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The functional profile and antioxidant capacity of tomato fruits are modulated by the interaction between microbial biostimulants, soil properties, and nitrogen regimen

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Abstract: The application of microbial biostimulants to plants has revealed positive effects related to nutrients uptake, stress tolerance, root development and phenological growth. However, little information is available exploiting the potential synergistic biostimulant action of microbes on the functional quality of the yields. The current research elucidated the effect of single or coupled action of biostimulants, associated with either optimal or reduced nitrogen application, on the functional quality of tomato berries. Chemical assays and untargeted metabolomics were applied to investigate *Rhizoglomus irregulare* and *Funneliformis mosseae* administration (both being arbuscular mycorrhiza, AMF), under optimal or low N input conditions, alone or coupled to *Trichoderma atroviride* application. The coupling of AMF and *Trichoderma* fungal inoculations resulted in a synergistic biostimulant effect on tomato fruits, revealing increased concentrations of antioxidant compounds (flavonoids, lignans and small-molecular-weight phenolics) at a higher rate than the sole AMF application. However, a strong dependence of the biostimulant effect on nitrogen availability was also noticed, reflecting significant increment in antioxidant activity under sub-optimal fertility conditions and low nitrogen levels.

Keywords: phenolic compounds; carotenoids; microbial biostimulants; antioxidant activity; metabolomics.

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1. Introduction

The use of plant biostimulants in agriculture has increased significantly over the last 10 years, mainly due to the successful advances in research that showed beneficial effects on plants, especially in terms of nutrient use efficiency, abiotic stress tolerance, quality traits and the availability of limited nutrients in the soil and rhizosphere [1-3].

More recently, an increasing number of experimental studies have addressed research evaluating the combined use of plant biostimulants with the aim of ensuring global food security and environmental sustainability without increasing the rate of nutrient use [4,5]. Antagonistic, additive, or rather synergistic interactions among different plant biostimulants categories have been described, depending on whether the combined effect

was respectively less, equal or greater than the effect obtained by each biostimulant individually [5]. In most cases, the combined application of microbial and non-microbial biostimulants has reflected a synergistic action on plants, revealing an increase in nutrient uptake, stress tolerance, root development and phonological growth [6-9]. However, only limited scientific literature is available regarding the effect of combined applications of biostimulants on fruit quality.

Tomato plant (*Solanum lycopersicum* L.) represents one of the most grown and consumed crops worldwide, mainly due to key role in the Mediterranean human diet as fresh or processed product. Given the commercial importance of this crop, research is addressing efforts targeted to improve the biochemical composition of the fruit, including the content of potentially health-beneficial components such as antioxidants (vitamin C, lycopene etc.). For this reason, various metabolomics approaches have been extensively adopted to gain a better understanding of the biochemical determinants concerning of fruit growth and quality, both under and presence and the absence of abiotic and biotic stress factors [10].

In this context, biostimulant products, which have been abundantly investigated for their multiple benefits for plants - including nutrient uptake and use efficiency stimulation, and abiotic/biotic stress tolerance increment -, have been recently evaluated as a sustainable approach to improve food nutritional/functional values [11,12]. Concerning tomato, latest advances in metabolomics have allowed to elucidate the physiological processes involved in fruit response to biostimulant application, which seem to be linked to higher levels of antioxidants, mineral nutrients (N, P, Ca, Na, Fe, Mn and Zn), total vitamin C and phenolics [13,14]. Nevertheless, the combined action of two or more biostimulants on tomato fruit quality remains few explored.

Given this premise, this paper gets insight into the effect of single or coupled action of biostimulants, associated with a high or low nitrogen (N) application, on the functionalquality of tomato berries. Specifically, the work makes use of chemical assays and untargeted metabolomics to describe and compare the impact of no-treatment and low N input, with *Rhizoglomus irregulare* and *Funneliformis mosseae* administration, in conventional or low N input condition, either in single or coupled to *Trichoderma atroviride* application.

2. Materials and Methods

2.1 Growth conditions and experimental design

Two field experiments were conducted between May and August 2020 at Pizzacchera S.n.c. and Felletti Luca farms, respectively located near Parma (44°50'55.7"N 10°15'34.4"E) and Ferrara (44°49'49.8"N 12°07'07.6"E), Emilia-Romagna Region, Northern Italy. The two sites were selected as optimal (Pizzacchera) and sub-optimal (Felletti) soil fertility conditions. Initial soil properties in the 0-30 cm soil layer at optimal fertility were: % sand 18.2, % silt 48.5, % clay 33.3, organic matter 40.13 g kg^{-1}, pH(H_2O) 7.98, pH($CaCl_2$) 7.35, % $CaCO_3$ 9.7, electrical conductivity (µS cm^{-1}) 295, organic carbon (g kg^{-1}) 23.28 and total soil Kjeldahl nitrogen (g kg^{-1}) 2.55. Same properties in the same soil layer at sub-optimal fertility were: % sand 93, % silt 6, % clay 1, organic matter 8.43 g kg^{-1}, pH(H_2O) 7.54, pH($CaCl_2$) 7.09, % $CaCO_3$ 5.8, electrical conductivity (µS cm^{-1}) 346, organic carbon (g kg^{-1}) 4.89 and total soil Kjeldahl nitrogen (g kg^{-1}) 0.60.

In both farms, the experiment was performed on non-randomized plots with four pseudo-replicates, arranged to test biostimulant-based treatments on tomato berries quality under conventional and low N input conditions. The single plot size was 144 m^2 (30 m x 1.6 m). In details, five different treatments were compared: 1) conventional N input without microbial treatment (Control); 2) low N input (LowN); 3) mycorrhizal treatment (*R. irregulare* BEG72 and *F. mosseae* BEG234, 700 sp g^{-1} each species) under conventional N input (AMF); 4) mycorrhizal treatment under low N input (AMF+LowN); 5) mycorrhizal

treatment coupled with *T. atroviride* application under low N input (AMF+*T.atroviride*+LowN). The microbial biostimulants were formulated commercial products supplied by Agrotecnogias Naturales (Tarragona, Spain), inoculated at transplanting according to label recommendations. 97 98 99 100

At harvest, tomato yields were determined by weighting tomato fruits from four randomly selected areas (25 m^2 each) from each plot. In details, commercial (red ripe berries), immature (green underripe barriers), overripe (homogenously rotten barriers) fruits, as well as fruits with apical rot fruits, were separated after removal of fruits from plant stems, and weighted separately. Then, yields were expressed as t/ha. The resulting values were divided by the total yield (t/ha) to respectively obtain the percentages of commercial, immature, rotten and apical rot yield. Total refractometric (Kg°Brix/ha) and optical residue (°Brix) as qualitative parameters on berries and fruit's juice were also measured. Finally, the mature yield of 5 plants per condition was collected and immediately frozen at -20 °C. Successively, the fruits were ground with liquid nitrogen using pestle and mortal for the following chemical analysis. 101 102 103 104 105 106 107 108 109 110 111

2.2 Carotenoids determination 112

Carotenoids were determined by high performance liquid chromatography with diode array detection–mass spectrometry, as previously reported [15]. Briefly, a binary elution using (A) methanol/acetonitrile/water (84:14:4, v/v/v) and (B) dichloromethane, with a 45 min gradient run at 25 °C, together with a polymeric C30 column were employed following extraction in ethanol:*n*-hexane (60:40, v/v)[16]. Detection was then set at the wavelengths 450, 348 and 286 nm and quantification done against pure reference standards. 113 114 115 116 117 118 119

2.3 Total phenolics and phenolic profile 120

Folin-Ciocalteu and $AlCl_3$ assays, respectively, were utilized to determine the total phenolic and flavonoid contents [17]. Results were expressed respectively as gallic acid equivalents (mg GAEs/g extract) and rutin equivalents (mg REs/g extract). 121 122 123

2.3.1 Determination of antioxidant and enzyme inhibitory effects 124

The antioxidant and enzyme inhibitory activity of the extracts was determined according to previously described methods [18]. DPPH and ABTS radical scavenging activity, cupric ion reducing antioxidant capacity (CUPRAC), and ferric ion reducing antioxidant power (FRAP) were expressed as mg Trolox equivalents (TE)/g extract. The metal chelating ability (MCA) was reported as mg EDTA equivalents (EDTAE)/g extract, whereas the total antioxidant activity (phosphomolybdenum assay, PBD) was expressed as mmol TE/g extract. AChE and BChE inhibitory activities were given as mg galanthamine equivalents (GALAE)/g extract; tyrosinase inhibitory activity was expressed as mg kojic acid equivalents (KAE)/g extract, and amylase inhibitory activities were presented as mmol acarbose equivalents (ACAE)/g extract. 125 126 127 128 129 130 131 132 133 134

2.4 UHPLC-ESI/QTOF-MS untargeted profiling of tomato berries polyphenols 135

Starting from the grounded samples, tomato berries were extracted as previously described by [19]. Briefly, six replicates per thesis (2 gr each) were extracted in 20 ml of 80% methanol (v/v) acidified with 0.1% formic acid (v/v), using an Ultra-turrax (Ika, T25, Staufen, Germany). Later, the extracts were centrifuged (12000 × *g*) and 1 ml of the resulting supernatants was transferred into vials for the analysis. 136 137 138 139 140

Metabolite screening of tomato berries was performed with an untargeted metabolomics approach, throughout a hybrid quadrupole-time-of-flight mass spectrometer coupled to an ultra-high performance liquid chromatographic system (UHPLC/QTOF). Specifically, A 1290 liquid chromatograph system, equipped with a binary pump and a Dual 141 142 143 144

Electrospray JetStream ionization system, coupled to a G6550 mass spectrometer detector (Agilent technologies, Santa Clara, CA, USA) was used. The mass spectrometer worked to acquire positive ions in the 100–1200 m/z rang, while a C18 column and a binary gradient consisting of 5% to 90% methanol in water (in 30 min) was used for reverse phase chromatographic separation. The volume of injection was 6 μL for each replicate and the flow rate was 200 μL/min, according to [20].

The Agilent Profinder B.07 software (Agilent Technologies, Santa Clara, CA, USA) was used to process the raw data mass features, according to a targeted 'find-by-formula' algorithm and basing on the Phenol-Explorer 3.6 database (http://phenol-explorer.eu). Following mass and retention time alignment, compound identification was based on both monoisotopic accurate mass and isotope pattern (accurate spacing and isotope ratio), adopting a mass tolerance of 5-ppm. Features which were not present in 100% of replications within at least one treatment were discarded.

According to the Phenol-Explorer subclass information, phenols were classified in the phenolic subclasses, whose cumulative intensities were calculated and converted in mg L-1 equivalent using standard solutions, as previously described by [21].

2.5 Statistical analyses

One and Two-way analysis of variance (ANOVA) were carried out in Rstudio software in order to determine any statistically differences among tomato berries when comparing different treatments, within and between the two farms. The elaboration was performed both for qualitative, productive, and chemical parameters. In the presence of statistically difference, Duncan test was conducted for multiple comparisons between pairs of treatments.

The statistical analyses on the metabolomics dataset were performed using Mass Profiler Professional B.12.06 (Agilent technologies) software. Compound abundance was Log2 transformed and normalized at 75th percentile and baselined against the median. Firstly, unsupervised hierarchical cluster analysis (Squared Euclidean distance, Ward's linkage rule) was carried out using the fold-change based heat map, to highlight the relatedness among treatments and the two farms.

Successively, for both farms, one-way ANOVA and Duncan test were carried out on semi-quantitative polyphenol results to determine whether there were any statistically significant differences between all and pairs of treatments.

3. Results

3.1 Productive and qualitative parameters

Results on qualitative and quantitative parameters of tomato production at sub-optimal fertility (Table 1) didn't show any significant difference among treatments, except for the apical rot which proved to be considerably higher in the control samples (2.1 t/ha).

On the contrary, at optimal fertility, the reduction of nitrogen doses for LowN, AMF+LowN and AMF +*T. atroviride*+LowN led to a remarkable decrease in marketable production (Table 2). Additionally, microbial treatment didn't reveal an increase of the total yield, having obtained the highest value for the control condition (104.9 t/ha).

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Table 1. One-way analysis and Duncan test for productive and qualitative parameters at sub-optimal fertility. 187

	Yield										Juice
	Total refractometric (Kg°brix/ha)	**Marketable** (t/ha)	**Immature** (t/ha)	**Rotten** (t/ha)	**Apical rot** (t/ha)	**Total yield** (t/ha)	**Commer-cial** (%)	**Immature** (%)	**Rotten** (%)	**Apical rot** (%)	**Optical residue** (°brix)
Treatment (T)											
LowN	3.339	68	5.4	1.4	1.2	76.1	89.3[a]	7.1	1.9	1.5	4.91
AMF	3.321	65.8	3.8	1.1	0.8	71.5	92[a]	5.3	1.5	1.2	5.05
AMF+LowN	3.625	70.7	4.8	1.4	1.5	78.4	90.2[a]	6.1	1.8	1.9	5.13
AMF+*T.atr.*+LowN	3.698	71.9	10.1	1.4	1.5	84.9	84.6[b]	11.9	1.6	1.8	5.15
Control	3.573	70.2	7.9	0.8	2.1	81	86.7[b]	9.8	1	2.6	5.09
Significance											
T	n.s.	n.s.	n.s.	n.s.	*	n.s.	*	n.s.	n.s.	n.s.	n.s.

*Different letters show significant difference at the 0.05 probability level. 188

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Table 2. One-way analysis and Duncan test for productive and qualitative parameters at optimal fertility. 191

	Yield										Juice
	Total refractometric (Kg°brix/ha)	**Marketable** (t/ha)	**Immature** (t/ha)	**Rotten** (t/ha)	**Apical Rot** (t/ha)	**Total Yield** (t/ha)	**Commercial** (%)	**Immature** (%)	**Rotten** (%)	**Apical Rot** (%)	**Optical residue** (°brix)
Treatment (T)											
LowN	3.803	82.7[b]	13	2.1	0.4	98.2[b]	84.1	13.2	2.2	0.5	4.6
AMF	4.124	88.8[a]	12.3	3.1	0.5	104.7[a]	84.8	11.8	3	0.5	4.65
AMF+LowN	3.548	78.6[b]	7.4	1.9	0.5	88.5[b]	88.9	8.3	2.2	0.6	4.51
AMF+*T.atr*.+LowN	4.215	88[a]	10.2	2.7	1	101.9[a]	86.4	10	2.6	1	4.79
Control	4.082	89[a]	12.1	3.1	0.8	104.9[a]	84.8	11.5	2.9	0.7	4.59
Significance											

T	n.s.	*	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.

*Different letters show significant difference at the 0.05 probability level. 192

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3.2 Carotenoids determination 194

Carotenoid content in tomato berries was significantly different in the two farms, highlighting –except for B-carotene- higher concentrations of each carotenoid compound in optimal fertility samples (Table S1). However, for both cultivation sites, all-trans lycopene content was the highest, reaching the maximum average concentration of 12.586 ± 1.511 and 16.781 ± 1.797 mg/100 g extract respectively in AMF+*T.atroviride*+LowN-treated sub-optimal fertility samples and LowN-treated optimal fertility samples (Tables 3 and 4). Interestingly, biostimulant application did not enhance carotenoid content in optimal fertility tomatoes, having found that, except for Z-carotene, LowN and Control treatments determined the greatest amounts of carotenoid compounds (Table 4). Contrary, in sub-optimal fertility samples, AMF+*T.atroviride*+LowN application showed the upmost concentrations of B-carotene, Z-carotene, All-E-y-carotene, 13-z-lycopene, while Cis-lycopene was mainly increased by AMF+LowN treatment (Table 3). 195 196 197 198 199 200 201 202 203 204 205 206

3.3 Total bioactive compounds determination 207

The total content of phenolics and flavonoids in tomato berries is provided in Tables 5 and 6. Focusing on sub-optimal+optimal fertility samples, total phenolic content values ranged from 10.2 mg GAE/g extract to 15 mg GAE/g extract and the greatest amount (13.1 ± 2.2 mg GAE/g extract) was observed with Low N application, followed by AMF+*T.atroviride*+LowN treatment (12.7 ± 2.3 mg GAE/g extract) (Table S2). Concerning total flavonoid content, two-way ANOVA revealed statically differences among treatments, ascribing to Control and AMF+LowN the highest concentrations, respectively of 0.9 ± 0.3 and 0.9 ± 0.2 mg GAE/g extract. Optimal fertility samples showed the topmost average value for both phenolics and flavonoids (Table 6). 208 209 210 211 212 213 214 215 216

3.4 Total antioxidant activity 217

The total antioxidant activity calculated via phosphomolybdenum [22] is provided in Tables 5 and 6. Concerning this assay, total antioxidant abilities of optimal fertility tomato samples were significantly higher (0.9 ± 0.1 mmol TE/g extract) than those of sub-optimal fertility (0.8 ± 0.1 mmol TE/g extract) (Table S2). However, keeping together the two farms, the study did not reveal any consistent difference among treatments. Curiously, looking at one-way ANOVA results, sub-optimal fertility samples enlightened a significant increment of total antioxidant activity following AMF+*T.atroviride*+Low N treatment (Table 5), while no treatment effect was pointed out for optimal fertility (Table 6). 218 219 220 221 222 223 224 225 226

3.5 Radical scavenging activity 227

The free radical scavenging activity of tomato berries was determined using DPPH and ABTS arrays and the results were presented in Tables S2, 5 & 6. Both assays are based on the quenching of these radicals through the transfer of either an electron or a hydrogen atom by antioxidant compounds. Concerning two-way ANOVA on DPPH assay data, AMF+ 228 229 230 231 232

T.atroviride+LowN treatment exhibited the highest activity (2.5 ± 2.9 mg TE/g extract) and no difference was observed between sub-optimal and optimal fertility farms (Table S2). Contrary, two-way ANOVA on ABTS assay data demonstrated a remarkable higher radical scavenging activity for optimal fertility (25.7 ± 3.4 mg TE/g extract) and for LowN application (26.4 ± 4.2 mg TE/g extract), which was significantly different from AMF treatment. 233 234 235 236 237 238

3.6 Reducing power 239

The reductive ability reflects to the electron-donation ability of antioxidant compounds. The reductive ability of tomato extracts was measured with FRAP and CUPRAC assays, respectively aimed at quantifying the potential for reducing ferric to ferrous and cupric to cuprous ions [23]. Two-way ANOVA results showed a similar tendency for both assays, highlighting a superior reducing power for AMF+*T.atroviride*+LowN (21.6 ± 4.9 mg TE/g extract for FRAP and 35.1 ± 6.2 mg TE/g extract for CUPRAC) (Table 4). Regarding CUPRAC, this last treatment significantly differed from AMF+LowN, which revealed the lowest value (30.5 ± 2.4 mg TE/g extract). However, no significance was found for the farm factor, revealing a lack of effect linked due to the cultivation area.

3.7 Metal chelating activity on ferrous ions

Ferrous chelating activity, based on the measure of the ferrous ion-ferrozine complex formation, was used as an indicator of tomato berries antioxidant activity and the results were presented in Tables 4, 5 and 6. Without detaching sub-optimal and optimal fertility samples, ferrous chelating activity data ranged from 10.2 to 16.4 mg EDTAE/g extract, confirming the highest and lowest average value respectively for LowN and AMF+LowN application (Table 4). In addition, a markedly increase for sub-optimal fertility values (15.2 ± 1.4 mg EDTAE/g extract) was revealed, when compared to optimal fertility (12.8 ± 2.6 mg EDTA/g extract).

3.8 Enzyme inhibitory activity

The results for the inhibitory activity of tomato samples on α-amylase, AChE, BChE and tyrosinase were depicted in Table S2. In general, no statistical difference between enzyme inhibition treatments was observed when comparing sub-optimal and optimal fertility (Table 4). Particularly, anti-α-Amylase activity exhibited the same values for all the treatments (0.2 ± 0.1 mmol ACAE/g extract). However, AMF+*T.atroviride*+LowN and control samples values tended to be great both for BChE (2.7 ± 0.5 and 2.7 ± 0.7 mg GALAE/g extract) and tyrosinase (57.4 ± 4.9 and 56.9 ± 6.1 mg KAE/g extract), while AMF+T. atroviride+Low N and AMF treatments confirmed the highest AChE inhibitory activity (2.4 ± 0.1 and 2.4 ± 0.2 mg GALAE/g extract). Except for tyrosinase, farm factor was crucial to determine dissimilarity between sub-optimal and optimal fertility sample, indicating a greater average value for cholinesterase (AChE and BChE) and Tyrosinase, respectively. Nevertheless, Table 5 & 6 revealed a different trend within 2 farms: within sub-optimal fertility, AChE and tyrosinase were significantly affected by treatment, while no differences were observed for any enzyme inhibitory activity within optimal fertility.

Table 3. One-way analysis and Duncan test for carotenoid content in tomato berries of sub-optimal fertility.

Source of variance	Phytoene mg/100g	Phytofluene mg/100g	Z-b-carotene mg/100g	B-carotene mg/100g	Z-carotene mg/100g	All-E-y-carotene mg/100g	13-z-lycopene mg/100g	7-z-lycopene mg/100g	9-z-lycopene mg/100g	Cis lycopene mg/100g	All trans lycopene mg/100g
Thesis(T)											
LowN	3.278±0.649[a]	1.233±0.015[a]	0.131±0.141	0.598±0.262	0.020±0.001	0.063±0.001[a]	0.126±0.061	0.151±0.06	0.020±0.001[a]	0.247±0.023	11.982±4.985
AMF	1.821±1.136[c]	0.599±0.138[c]	0.044±0.021	0.433±0.103	0.006±0.009	0.045±0.004[b]	0.103±0.020	0.112±0.022	0[b]	0.175±0.043	9.451±1.763
AMF+LowN	2.521±0.475[b]	0.839±0.141[b]	0.033±0.010	0.543±0.088	0.008±0.014	0.063±0.010[a]	0.142±0.037	0.175±0.036	0.008±0.012[b]	0.256±0.044	12.415±1.648
AMF+T.atr.+LowN	2.599±0.424[b]	0.856±0.293[b]	0.038±0.011	0.647±0.243	0.021±0.021	0.063±0.022[a]	0.145±0.052	0.154±0.037	0.007±0.012[b]	0.233±0.051	12.586±1.511
Control	3.426±0.424[a]	1.042±0.099[ab]	0.082±0.079	0.486±0.189	0.014±0.021	0.071±0.015[a]	0.135±0.053	0.191±0.074	0.008±0.012[b]	0.215±0.017	10.435±4.551
Significance											
T	***	***	n.s.	n.s.	n.s.	*	n.s.	n.s.	*	n.s.	n.s.

Different letters show significant difference at the 0.05 (*), 0.01 (**) and 0.001 (***) probability levels

Table 4. One-way analysis and Duncan test for carotenoid content in tomato berries of optimal fertility.

Source of variance	Phytoene mg/100g	Phytofluene mg/100g	Z-b-carotene mg/100g	B-carotene mg/100g	Z-carotene mg/100g	All-E-y-carotene mg/100g	13-z-lycopene mg/100g	7-z-lycopene mg/100g	9-z-lycopene mg/100g	Cis lycopene mg/100g	All trans lycopene mg/100g
Thesis(T)											
LowN	3.718±0.649	1.042±0.099[a]	0.315±0.022	0.436±0.070[bc]	0[b]	0.079±0.008	0.215±0.020	0.262±0.037	0.024±0.002	0.418±0.0766	16.781±1.797
AMF	3.310±1.182	1.056±0.280[ab]	0.255±0.055	0.332±0.099[c]	0.008±0.013[ab]	0.061±0.022	0.170±0.069	0.182±0.073	0.015±0.012	0.274±0.122	12.201±3.562
AMF+LowN	2.611±0.291	0.827±0.088[a]	0.279±0.034	0.573±0.082[a]	0.020±0.002[a]	0.072±0.014	0.179±0.039	0.191±0.043	0.019±0.003	0.315±0.085	13.694±2.439
AMF+*T.atr.*+LowN	2.763±1.997	1.051±0.087[ab]	0.295±0.018	0.647±0.243[c]	0.009±0.014[ab]	0.072±0.011	0.200±0.206	0.206±0.005	0.021±0.001	0.349±0.027	14.549±0.225
Control	3.563±1.263	1.190±0.263[a]	0.310±0.041	0.552±0.158[ab]	0.009±0.014[b]	0.079±0.021	0.220±0.060	0.278±0.113	0.023±0.006	0.468±0.235	16.072±4.187
Significance											
T	n.s.	*	n.s.	**	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Different letters show significant difference at the 0.05 (*) and 0.01 (**) probability levels

Table 5. One-way analysis and Duncan test for chemical assays in tomato berries of sub-optimal fertility.

Source of variance	Total phenolic content mgGAE/g	Total flavonoid content mgRE/g	DPPH mgTE/g	ABTS mgTE/g	CUPRAC mgTE/g	FRAP mgTE/g	Metal chelating mgEDTAE/g	Phosphomolybdenum mmolTE/g	AChe inh. mgGALAE/g	BChe inh. mgGALAE/g	Tyrosinase mgKAE/g	Amylase mmolACAE/g
Thesis(T)												
LowN	11.9 ± 2.0	0.8 ± 0.1^{b}	1.6 ± 0.4	23.0 ± 2.9	33.3 ± 3.8^{ab}	20.4 ± 1.1^{ab}	15.4 ± 1.4	0.8 ± 0.1^{ab}	2.3 ± 0.1	2.4 ± 0.3	56.9 ± 4.9^{c}	0.21 ± 0.0
AMF	11.9 ± 2.2	0.9 ± 0.1^{ab}	1.4 v 1.3	21.6 ± 4.4	33.4 ± 5.0^{ab}	20.6 ± 2.5^{ab}	13.6 ± 1.8	0.9 ± 0.1^{a}	2.4 ± 0.1	2.2 ± 0.6	54.9 ± 4.2^{bc}	0.20 ± 0.0
AMF+LowN	10.6 ± 1.1	0.9 ± 0.2^{a}	1.6 ± 0.7	20.3 ± 2.5	29.5 ± 1.9^{b}	19.6 ± 0.8^{b}	14.0 ± 2.8	0.7 ± 0.1^{b}	2.2 ± 0.1	2.4 ± 0.4	56.9 ± 6.2^{abc}	0.21 ± 0.0
AMF+T.atr.+LowN	12.9 ± 2.9	0.8 ± 0.1^{ab}	3.4 ± 3.7	24.5 ± 8.3	37.4 ± 7.6^{a}	23.6 ± 6.3a	15.2 ± 1.6	0.9 ± 0.2^{a}	2.4 ± 0.1	2.3 ± 0.4	62.2 ± 3.1^{ab}	0.21 ± 0.0
Control	11.2 ± 1.3	0.8 ± 0.1^{b}	1.2 ± 0.4	21.2 ± 0.8	31.2 ± 1.8^{b}	19.2 ± 1.0^{b}	14.4 ± 2.5	0.8 ± 0.1^{ab}	2.2 ± 0.1	2.7 ± 0.9	63.5 ± 4.6^{a}	0.21 ± 0.0
Significance												
T	n.s.	*	n.s.	n.s.	*	*	n.s.	*	**	n.s.	*	n.s.

Different letters show significant difference at the 0.05 (*) and 0.01 (**) probability levels

Table 6. One-way analysis and Duncan test for chemical assays in tomato berries of optimal fertility. 282

Source of variance	Total phenolic content mgGAE/g	Total flavonoid content mgRE/g	DPPH mgTE/g	ABTS mgTE/g	CUPRAC mgTE/g	FRAP mgTE/g	Metal chelating mgEDTAE/g	Phosphomolybdenum mmolTE/g	AChe inh. mgGALAE/g	BChe inh. mgGALAE/g	Tyrosinase mgKAE/g	Amylase mmolACAE/g
Thesis(T)												
LowN	14.4 ± 1.7[a]	0.9 ± 0.1[bc]	3.2 ± 1.6[a]	29.8 ± 1.7[a]	35.6 ± 5.7	21.8 ± 2.2[a]	14.9 ± 1.4[a]	0.9 ± 0.1	2.4 ± 0.2	2.8 ± 0.7	53.9 ± 4.7	0.19 ± 0.0
AMF	12.7 ± 0.4[b]	0.8 ± 0.1[c]	1.4 ± 05[b]	23.2 ± 1.4[c]	32.5 ± 1.6	19.5 ± 0.6[bc]	12.5 ± 1.2[b]	0.9 ± 0.1	2.4 ± 0.2	3.0 ± 0.7	54.7 ± 3.5	0.18 ± 0.0
AMF+LowN	12.4 ± 0.8[b]	1.0 ± 0.2[ab]	1.5 ± 0.2[b]	23.6 ± 0.9[c]	31.6 ± 2.5	19.2 ± 0.9[c]	11.5 ± 1.7[b]	0.8 ± 0.1	2.3 ± 0.1	2.9 ± 0.4	53.8 ± 3.2	0.19 ± 0.0
AMF+T.atr.+LowN	12.5 ± 1.7[b]	0.8 ± 0.2[bc]	1.6 ± 1.4[b]	24.8 ± 3.1[bc]	32.9 ± 3.6	19.7 ± 1.4[bc]	12.7 ± 2.7[b]	0.9 ± 0.1	2.4 ± 0.1	3.1 ± 0.3	53.9 ± 4.1	0.19 ± 0.0
Control	13.4 ± 0.6[ab]	1.2 ± 0.3[a]	1.8 ± 0.1[b]	27.1 ± 3.8[b]	34.7 ± 2.1	20.7 ± 1.2[ab]	12.4 ± 1.7[b]	0.9 ± 0.1	2.4 ± 0.1	2.7 ± 0.6	52.9 ± 2.3	0.19 ± 0.0
Significance												
T	**	**	**	***	n.s.	**	**	n.s	n.s.	n.s.	n.s.	n.s.

Different letters show significant difference at the 0.05 (*), 0.01 (**) and 0.001 (***) probability levels 283

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Table 7. One-way analysis and Duncan test for phenolic classes in tomato berries under sub-optimal fertility conditions. 285

Source of variance	Flavonoids mg eq. g^{-1} DM	Lignans mg eq. g^{-1} DM	Other polyphenols mg eq. g^{-1} DM	Phenolic acids mg eq. g^{-1} DM	Stilbenes mg eq. g^{-1} DM
Thesis (T)					
LowN	4.7 ± 1.8[b]	13.2 ± 1.2[a]	52.6 ± 14.9[a]	27.7 ± 4.5	0.4 ± 0.2
AMF	5.2 ± 0.8[ab]	11.7 ± 3.3[ab]	37.7 ± 15.9[ab]	28.2 ± 5.6	0.4 ± 0.1
AMF+LowN	6.7 ± 1.4[a]	6.4 ± 1.8[c]	22.1 ± 4.6[b]	21.9 ± 8.7	0.5 ± 0.1
AMF+T.atr.+LowN	4.5 ± 1.1[b]	8.4 ± 3.7[bc]	24.8 ± 13.3[b]	17.1 ± 9.7	0.5 ± 0.1
Control	6.1 ± 0.9[ab]	11.4 ± 2.7[ab]	30.9 ± 14.1[b]	21.9 ± 8.7	0.5 ± 0.1
Significance					
T	*	**	**	n.s.	n.s.

Different letters show significant difference at the 0.05 (*) and 0.01 (**) probability levels 286

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Table 8. One-way analysis and Duncan test for phenolic classes in tomato berries under optimal fertility conditions. 288

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Source of variance	Flavonoids	Lignans	Other polyphenols	Phenolic acids	Stilbenes
	mg eq. g^{-1} DM	mg eq. g^{-1} DM	mg eq. g^{-1} DM	mg eq. g^{-1} DM	mg eq. g^{-1} DM
Thesis (T)					
LowN	3.5 ± 0.7	13.0 ± 1.9	55.0 ± 11.5	30.8 ± 7.2	0.5 ± 0.2
AMF	2.4 ± 0.3	12.8 ± 3.1	44.2 ± 16.2	32.4 ± 6.2	0.5 ± 0.1
AMF+LowN	3.1 ± 1.0	10.9 ± 4.9	31.9 ± 13.6	24.0 ± 11.7	0.5 ± 0.2
AMF+T.atr.+LowN	3.7 ± 1.0	11.2 ± 4.0	37.9 ± 19.3	27.3 ± 12.8	0.6 ± 0.1
Control	3.8 ± 1.4	12.7 ± 2.8	37.4 ± 15.7	27.7 ± 9.6	0.6 ± 0.2
Significance					
T	n.s.	n.s.	n.s.	n.s.	n.s.

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3.9 Metabolomics untargeted analysis of the phenolic composition of tomato berries 294

The metabolomic analysis with the UHPLC/QTOF investigated the phenolic compositions of tomato berry sample subjected to different biostimulant treatments. Overall, when considering all the samples analyzed, 271 phenolic compounds were putatively annotated (Table S3), mostly consisting of flavonoids (115). Moreover, 21 lignans, 62 phenolic acids and 9 stilbenes were identified, while alkylmethoxyphenol, alkylphenol, curcuminoid, furanocoumarin, hydroxybenzaldehyde, hydroxybenzochetone, hydroxybenzoketone, hydroxycinnamaldehyde, hydroxycoumarin, hydroxyphenylpropene, methoxyphenol, naphtoquinone, phenolic terpene and tyrosol compounds were grouped together as "Other polyphenols" class (64). 295–303

An unsupervised multivariate approach, consisting of a fold-change-based hierarchical clustering, was carried out in order to gain insight into the biochemical processes of tomato berries which appeared to be regulated following treatments. The analysis showed a clear division between sub-optimal and optimal fertility tomatoes, describing the farm as the unique factor affecting samples arrangement, since no clusters were formed for treatment factor (Fig. 1). 304–309

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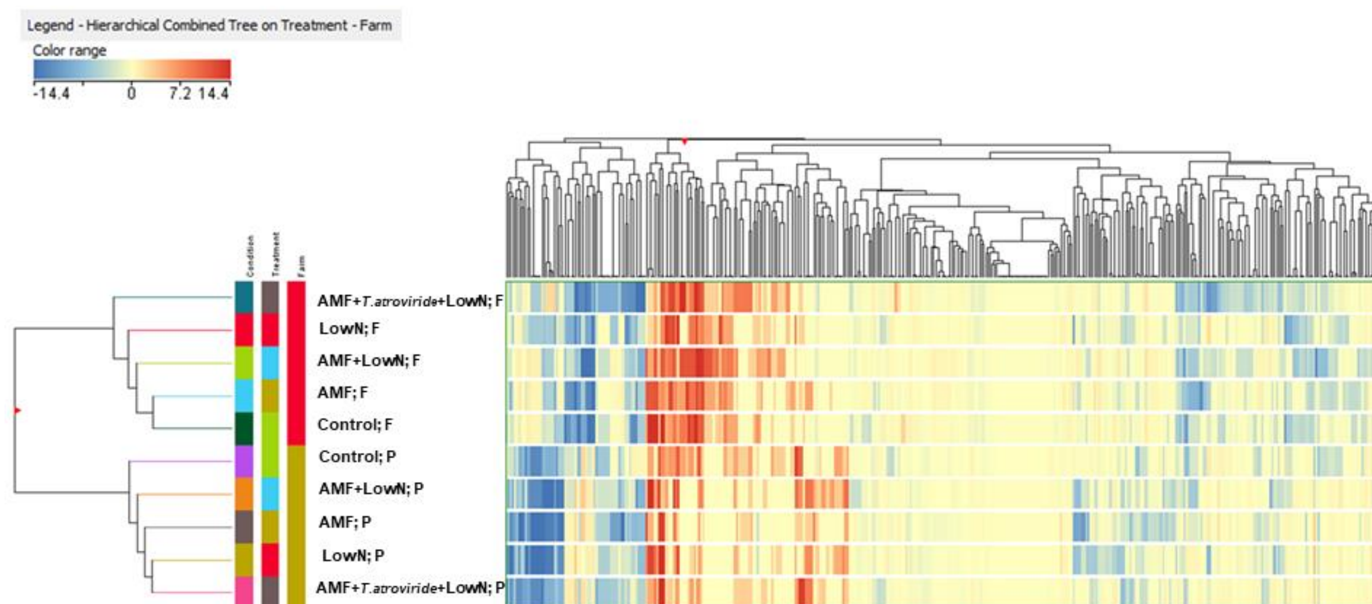

311

Fig. 1 Unsupervised hierarchical cluster analysis (Euclidean distance; linkage rule: Ward) of tomato berries phenolic profiles amid Control, LowN, AMF, AMF+LowN and AMF+*T.atroviride*+LowN treatments at optimal (P) and sub-optimal (F) fertility. Metabolites were obtained by UHPLC-ESI/QTOF-MS untargeted analysis, and their intensities were used to create the fold-change heatmap provided here. 312–316

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Consequently, the hierarchical clustering analysis was repeated keeping the two farm samples separated in order to better achieve similarities and distances across treatments within the same pedoclimatic conditions (Fig. 2). Regarding sub-optimal fertility, control samples were clustered together with AMF-treated tomatoes but highlighted a markedly different metabolomic profile from those samples obtained with the coupled action of AMF and *T. atroviride* under low N input (Fig. 2A). Generally, treatments under low N rates appeared to be more distant from high N-treatments, thus showing LowN 319–325

closer to AMF+LowN and AMF+*T.atroviride*+Low N, and further away from AMF and control. On the contrary, the nitrogen level within the treatment did not represent a discerning factor affecting clustering for optimal fertility, since no-separation N level-depending was achieved between samples (Fig. 2B). Here, two clusters –respectively formed by Control and AMF+*T.atroviride*+Low N, and by AMF and AMF+LowN were observed.

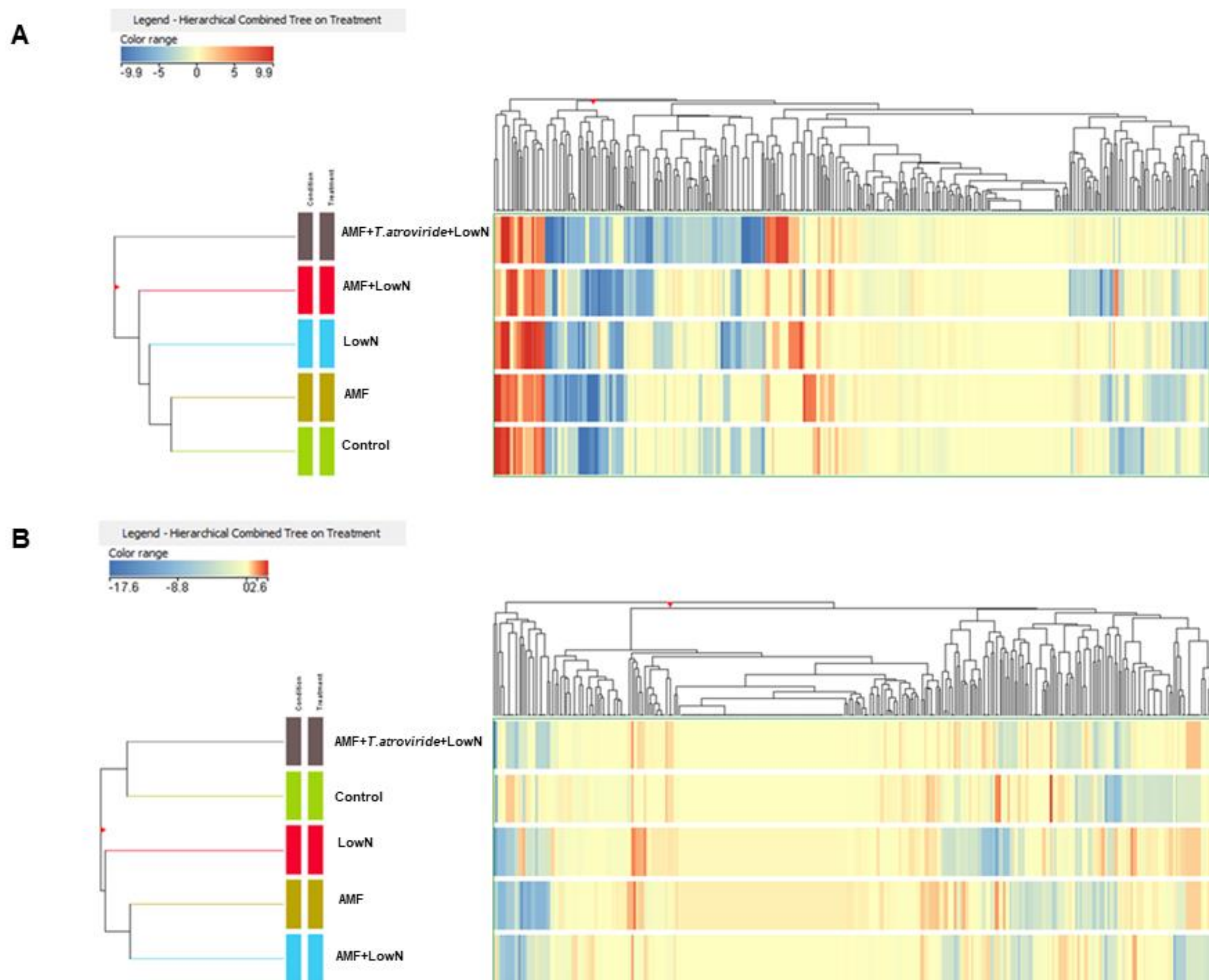

Fig. 2 Unsupervised hierarchical cluster analysis (Euclidean distance; linkage rule: Ward) of sub-optimal fertility (A) and optimal fertility (B) tomato berries phenolic profiles under LowN, AMF, AMF+LowN, AMF+*T.atroviride*+Low N and Control conditions. Metabolites were obtained by UHPLC-ESI/QTOF-MS untargeted analysis, and their intensities were used to create the fold-change heatmap provided here**.**

Finally, ANOVA and Duncan test results for phenolic compounds as equivalents per class were provided in Table 7 & 8. Regarding sub-optimal fertility, the treatment factor was statistically determinant for flavonoid class content, which varied from 2.9 to 8.1 mg eq. g-1 DM and showed the highest value (6.7 ± 1.4 mg eq. g-1 DM) with AMF+LowN application. Equally, a considerable treatment effect was remarked for lignan and other polyphenol classes, whose amounts were both mostly improved by AMF treatment (respectively 11.7 ± 3.3 and 37.7 ± 15.9 mg eq. g-1 DM). In contrast, in optimal fertility samples, none of the phenolic classes were significantly affected by the type of treatment.

4. Discussion

Finding new sustainable technologies to improve the functional and nutraceutical values of food products while improving yield and pomological traits has become a major research challenge due to ambitious objectives of the EU "Farm to fork" strategy [24]. In this context, the present study indicated that the use of biostimulants in agriculture may lead to a general increase of fruit quality-related compounds in *S. lycopersicum* L. On the contrary, we did not find a specific pattern in terms of tomato yield response to selected biostimulants.

Notably, mycorrhizal treatment revealed remarkable accumulations of carotenoids and phenols in tomato berries, confirming the previous findings in literature [12,25,26]. Indeed, AMF have been proved to support plant accumulation of those secondary metabolites which are involved in the response to abiotic stresses and pathogens. This results in a concrete help for the plant to counteract the negative effects of the stress and, on the other hand, in the enhancement of the functional quality of edible plant parts. Specially carotenoids, implicated in plants' defense mechanisms as antioxidants and photo-protecting molecules, play a key role against human cancer development, thus concurring to the nutraceutical quality of plant-based foods [27]. Likewise, phenolic compounds, involved in plant responses to environmental stress including wounding, pathogen attack, mineral deficiencies, and temperature stress, have been linked to a reduced risk of cardiovascular mortality for humans thanks to their high antioxidant potential [28,29].

More in details, our study highlights that a synergic biostimulant effect may be observed at the field level with the coupled inoculation of AMF and *T. atroviride*, which revealed increases in concentration for most compounds at higher rate than those due to single mycorrhizal application in our experiment. It has been reported that *T. atroviride* acts as a biocontrol agent against a many aerial and soilborne plant pathogens, by activating different mechanisms, including competition for nutrients, production of useful secondary metabolites, modification of the rhizosphere, and mycoparasitism [30]. Our results corroborate recent research, which previously showed the synergetic potential of AMF and *T. atroviride* co-inoculation, with increased plant growth, yield, nutrient uptake and stress-tolerance [8,31,32]. Trichoderma-plant associations take place following the fungus secretion of proteins which are recognized by plant receptors. Successively, the following transient suppression of plant defenses promotes the Trichoderma penetration and, in case of co-inoculation, the concomitant access to AMF [33,34]. Similarly, it has been ascribed to AMF a corresponding help in Trichoderma conidia germination [35].

In the present study, AMF+*T.atroviride*+Low N- treated samples of sub-optimal fertility showed higher values of carotenoid concentrations -including B-carotene, z-carotene, all-e-y carotene, 13-z-lycopene and all trans lycopene- and total phenolic content. Similarly, the same treatment highlighted the strongest antioxidant abilities in DPPH, ABTS, phosphomolybdenum, reducing power and enzyme inhibitory assays. This suggests that the synergic biostimulant effect of AMF and *T. atroviride* is highly related to soil fertility status (as revealed here by our initial analyses on soil OM and total N), as confirmed by our results on optimal fertility showing no treatment differences in fertile soils.

Plant N uptake is greatly aided by mutualistic association with AMF, which grow and extend their hyphae in the surrounding soil. Many studies have indicated an increased inflow of N (and other nutrients) in mycorrhizal root and, consequently, associated plants have been shown higher N concentrations than non-mycorrhized plants [36-38]. Nevertheless, several previous works have reported a remarkable decrease in the colonization intensity and diversity of mycorrhizal fungi in high-quality agricultural lands, leading to a weakening of the mutually beneficial symbiosis between AMF and plant roots [39-41]. Particularly, limited benefits of mycorrhizal inoculation benefits for agricultural production have been found under high levels of N-fertilization, denoting a better promising in low-quality lands [42,43].

In the current study, results from mycorrhized berries showed evidence of improved levels of carotenoid and bioactive compound exclusively in sub-optimal fertility, confirming the plant stronger benefit from a symbiotic relationship with AMF in soil nutrient-scarce environments than in soil nutrient-rich environments [44-47].

5. Conclusions

The present field experiment suggested the single and coupled use of microbial biostimulants to improve the quality of tomato fruits. However, the results obtained highlighted a strong dependence of the biostimulant effect on soil fertilization, reflecting significant increment in antioxidant properties only under sub-optimal conditions. Further and more in-depth studies should be carried out to fully understand the molecular and biochemical processes underlying the plant-fungi associations and the resulting changes in the functional value of fruits.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1. Table S1: Two-way analysis and Duncan test for carotenoid content in tomato berries of sub-optimal and optimal fertility. Table S2: Two-way analysis and Duncan test for chemical assays in tomato berries of sub-optimal and optimal fertility. Table S3: List of metabolites revealed with the untargeted UHPLC-ESI/QTOF-MS analysis on tomato berries.

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