giagiù’) and red-pigmented type (‘Lucariello’) landraces of ‘Pomodorino del Piennolo del Vesuvio’ (PPV) cultivated in Campania region under rainfed conditions. Values are the means of three replicates \pm SD. Different letters indicate significant differences according to Duncan’s test ($p = 0.05$).

Table 3. Significance of the main effects and the interactions on total soluble solids (TSS) content, dry matter, lipophilic and hydrophilic (LAA and HAA), starch, lycopene and sucrose. Values represent means of three replicates.

Source of Variation	TSS (°Brix)	DM (%)	LAA (mmol Trolox 100 g ⁻¹ dw)	HAA (mmol AA 100 g ⁻¹ dw)	Starch (µmol g ⁻¹ dw)	TAA (mg 100 g ⁻¹ fw)	Lycopene (mg 100 g ⁻¹ dw)	Sucrose (µmol g ⁻¹ dw)
Mycorrhiza (M)	*	**	*	*	**	***	**	*
Landraces (L)	ns	ns	**	*	ns	ns	***	**
M × L	ns	ns	ns	ns	ns	**	ns	*
Main effect—AMF								
−AMF	8.16	9.83	17.24	10.43	15.60	110.2	26.23	13.52
+AMF	8.95	10.78	19.31	11.33	20.50	141.5	38.48	11.56
Main effect—Landrace								
‘Giagiù’	8.67	10.37	19.75	10.67	18.22	127.0	5.89	11.59
‘Lucariello’	8.45	10.23	16.79	11.08	17.88	124.7	58.82	13.49

ns, *, **, *** Nonsignificant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. TAA: total ascorbic acid.

3.4. Amino Acids Profiling of two Tomato Landraces as Affected by AMF Inoculation

The HPLC analysis showed that the average total amino acids content differed significantly between the two PPV landraces as it ranged from 602.53 $\mu\text{mol g}^{-1}$ dw in 'Giagiù' to 954.3 $\mu\text{mol g}^{-1}$ dw in 'Lucariello' (+58.3%) (Table 4). The effect of AMF was also significant as the total amino acid content increased, on average, from 672.1 to 884.7 $\mu\text{mol g}^{-1}$ dw (+31.6%) with the application. The effect of mycorrhiza and landrace was significant on nearly all amino acids. In all cases, amino acid concentration was higher with AMF application and with landrace 'Lucariello'. The application of AMF increased the total amino acid content of both 'Giagiù' and 'Lucariello' by 27.3% and 34.4%, respectively. Alanine, GABA, Ile, Leu, Met, Ser, Trp, Val and branched-chain amino acids (leucine, isoleucine and valine; BCAAs) were significantly influenced by Landrace \times AMF inoculation interaction (Table 4). Of the mean total amino acid content of both landraces, it was observed that Glu, Gln, GABA and Asn accounted on average for 25.5%, 17.3%, 16.1% and 12.7%, respectively (Table 4). Correspondingly, the essential amino acids (the sum of Arg, His, Ile, Leu, Lis, Met, Phe, Thr, Trp and Val) accounted for 11.4% of total amino acids (Table 4; Figure 3E). The highest values of Ala, GABA, Ile, Leu, Met, Ser, Trp, Val and BCAAs were recorded in 'Lucariello' tomato fruit inoculated with AMF (Table 4, Figure 3). In fact, the AMF treatment on 'Lucariello' enhanced Ala (+162%), GABA (+100.1%), MEA (+61.8%) and essential amino acids (+36.2%), of which in particular tryptophan (+106.3%), methionine (+67.4%) and BCAAs (+52.6%; Table 4; Figure 3). By contrast, the beneficial effect of microbial inoculant on 'Giagiù' amino acids was only observed on Asp (+69.6%), GABA (+53.1%) and essential amino acids (+33.4%), of which in particular Arg (+57.7%) and Lys (+45.3%).

3.5. Hierarchical Cluster Analysis

The hierarchical cluster analysis of amino acids accounted for in Table 4 is presented in Figure 4 and clearly shows landrace differences in amino acid concentrations. The application of AMF had a stronger effect on 'Lucariello' than on 'Giagiù'. In particular, AMF not only enhanced the concentration of total amino acids, but it also reshaped their profiles as evidenced in Section 3.4 and summarized in the pathway map shown in Figure 5, which includes also the metabolites presented in Table 3. The different response of the two landraces explains the observed interactions.

Cluster analysis based on the traits listed in Tables 1–3 and total amino acid content clearly shows a strong effect of AMF application on the overall performance of both landraces (Figure 4). Despite that, the absence of significance in the $M \times L$ interactions for most traits, overall, the two landraces differed in their response to the AMF application, as shown by the widely differing distance between their +AMF and -AMF clusters (Figure 6). Therefore, the discrimination of the two landraces based on morpho-physiological and metabolic parameters is accentuated by the AMF application.

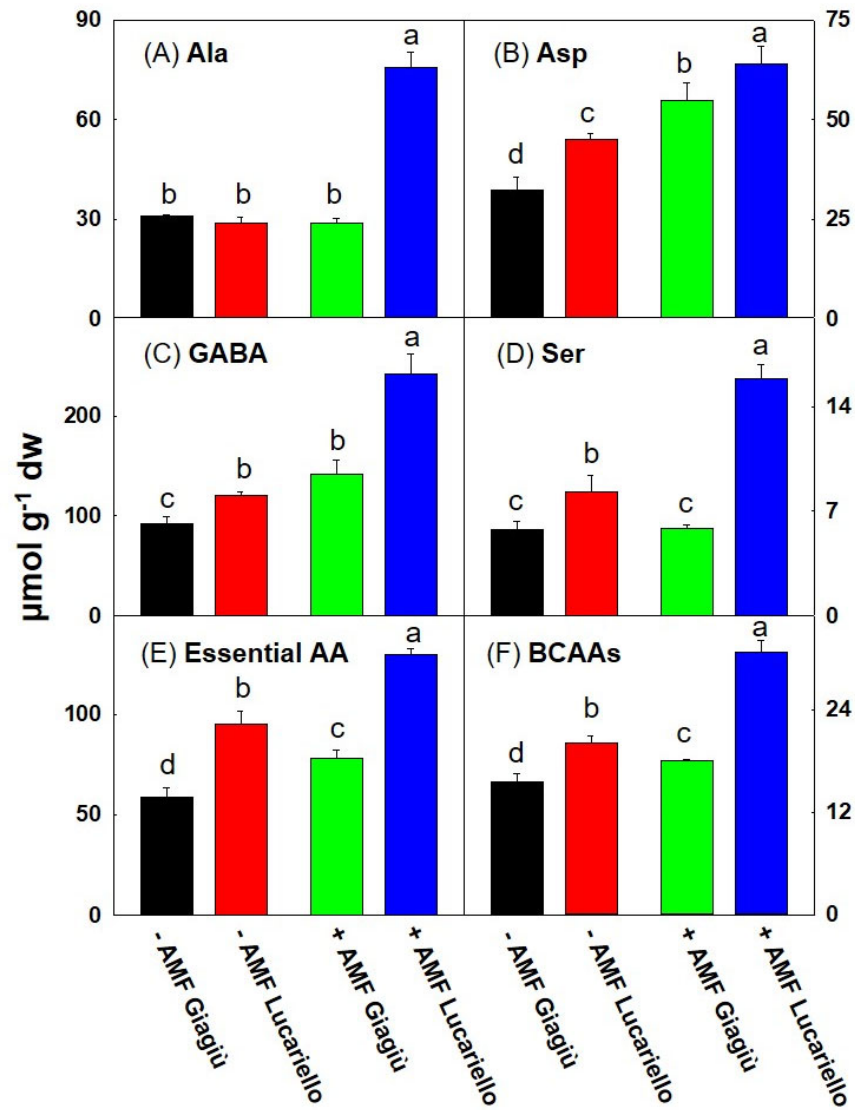


Figure 3. Effects of arbuscular mycorrhizal fungi (AMF) on alanine (A), aspartate (B), GABA (C), serine (D), essential amino acids (E) and branched-chain amino acids (BCAAs) (F) of yellow-pigmented type ('Giagiù') and red-pigmented type ('Lucariello') landraces of 'Pomodorino del Piennolo del Vesuvio' (PPV) cultivated in the Campania region under rainfed conditions. Values are the means of three replicates \pm SD. Different letters indicate significant differences according to Duncan's test ($p = 0.05$).

Table 4. Significance of the main effects and their interactions on free amino acids profile and total amino acids content. Values represent means of three replicates.

Amino Acids ($\mu\text{mol g}^{-1}$ dw)	Main Effect Mycorrhiza		Main Effect Landraces		Significance		
	-AMF	+AMF	'Giagiù'	'Lucariello'	Mycorrhiza (M)	Landraces (L)	M x L
Ala	29.86	52.22	29.80	52.27	***	***	***
Arg	26.10	36.82	24.86	38.06	**	***	ns
Asn	89.54	91.37	58.75	122.16	ns	**	ns
Asp	38.61	59.36	43.50	54.47	***	*	ns
GABA	106.4	191.4	116.8	181.0	***	**	*
Gln	117.6	118.7	87.08	149.3	ns	**	ns
Glu	169.9	213.4	157.7	225.6	ns	*	ns
Gly	12.00	12.66	9.93	14.73	ns	*	ns
His	8.06	11.15	8.04	11.18	**	*	ns
Ile	7.32	10.27	7.12	10.47	**	***	*
Leu	5.91	8.33	5.39	8.84	***	***	**
Lys	11.82	16.86	9.19	19.49	*	**	ns
MEA	3.89	5.82	4.48	5.23	*	ns	ns
Met	1.06	1.58	0.78	1.85	**	***	**
Orn	2.38	3.02	2.02	3.39	*	**	ns
Phe	6.02	4.73	3.62	7.13	*	**	ns
Pro	13.32	17.15	15.18	15.30	*	ns	ns
Ser	7.01	10.85	5.81	12.04	***	***	**
Thr	3.42	4.11	2.74	4.78	ns	*	ns
Trp	2.45	4.11	2.22	4.33	**	**	**
Tyr	4.79	4.93	3.18	6.55	ns	*	ns
Val	4.61	5.79	4.28	6.11	**	**	*
Essential AA	76.77	103.7	68.26	112.3	***	***	ns
BCAAs	17.84	24.38	16.80	25.42	***	***	**
Total AA	672.1	884.7	602.53	954.3	**	***	ns

ns, *, **, *** Nonsignificant or significant at $p \leq 0.05, 0.01$ and 0.001 , respectively.

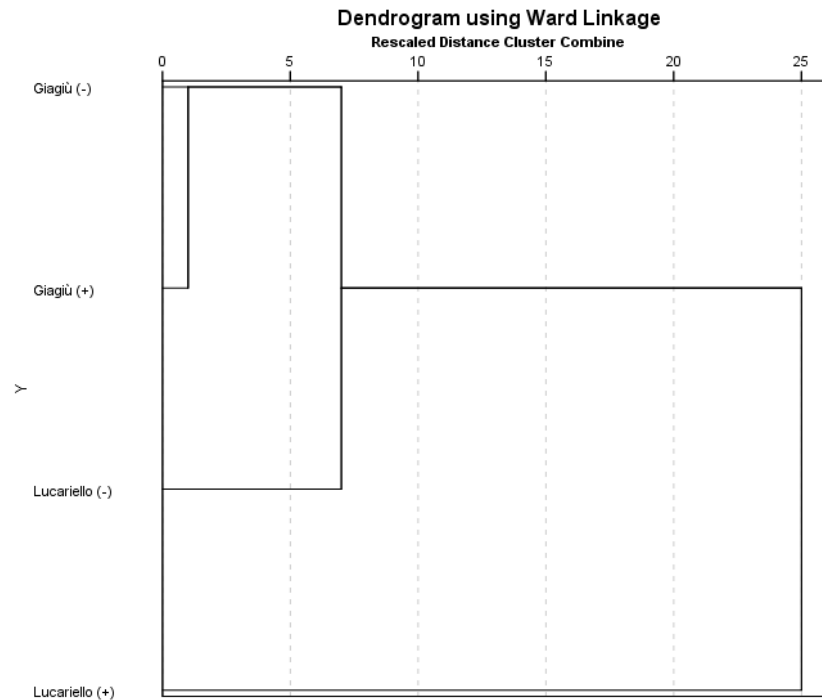


Figure 4. Hierarchical cluster analysis based on free amino acids profile and total amino acids content.

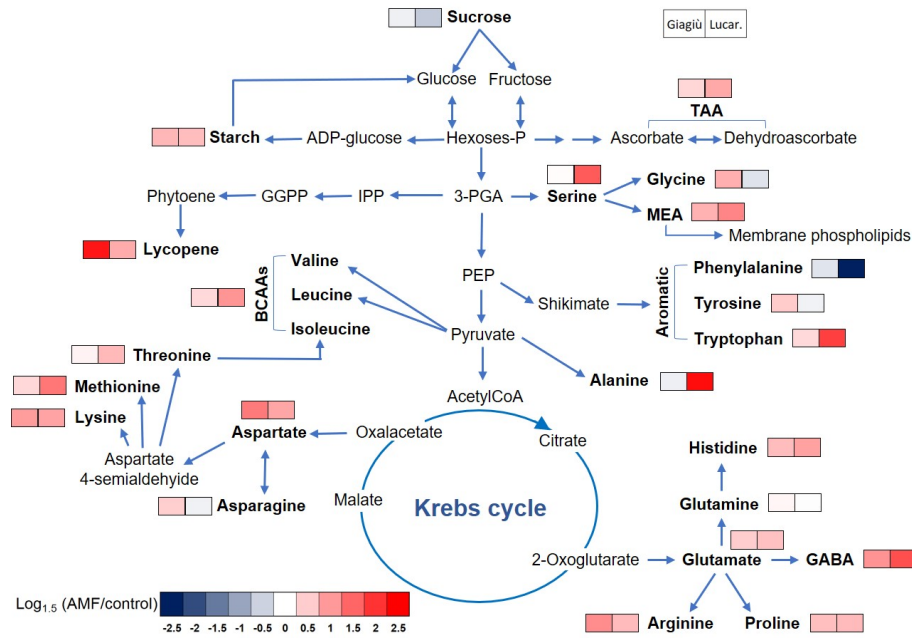


Figure 5. Pathway map summarizing the effect of arbuscular mycorrhizal fungi (AMF) on starch, sucrose, total ascorbic acid (TAA) and free amino acids, including branched-chain amino acids (BCAAs), and methyl ethanolamine (MEA) of yellow-pigmented type ('Giagiù') and red-pigmented type (Lucar.) landraces of Pomodorino del Piennolo del Vesuvio (PPV) cultivated in Campania region under rainfed conditions. The heat map results were calculated as Logarithm base 1.5 (Log_{1.5}) of AMF/Control values and visualized using a false-color scale, with red indicating an increase and blue a decrease of values.

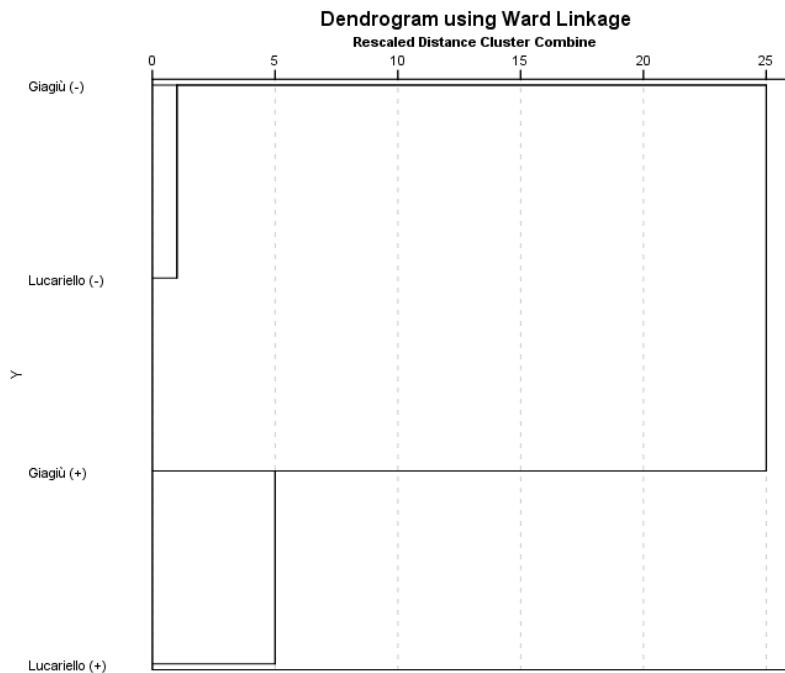


Figure 6. Hierarchical cluster analysis based on total yield, number of fruits, fruit mean weight, total soluble solids (TSS) content, dry matter, lipophilic and hydrophilic (LAA and HAA), starch, lycopene, sucrose, macro (phosphorus, potassium, calcium and magnesium) and micro (sodium, iron, copper, manganese and zinc) mineral content and total amino acids content.

4. Discussion

The use of synthetic fertilizers and pesticides together with irrigation has certainly provided farmers with great flexibility in the management of agricultural cropping systems and a potential for boosting yield and productivity, particularly in the horticultural industry [28]. However, intensive agriculture has posed serious threats to the environment and the different compartments of the agroecosystem, leading to water, soil and air pollution and ultimately causing a decline in crop productivity while aggravating risks to human health [29]. Therefore, the upcoming challenge facing agriculture is that of identifying alternative strategies and/or tools that can assist in increasing plant NUE and yield in order to feed the growing world population, while sustainably reducing conventional inputs and pollution [30–32]. Indeed, beneficial microbes could be used as PBs to achieve this multifaceted goal [8,33,34]. According to this view, the present study appraised the application of AMF on the yellow-pigmented type ('Giagiù') and red-pigmented type ('Lucariello') PPV landraces in order to determine if it could positively affect tomato growth and marketability, while also furthering our understanding of the effects of AMF on nutritional and nutraceutical quality of these horticultural products.

Our findings indicate that mycorrhization elicited systemic effects expressed on tomato fruits from PPV plants by inducing phenological, physiological and metabolic variations mainly evident in 'Lucariello', but also in 'Giagiù'. In fact, AMF was able to colonize both cherry tomato landraces, increasing the fruit number per plant but not the fruit mean mass, yet resulting in a significant increase in fruit yield. AMF also enhanced in both landraces the capacity to accumulate starch, which constitutes a feature of increased photosynthetic efficiency, and free amino acids [31]. The beneficial effect of AMF on tomato plants has been previously reported [6,35–37], and often linked to the higher efficiency of mycorrhizal plants in taking up soil nutrients, phosphorus in particular [35,37]. However, in our case, phosphorus was not the only ion significantly increased under AMF treatment. Magnesium and copper increased and sodium decreased in both landraces; whereas, in 'Giagiù' only, calcium and zinc also were strongly increased.

Calcium has a key role in plant membrane stability, the functional integrity of cell wall and osmoregulation, and acts as second messenger allowing plants to respond to environmental stimuli, regulating and adapting developmental processes accordingly [38]. Therefore, calcium low levels can negatively affect plant growth and yield [39]. Moreover, low calcium plant tissues and in particular fleshy fruits like tomatoes are more susceptible to biotic stresses (e.g., parasitic diseases) and rapid loss of quality during storage [40]. Studies aimed to increase the content of calcium in tomatoes for coping with osteoporosis in women have led to the discovery that the constitutive expression of the vacuolar $\text{Ca}^{2+}/\text{H}^{+}$ antiporter *AtCAX₁* in transgenic tomatoes by removal of its autoinhibitory region, is able to increase calcium vacuolar transport and its fruit concentration [40]. However, the modification of *AtCAX₁* expression causes also the depletion of calcium in apoplast and cytosol, facilitating membrane leakage and increasing plant susceptibility to blossom end rot [40,41]. Indeed, genetic alteration of calcium transporters affects calcium nutrition and signaling pathways, causing still unknown consequences on fruit development and ripening [42]. According to these data, the AMF related higher calcium concentration in 'Giagiù' is particularly relevant because it does not cause negative consequences on fruit tissues, and on the contrary, it contributes to the increase in fruit number and yield. Moreover, the increase in calcium contributes to maintaining the turgor and firmness of fruit tissues, to extending fruit shelf life and, moreover, to reducing the risk of osteoporosis in humans [40]. The comprehension of the mechanisms by which AMF can increase calcium content in 'Giagiù' without creating physiological imbalances in the plant is of pivotal importance toward establishing in practice the use of this PB for naturally increasing calcium content in tomatoes but also in other types of fruit or vegetables.

Zinc too was strongly increased by AMF in 'Giagiù' fruits. This ion is essential for the synthesis and configuration of proteins and for starch synthesis [43]. It acts as an essential cofactor for thousands of proteins having structural, regulatory and functional roles in plant cells [44]. Zinc is involved in the scavenging of superoxide radicals, and contributes to membrane integrity as well as the synthesis of proteins and the plant hormone auxin [45]. It enters the structure of zinc finger

transcription factors, regulating the expression of genes involved in cell expansion thus playing a key role in the correct development of tomato fruits [46]. In addition, the application of zinc to tomato fruits may reduce their rate of respiration and transpiration, decrease ethylene production and extend their shelf life [47]. Zinc is also essential for human nutrition since it plays a central role in cellular growth, differentiation and metabolism [48]. In particular, zinc is important during the prenatal and infancy stages of rapid growth [48,49]. Furthermore, it is essential in tissues with rapid cell turnover, such as the immune system and the gastrointestinal tract. Thus nutritional deficiencies of zinc can affect the outcome of pregnancy, physical growth, susceptibility to infections and neurobehavioral development [50].

Nitrogen is another key element for plants whose uptake is enhanced by AMF, as evidenced by the strong increase in free amino acids in the two PPVs. Govindarajulu, et al. [51] demonstrated through stable isotope labeling experiments that the nitrogen absorbed by AMF extraradical mycelium is immediately assimilated into amino acids, mainly Arg, then transferred to the intraradical mycelium, where it is, for the most part, converted into different low-carbon containing nitrogen forms, probably ureides, made available to the host plants [37]. Besides, Arg content significantly increased in tomato fruits, particularly of 'Giagiù', suggesting that not all Arg was broken down and transformed to different compounds released from the mycelium to the host plant. It has also been suggested that amides (glutamine and asparagine) could be the low-carbon forms by which N is exported from AMF mycelium and then accumulated in tomato fruits [52]. However, the fruits of the two PPVs did not show any significant increase in the content of the two amides.

Whatever the carbon form used for the export of nitrogen from intraradical mycelium to the plant, it is interesting that the surplus of this nutrient acquired by means of AMF was mainly used to synthesize and accumulate amino acids with antioxidant properties. The AMF-dependent increase of free amino acids was manifested in both landraces, even if the effect on 'Lucariello' was more evident for the higher constitutive content of these metabolites in the untreated control. The impact of AMF on amino acids profiling has been previously reported by Salvioli et al. [37] as an either direct or indirect mechanism. The direct effect may depend on the systemic modulation of the transcription exerted by some tomato leaf microRNAs induced by the AMF symbiont [53]; while the indirect effect could depend on the beneficial effect of symbiosis on nutrient availability, since AMF can directly solubilize plant nutrients in the soil rhizosphere (e.g., rock P, Fe, Cu and divalent ions) or produce siderophores, making nutrients directly available to the plant [7].

The AMF treatment allowed a strong increase in Asp content of both landraces, while maintaining a high unchanged Glu value, independent of landraces and treatments. Since umami taste is elicited by many small molecules, including Glu and Asp in addition to nucleotides (inosine 5'-monophosphate and guanosine-5'-monophosphate), the concentrations of Asp and Glu improve tomato flavor and fruit palatability [16,54]. Moreover, Asp is very important in plant metabolism because from this amino acid starts the pathway that leads to the synthesis of the four essential amino acids Ile, Lys, Met and Thr [55]. Accordingly, an increase in these latter amino acids was also elicited, with the exception of Thr in 'Giagiù'. The increase of Lys was the most relevant result because it is considered nutritionally as the most important essential amino acid because its concentration is low in cereal grains, which are the main source of staple plant foods and feeds worldwide [55]. The attempts to increase its synthesis and accumulation or reduce its catabolism by metabolic engineering have failed because this approach has the drawback of decreasing tricarboxylic acid (TCA) cycle metabolites which reduce cellular energy and delay seed germination [55]. AMF action increased this amino acid in both landraces without consequences on plant physiology and metabolism.

'Lucariello' differed from 'Giagiù' also in that it underwent a strong increase of BCAAs. These amino acids are used by plants both as osmolytes to counteract the effects of water stress and as alternative electron donors for the mitochondrial electron transport chain [16]. BCAAs have been found to play also a ROS scavenging role in rats and mice through a still unknown underlying mechanism [27].

Serine almost doubled in 'Lucariello', while it remained unvaried in 'Giagiù'. The increase of Ser, in agreement with the findings of Salvioli et al. [37] in mycorrhizal plants, could be due to an

increase of photorespiration. However, this event must not be seen as negative because, on the one hand, AMF are able to alter stomatal morphology and stomatal density enhancing stomatal conductance and transpiration to increase xylem nutrients uptake and fluxes during active growth, as found in mycorrhizal wheat leaves [56]; on the other hand, AMF allow whenever necessary a strict stomatal control that has as consequence the increase of photorespiration but also the synthesis of specific metabolites for alleviating its negative effects. Certainly, the reason why this is manifested in 'Lucariello' but not in 'Giagiù' should be further investigated. In fact, the strong increase of serine is accompanied by an equally sharp increase of alanine and the nonprotein amino acid GABA in the same landrace. Their syntheses, starting from the decarboxylation of malate to pyruvate as a result of malic enzyme activity for alanine and decarboxylation of glutamate to GABA, are proton-consuming reactions, useful for buffering cytosolic acidosis during photorespiration [31,57,58]. Alanine after transamination to pyruvate can be converted to acetyl-Coenzyme A (acetyl-CoA) in the mitochondria, while the GABA shunt can supply succinate to the tricarboxylic acid (TCA) cycle and NADH directly to respiratory electron chain to increase the production of ATP [59,60]. The synthesis of GABA, catalyzed by the enzyme glutamate decarboxylase (GAD), releases also stoichiometric amounts of CO₂, which can be used by RUBISCO to reactivate the Calvin cycle reducing the pressure on the photosynthetic electron chain, and decreasing photo-oxidative stress and photodamage in plant tissues when stomata are closed [60,61]. Moreover, GABA is also a compatible osmolyte able to balance the decrease in water potential during cellular dehydration due to drought stress, and acting also as an antioxidant for stabilizing and protecting membranes and macromolecules [32]. Nonetheless, GABA can exert beneficial effects on human health; in fact, it can act as a hypotensive and enhancer of the immune system under stress, and can contribute to cancer and diabetes prevention and the control of blood cholesterol levels [62,63].

'Lucariello' under AMF treatments showed also the highest contents of lycopene and TAA. Lycopene is the main lipophilic antioxidant generally present in tomatoes [64], with the exception of yellow-fleshed genotypes like 'Giagiù'. It is able to scavenge hydroxyl radicals and activate enzymes like superoxide dismutase, glutathione peroxidase and glutathione reductase, preventing the formation of ROS, and therefore protecting membranes and macromolecules in plant tissues [65]. Lycopene present in tomato products can play an important role in protecting against chronic disorders such as cardiovascular disease, and respiratory and digestive epithelial cancers [64]. It seems to exert cardiovascular protection by decreasing HDL-associated inflammation and modulating HDL functionality [66]. Lycopene has been recognized as a safe food supplement by the US Food and Drug Administration [67]. Lucarini et al. [68] reported that the average per capita intake of this carotenoid in Italy is 7.4 mg/d, greater than in other European countries except for Poland (7–7.5 mg/d). Clinical studies aimed to evaluate the beneficial effects of lycopene rarely exceed doses of 10 mg/d, even if it has been demonstrated that in cancer patients daily doses of up to 75 mg lycopene contribute to reducing a further progression of the disease [69]. 'Lucariello' under control and AMF treatments show a content of lycopene 18% and 85% higher than that of other commercial tomato varieties (4.7 and 7.4 mg/100 g fw, respectively) [70]. This means that eating 160 or 100 g of control or AMF treated 'Lucariello' tomatoes per day could be sufficient for getting the suggested amount of lycopene per day. However, this carotenoid is not the only antioxidant present in these tomatoes. In fact, these landraces contain also very high concentrations of AA (vitamin C) (on average 110 and 140 mg 100 g⁻¹ fw in control and AMF PPVs, respectively), which is considered to be one of the most abundant antioxidants found in plant tissues [71]. According to the Recommended Dietary Allowance (RDA), the intake of AA should be of 90 mg/d for adult men and 75 mg/d for adult women. Therefore, for an adult man it would be sufficient to eat only 82 g or 64 g of tomato fruits from control or AMF treated PVVs, respectively, to get the RDA for AA. This vitamin is a multifunctional molecule necessary for both plants and animals. It plays primary roles as redox buffer and cofactor for enzymes involved in multiple cell processes, among which hormone biosynthesis, photosynthesis, respiration and regeneration of other different antioxidants. Moreover, it has a role in signal transduction and is involved in cell cycle progression and plant growth [72]. Lycopene together with the high levels of

TAA observed under AMF treatment can also protect fruit tissues from oxidative stress and extend the postharvest conservation of ‘Lucariello’ tomatoes [16,17].

5. Conclusions

Biostimulation through AMF application represents a promising strategy for improving yield and quality of horticultural products, such PPV tomatoes, moreover it constitutes a sustainable approach toward achieving these goals. This microbial biostimulant was able to increase the content of both PPV landraces in ions and/or metabolites that contribute not only to shelf life extension but also to nutritional and nutraceutical quality rendering them desirable from a palatability and healthiness point of view. In addition, it is important to underline that while the main changes caused by AMF were more readily manifested in ‘Lucariello’, this microbial PB was also able to confer great improvement to the quality of ‘Giagiù’, exalting its taste and nutritional quality. In fact, the AMF treatment not only enhances the place of ‘Giagiù’ in the niche market of haute cuisine, in which it is already entrenched, but also opens up a new market for it, which is that of premium products that are nonallergenic but highly palatable, due to the high contents of umami-linked Glu and Asp, and also health-promoting due to the high contents of Ca, Zn, GABA, Arg and Lys. In fact, thanks to its very low lycopene content ‘Giagiù’ is also suitable for people suffering from intolerance or allergic reaction to dietary lycopene, which may trigger serious disorders like diarrhea, nausea, stomach pain or cramps, gas, vomiting, and loss of appetite [73].

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