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ORIGINAL ARTICLE

Arbuscular mycorrhizal fungi and plant growth-promoting pseudomonads improve yield, quality and nutritional value of tomato: a field study

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Abstract The aim of this work was to assess the effects of plant-beneficial microorganisms (two *Pseudomonas* strains and a mixed mycorrhizal inoculum, alone or in combination) on the quality of tomato fruits of plants grown in the field and subjected to reduced fertilization. *Pseudomonas* strain 19Fv1T was newly characterized during this study. The size and quality of the fruits (concentration of sugars, organic acids and vitamin C) were assessed. The microorganisms positively affected the flower and fruit production and the concentrations of sugars and vitamins in the tomato fruits. In particular, the most important effect induced by arbuscular mycorrhizal (AM) fungi was an improvement of citric acid concentration, while bacteria positively modulated sugar production and the sweetness of the tomatoes. The novelty of the present work is the application of soil microorganisms in the field, in a real industrial tomato farm. This approach provided direct information about the application of inocula, allowed the reduction of chemical inputs and positively influenced tomato quality.

Keywords Tomato · PGPB · Pseudomonads · Arbuscular mycorrhizae · Fertilization · Fruit quality

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most studied fleshy fruits, and it has assumed the status of "functional food" because of the epidemiological evidence as regards the reduced risk of cancer and cardiovascular diseases in relation to its consumption (Giovannetti et al. 2012). Tomato fruits contain high concentrations of antioxidant molecules such as lycopene, ascorbic acid and carotenoids (Giovannetti et al. 2012). Tomato fruit taste and antioxidant content vary according to cultivar, growing conditions, production methods, harvest time and storage (Loiudice et al. 1995; Langlois et al. 1996).

Among suitable practices for sustainable management of agricultural soils, the use of biofertilizers such as arbuscular mycorrhizal (AM) fungi and plant beneficial bacteria can lead to yield enhancement and fruit quality improvement (Copetta et al. 2006; Baslam et al. 2011a; Baslam et al. 2011b; Lingua et al. 2013; Berta et al. 2014; Bona et al. 2015; Bona et al. 2016). Arbuscular mycorrhizae are symbiotic associations between plants and soil fungi (Smith and Read 2008) of the phylum Glomeromycota (Schüβler et al. 2001). AM fungi colonize plant roots, but the symbiosis can affect the whole of plant physiology with detectable effects on shoots and fruits (Guerrieri et al. 2004; Copetta et al. 2006; Lingua et al. 2013, Berta et al. 2014; Bona et al. 2015).

Plant growth-promoting bacteria (PGPB) are free-living microorganisms that interact with plant roots and induce beneficial effects in plants by improving mineral nutrition, producing phytohormones and synthesizing antibiotics involved

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in plant disease suppression (Glick 1995; Ordookhani et al. 2010; Gamalero and Glick 2011). The amount and quality of plant secondary metabolites can be modulated by plant inoculation with PGPB and AM fungi, as demonstrated for different important crops (Copetta et al. 2006; Aimo et al. 2010; Ordookhani et al. 2010; Lingua et al. 2013; Bona et al. 2015).

The aim of this study was to investigate, directly in the field, the effects of two selected strains of Pseudomonads, alone and/or in combination with a mix of AM fungi, on tomato plants grown under a reduced fertilization regimen. Special attention was devoted to the effects of these microorganisms on the industrial and nutritional features of tomato fruits.

Materials and methods

Microorganisms

A mycorrhizal inoculum produced in the greenhouse on sorghum (*Sorghum bicolor*) and consisting of fragments of colonized roots, spores and hyphae of *Rhizophagus intraradices*, *Glomus aggregatum*, *Glomus viscosum*, *Claroideoglomus etunicatum* and *Claroideoglomus claroideum* was provided by Mybasol s.r.l (Alessandria, Italy). The inoculum potential, tested by the provider before the experiment, was about 85,000 infective propagules/l of inoculum.

Two bacterial strains were used to inoculate the plants:

Pseudomonas fluorescens bv. II strain C7 (briefly: C7), isolated from the rhizosphere of *Linum usitatissimum* from Châteaurenard soil (Eparvier et al. 1991), was kindly provided by Dr. Philippe Lemanceau (ECOLDUR, INRA, Dijon, France). This strain is efficient in the suppression of *Fusarium* wilt disease in tomato and is able to reduce nitrate (Lemanceau and Alabouvette 1991; Olivain et al. 2004).

Pseudomonas sp. 19Fv1T (abbreviated 19Fv1T) was isolated from the rhizosphere of *Fragaria vesca* grown in a larch woodland located in Bellino (CN, Italy).

The characterization method of these strains is fully described in Bona et al. (2015). Genomic DNA of 19Fv1T was extracted using the Nucleo Spin tissue DNA purification kit (Macherey-Nagel, M-Medical, Cornaredo, Milan) according to the manufacturer's instructions. PCR amplification of 16S ribosomal DNA (rDNA) was performed using the primers fD1 (5′-ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG-3′) and RP2 (5′-cccgggatccaagcttACGGCTACCTTGTTAC GACTT-3′), as described by Weisburg et al. (1991). The PCR reaction was carried out as reported in Bona et al. (2015). The amplified PCR product was sequenced by BMR Genomics (Padua, Italy). Obtained DNA sequences were compared against all bacterial 16S rDNA reference sequences available at the NCBI World Wide Web database (http://www.ncbi.nlm.nih.gov/BLAST/). Bacterial 16S rDNA reference sequences of *Pseudomonas* sp. 19Fv1T are available at the NCBI World Wide Web database GenBank with the accession numbers KF752592.

Siderophore production by *Pseudomonas* sp. 19Fv1T and *P. fluorescens* C7 was assayed on universal chrome azurol S (CAS) agar (Schwyn and Neilands 1987). The bacterial strains were inoculated at the centre of each plate and incubated at 28 °C for 3 days. Siderophore production was indicated by a halo of colour change from blue to orange on the CAS medium and was measured in triplicate as the ratio between two diameters of the halo and two diameters of the colony.

Phosphate solubilization by *Pseudomonas* sp. 19Fv1T and *P. fluorescens* C7 was assayed according to Goldstein (1986), using two different media: one containing dicalcium phosphate (DCP) (NH_4Cl 4.25 g l^{-1}, NaCl 0.85 g l^{-1}, $MgSO_4$ $7H_2O$ 0.85 g l^{-1}, glucose 8.5 g l^{-1}, K_2HPO_4 2 g l^{-1}, $CaCl_2$ $2H_2O$ 4 g l^{-1}, agar 17 g l^{-1}) and one containing tricalcium phosphate (TCP) (NH_4Cl 5 g l^{-1}, NaCl 1 g l^{-1}, $MgSO_4$ 7 H_2O 1 g l^{-1}, glucose 10 g l^{-1}, $Ca_3(PO_4)_2$ 40 g l^{-1}, agar 20 g l^{-1}). The strains were inoculated at the centre of each plate and incubated at 28 °C for 15 days. DCP solubilization was indicated by a clarification halo around the colony; TCP solubilization was identified by colony growth on the medium.

Indole-3-acetic acid (IAA) production by *Pseudomonas* sp. 19Fv1T and *P. fluorescens* C7 was quantified according to De Brito et al. (1995). The bacterial strains were inoculated onto a nitrocellulose disc placed on trypticase soy agar (TSA) containing 10 % of L-tryptophan (5 mM) and incubated at 28 °C for 3 days. The membrane was then stained with the Salkowsky's reagent ($FeCl_3$ 2 % in perchloric acid 35 %); the presence of a red/pink halo around the colony indicated a positive reaction.

The two bacterial inocula were produced using overnight culture on TSA medium, resuspended on 0.1 M $MgSO_4$ and adjusted to 10^8 CFU/ml after optical density assay (λ 600 nm).

Experimental design and plant growth

The experiment aimed at assessing the effects of different inocula under conditions of reduced fertilization; therefore, it included seven treatments: (1) control full dose (CFD): uninoculated plants fertilized according to conventional practice; (2) control reduced dose (CRD): uninoculated plants; (3) 19Fv1T: plants inoculated with *Pseudomonas sp*. strain 19Fv1T; (4) C7: plants inoculated with *P. fluorescens* C7; (5) Myc: plants inoculated with the AM fungi; (6) Myc + 19Fv1T: plants inoculated with the AM fungi and with *Pseudomonas* sp. 19 Fv1T; and (7) Myc + C7: plants inoculated with the AM fungi and with *P. fluorescens* C7. All the plants belonging to the treatments from no. 2 to no. 7 were

subjected to reduced fertilization as detailed below. Sterilized tomato seeds of *S. lycopersicum* var. TC 2000 (Tomato Colors Soc. Coop., S. Agata Bolognese, BO, Italy) were germinated in alveolar boxes on sterilized soil and grown in a greenhouse for 3 weeks before transplanting. Myc, Myc + 19Fv1T and Myc + C7 plantlets were inoculated with 20 ml of mycorrhizal inoculum (85,000 infective propagules/L of inoculum) and with 10 ml of bacterial suspension (density about 10^8 CFU/ml) in the alveolar boxes. After 3 weeks (when Myc tomato plantlets reached about 3 % AM colonization), 168 tomato plantlets per treatment were transplanted into an open field, divided among three different parallel rows. The distance between adjacent plants within a row was 0.4 m, and the distance between the rows was 1.2 m (see Figure 1 supplementary material). Each set of plants involved in the experiment (control and inoculated plants) was separated from the others using two rows of plants not considered in our experimental plan. At the time of transplanting, 200 ml/plant of the same AM inoculum were added to all Myc treatments. The experiment was performed between April and August 2011, in open-field conditions located in Torre Garofoli (latitude 44° 88′ 84″ N, longitude 8° 79′ 92″ W, altitude 90 a.s.l.), close to Alessandria (Italy). According to its texture, the soil was classified as clay-loam (silt, 40 %; clay, 28 %; and sand, 32 %). The soil was alkaline (pH 8.2) and soil organic matter content was low (1.5 %). Before transplanting, the soil was fertilized with NH_4NO_3 (150 kg/ha), $(NH_4)_2HPO_4$ (300 kg/ha) and K_2SO_4 (330 kg/ha). In addition to the above, conventional fertilization consisted for the entire growing period of NH_4NO_3 (109.78 kg/ha), K_2O (65.70 kg/ha), CaO (13.65 kg/ha) and K_2SO_4 (121.84 kg/ha) and reduced fertilization consisted of NH_4NO_3 (84.44 kg/ha), K_2O (50.54 kg/ha), CaO (10.50 kg/ha) and K_2SO_4 (93.72 kg/ha). Fertilizers were managed and provided by Green Has Italia S.p.A. (Canale, CN, Italy). All the plants were fed with fertilizers weekly and watered when necessary using drip irrigation. During the growth period, diseases and insects were controlled according to standard, conventional practices.

Mycorrhizal colonization, growth parameters and qualitative analyses of fruits

The number of inflorescences per branch, number of fruits, plant collar diameter, internode length and roots were assessed at harvest time for 24 plants, randomly chosen (1 every 7 plants) among the 168 plants grown in the field per each treatment. The fruits produced by the 24 plants were harvested and used for further analyses.

For the assessment of mycorrhizal colonization, 40 randomly chosen 1 cm-long pieces were cut from each root system (24 plants per treatment) and cleared in 10 % KOH for 45 min at 60 °C, stained with 1 % methyl blue in lactic acid and mounted on a slide. Mycorrhizal colonization was estimated according to Trouvelot et al. (1986): frequency of mycorrhization (F%), mycorrhizal degree (M%) and frequency of arbuscules (A%) were calculated.

Fruit dry biomass, water percentage, pH, titratable acids, concentration of sugars (sucrose, glucose and fructose), nitrite and nitrate, organic acids (malic, citric, glutamic, tartaric, oxalic acid) and vitamins (ascorbic acid) were evaluated for eight replicates per treatment (comprising three plants per replicate).

To measure fruit dry biomass and water percentage, 5 g of fresh fruit homogenate were dried at 60 °C for 7 days and weighed. Fruit pH, titratable acidity, sugars (glucose and fructose) and ascorbic acid concentrations were determined according to Bona et al. (2015). The sweetness index of the fruits, an estimate of the total sweetness perception, was calculated according to the amount and sweetness properties of each individual carbohydrate in strawberry. Because fructose is 2.30 and sucrose 1.35 times sweeter than glucose, the sweetness index was calculated as (1.00 [glucose]) + (2.30 [fructose]) + (1.35 [sucrose]) (Keutgen and Pawelzik 2007). The ratio sweetness index to titratable acidity was calculated.

Nitrite and nitrate concentrations were determined using enzymatic analytical kits (UV method) (R-Biopharm, Roche, Germany) according to the manufacturer's instructions. Three grams of fruit homogenate were diluted to 100 ml with deionized water, previously heated to 60 °C. The suspensions were maintained at 60 °C for 15 min, filtered through Whatman filter paper (pore diameter 20 μm) and adjusted to pH 8. The clean colourless serum was used for the analyses.

Organic acid concentration was determined according to Keutgen and Pawelzik (2007) with some modifications as described in Bona et al. (2015). The results were expressed in g per unit (kg) of fresh mass. Five grams of fruit homogenate were diluted to 50 ml with deionized water. The suspensions were filtered through Whatman filter paper and adjusted to pH 8. The clean colourless serum was used for the analyses. Organic acids were identified by retention time of samples spiked with standards and quantified by external standards analysis. To monitor the retention times and the performance of the column before starting the HPLC analysis, a standard solution of organic acids was injected. Because malic and citric acid co-eluted with the other organic acids, concentrations of these acids were determined using enzymatic analytical kits (R-Biopharm, Roche, Germany) for the detection of these compounds in foods.

The analysis of carotenoids was carried out according to Oke et al. (2005) for the extraction in organic phase and to Hart and Scott (1995) for the chromatographic analysis. Briefly, 4 g of tomato pulp (including fruit skin) was put into 250-ml brown bottles. In each bottle ,100 ml of a 2:1:1 hexane/acetone/ethanol solution containing 0.1 % butylated hydroxytoluene (BHT) was added. The bottles were then shaken for 10 min at 250 rpm on an orbital shaker (Bibby Scientific

Limited, Stone, UK) followed by the addition of 15 ml of HPLC-grade water and shaken again for further 5 min. The organic phase was separated by centrifugation at 4000 rpm for 6 min. The organic extract was analysed with an HPLC instrument (Dionex, Sunnyvale, CA, USA). Carotenoids were separated on a pre-column Aphera C18 polymer 15 cm × 4.6 mm, 5 μm and an analytical column Aphera C18 polymer 25 cm × 4.6 mm, 5 μm at a flow rate of 1.5 ml/min. An eluent of acetonitrile/methanol (containing 0.05 M ammonium acetate) 80:20 added with 0.1 % BHT was used in isocratic flow. Carotenoids were detected at 450 nm (injection volume 20 μl), identified by retention time of samples spiked with standards and quantified by calibration curves, obtained by triplicate injection of standard solutions of the same organic acids. All reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). The results were expressed as μg/100 g of fresh mass.

Statistical analysis

Statistical analyses were performed with StatView 4.5 (Abacus Concepts).

Data were analysed by one-way ANOVA, using "treatment" as factor, followed by Fisher's probable least-squares difference test with cut-off significance at $P < 0.05$ to assess differences among treatments. Data excluding those for CFD plants were statistically analysed additionally by two-way ANOVA using "fungus" and "bacterium" as factors in order to test for interactions. Statistically significant differences based on two-way ANOVA are reported as F (fungus effect), B (bacterium effect) and F × B (interaction between fungus and bacterium). NS means not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Results

Identification and characterization of the bacterial strain 19Fv1T

Strain 19Fv1T is a Gram-negative, rod-shaped bacterium producing a yellow pigment on King B medium. The results of the nearest neighbours search against all bacterial 16S rDNA reference sequences, available at the NCBI World Wide Web database, showed that the strain belongs to the genus *Pseudomonas* (KF752592). *Pseudomonas* sp. 19Fv1T strain and *P. fluorescens* C7 physiological traits are summarized in Table 1. In particular, *Pseudomonas* sp. 19Fv1T strain synthesized siderophores (++), solubilized tricalcium phosphate (+) and produced the phytohormone indole acetic acid (IAA) (++++) and *P. fluorescens* C7 synthesized siderophores (+/−), solubilized dicalcium and tricalcium phosphate (+) and produced the phytohormone IAA (+).

Table 1 Analysis of functional traits of *Pseudomonas fluorescens* bv. II strain C7 and *Pseudomonas* sp. 19Fv1T

Strain	Origin	Physiological traits	Qualitative assay (qualitative assay)
Pseudomonas sp. 19Fv1T	Rhizosphere of *Fragaria vesca* grown in a larch woodland located in Bellino (CN, Italy).	Siderophore synthesis	++
		IAA	++++
		Phosphate solubilization of dicalcium phosphate (DCP)	−
		Phosphate solubilization of tricalcium phosphate (TCP)	+
Pseudomonas fluorescens bv. II strain C7[a]	Rhizospheric soil of *Linum usitatissimum* from Châteaurenard soil[a]	Siderophore synthesis	+/−
		IAA	+
		Phosphate solubilization of dicalcium phosphate (DCP)	+
		Phosphate solubilization of tricalcium phosphate (TCP)	+

[a] This strain was kindly provided by Dr. Philippe Lemanceau (ECOLDUR, INRA, Dijon, France). This strain is efficient in the suppression of Fusarium wilt disease

Mycorrhizal colonization

The mycorrhizal colonization of the inoculated tomato plant roots after 4 months of growth is shown in Fig. 1. While uninoculated, the plants showed occasional traces of mycorrhization (M% < 0.15); all the Myc treatments showed a significantly higher degree of colonization (M%) and frequency of arbuscules (A%). The two bacterial strains did not affect root colonization.

Plant parameters and flower production

Plant growth parameters and inflorescence production are shown in Table 2. Plant growth parameters were not

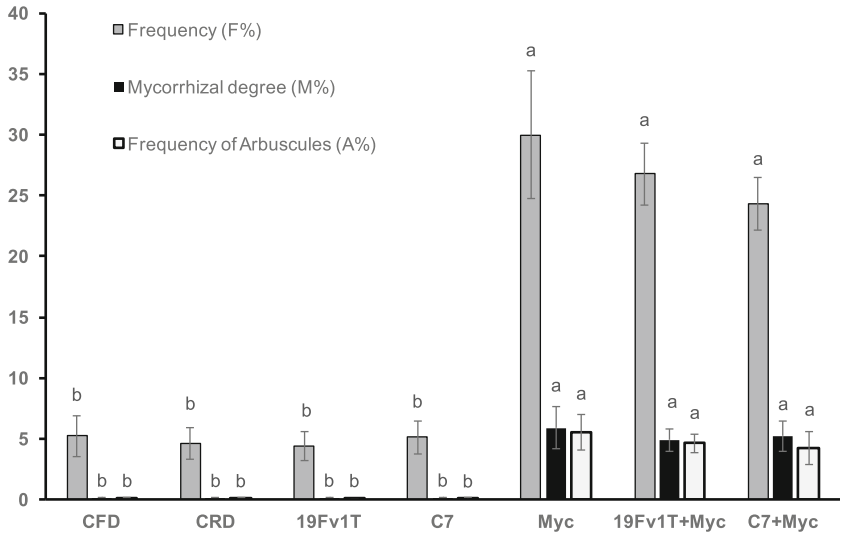

Fig. 1 Mycorrhizal colonization parameters. The values presented in the figure are means ± standard errors. *Bars* topped by the same letter within each parameter do not differ significantly at $P < 0.05$ by one-way ANOVA followed by Fisher's post hoc test. *CFD* control 100, uninoculated plants with traditional fertilization, *CRD* control 70, uninoculated plants with 70 % of the traditional fertilization, *19Fv1T* plants inoculated with *Pseudomonas* sp. 19 Fv1T with 70 % of the traditional fertilization, *C7* plants inoculated with *P. fluorescens* C7 with 70 % of the traditional fertilization, *Myc* plants inoculated with AMF and with 70 % of the traditional fertilization, *19Fv1T + Myc* plants inoculated with AMF and with *Pseudomonas* sp. 19 Fv1T with 70 % of the traditional fertilization, *C7 + Myc* plants inoculated with AMF and with *P. fluorescens* C7 with 70 % of the traditional fertilization

significantly affected by the reduction of fertilization, while the presence of AM fungi alone increased the collar diameter (in Myc plants) compared to the uninoculated plants. Moreover, Myc plants showed the highest number of inflorescences per branch, even higher than those produced by the CFD plants (+27 %). The two-way ANOVA indicated that the factor "fungus" significantly affected the internode length and the number of inflorescences per branch, the interaction between the factors fungus and "bacterium" significantly influenced the collar diameter and inflorescence per branch production. The factor bacterium alone did not affect any parameter.

Production and quality of tomato fruits

The parameters concerning tomato fruit production, size and quality are shown in Figs. 2 and 3 and in Table 3. Both fruit fresh weight and the number of marketable fruits were significantly higher in plants fed with reduced fertilization. The mixture of bacterial and fungal inoculum induced the production of heavier fruits (about + 35 % vs CFD). Moreover, plants inoculated with *P. fluorescens* C7 alone showed a higher production of marketable fruits (+160 %).

The size (Table 3) of the fruits produced by uninoculated plants was not inhibited by the reduced fertilization: on the contrary, the diameters of the fruits obtained by CRD plants

Table 2 Tomato plant parameters

Parameters	CFD	CRD	19Fv1T	C7	Myc	19Fv1T + Myc	C7 + Myc	F	B	FxB
Collar diameter (cm)	1.70 ± 0.06bc	1.53 ± 0.06c	1.63 ± 0.04c	1.90 ± 0.13ab	2.13 ± 0.03a	1.73 ± 0.09bc	1.53 ± 0.12c	NS	NS	***
Internode length (cm)	9.62 ± 0.58abc	9.12 ± 0.39bc	8.90 ± 0.44c	8.93 ± 0.29c	9.07 ± 0.38bc	10.31 ± 0.37ab	10.45 ± 0.56 a	**	NS	NS
Inflorescence per branch	6.06 ± 0.54bc	4.39 ± 0.57c	5.80 ± 0.74bc	5.80 ± 0.62bc	7.73 ± 0.71a	5.46 ± 0.27bc	6.43 ± 0.62ab	*	NS	*

The values presented in the table are means ± standard errors. The same letter in the row indicates not significantly different values based on one-way ANOVA and Fisher post hoc test ($P < 0.05$). Statistically significant differences based on two-way ANOVA are reported in columns F (fungus effect), B (bacterium effect) and F × B (interaction between fungus and bacterium). *NS* not significant * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

NS not significant, *CFD* control 100, uninoculated plants with traditional fertilization, *CRD* control 70, uninoculated plants with 70 % of the traditional fertilization, *19Fv1T* plants inoculated with *Pseudomonas* sp. 19 Fv1T with 70 % of the traditional fertilization, *C7* plants inoculated with *P. fluorescens* C7 with 70 % of the traditional fertilization, *Myc* plants inoculated with AMF and with 70 % of the traditional fertilization, *19Fv1T + Myc* plants inoculated with AMF and with *Pseudomonas* sp. 19 Fv1T with 70 % of the traditional fertilization, *C7 + Myc* plants inoculated with AMF and with *P. fluorescens* C7 with 70 % of the traditional fertilization

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

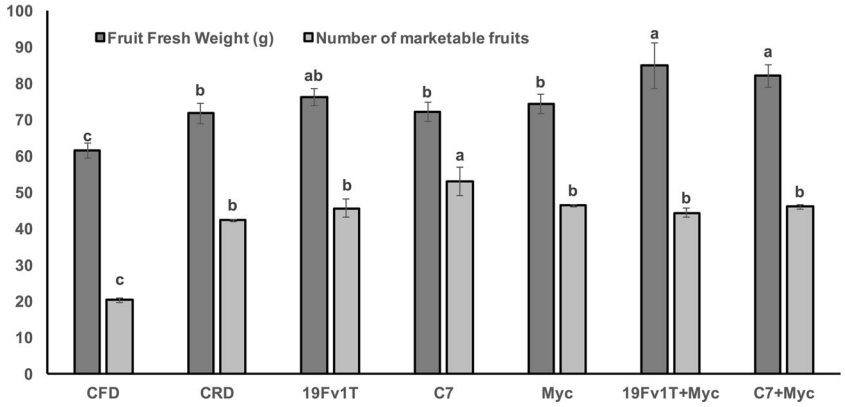

Fig. 2 Fruit fresh weight (g) and number of marketable fruits/plant*. The values presented in the figure are means ± standard errors. *Bars* topped by the same letter within a fruit attribute do not differ significantly at $P < 0.05$ by one-way ANOVA followed by Fisher's post hoc test. *By the term "marketable", the authors mean those fruits without defects considered of the first category for industrial production of tomatoes. *CFD* control 100, uninoculated plants with traditional fertilization, *CRD* control 70, uninoculated plants with 70 % of the traditional fertilization, *19Fv1T* plants inoculated with *Pseudomonas sp*. 19 Fv1T with 70 % of the traditional fertilization, *C7* plants inoculated with *P. fluorescens* C7 with 70 % of the traditional fertilization, *Myc* plants inoculated with AMF and with 70 % of the traditional fertilization, *19Fv1T + Myc* plants inoculated with AMF and with *Pseudomonas sp*. 19 Fv1T with 70 % of the traditional fertilization, *C7 + Myc* plants inoculated with AMF and with *P. fluorescens* C7 with 70 % of the traditional fertilization

were larger than those of fruits produced by CFD plants. 19Fv1T, C7 and Myc plants did not show a significant increase of fruit size. These parameters increased in plants inoculated with both bacterial strains and the AM fungi. The proportion of dry pulp (data shown in Table 3) was increased (+100 %) in fruits produced by plants inoculated with AM fungi alone or in combination with the bacterial strains.

Sucrose was not detected in tomato fruits. The fruits of plants grown at reduced fertilization contained a higher concentration of glucose and a lower concentration of fructose than those produced by CFD plants. The sugar concentration was modulated both by bacteria and by AM fungi, alone or in combination: while the amount of fructose increased, that of glucose decreased compared to CRD, as indicated also by the two-way ANOVA. Sweetness index is reported in Fig. 3. The reduction of fertilization induced a significant decrease of this quality parameter in uninoculated plants while the presence of the two bacterial strains induced a significant increase of the tomato sweetness. This effect was confirmed by two-way ANOVA ("F" *; "B" *** and "F×B" ***).

All the analysed fruits had a pH value lower than 4.5 (see Table 3). Fruits of 19Fv1T plants were characterized by significantly lower pH values (4.14) compared with the other treatments.

Values of titratable acidity ranged between 0.29 and 0.34. The reduction of fertilization induced a significant increase of the titratable acidity compared to CFD fruits, except for fruits from plants of C7, Myc and 19Fv1T + Myc. Two-way

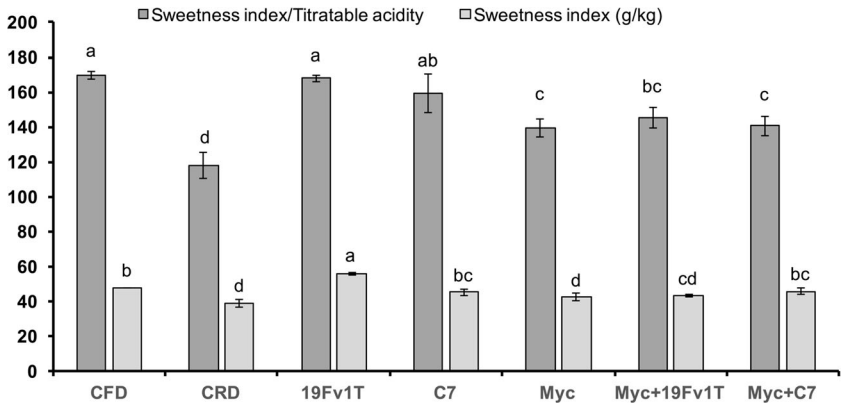

Fig. 3 Sweetness index to titratable acidity ratio and sweetness index (g/kg). The values presented in the figure are means ± standard errors. *Bars* topped by the same letter within a fruit attribute do not differ significantly at $P < 0.05$ by one-way ANOVA followed by Fisher's post hoc test. *CFD* control 100, uninoculated plants with traditional fertilization, *CRD* control 70, uninoculated plants with 70 % of the traditional fertilization, *19Fv1T* plants inoculated with *Pseudomonas sp*. 19 Fv1T with 70 % of the traditional fertilization, *C7* plants inoculated with *P. fluorescens* C7 with 70 % of the traditional fertilization, *Myc* plants inoculated with AMF and with 70 % of the traditional fertilization, *19Fv1T + Myc* plants inoculated with AMF and with *Pseudomonas sp*. 19 Fv1T with 70 % of the traditional fertilization, *C7 + Myc* plants inoculated with AMF and with *P. fluorescens* C7 with 70 % of the traditional fertilization

Table 3 Dimensions and qualitative parameters of tomato fruits

Parameters	CFD	CRD	19Fv1T	C7	Myc	19Fv1T + Myc	C7 + Myc	F	B	FxB
Fruit length (cm)	5.69 ± 0.10ab	5.53 ± 0.05b	5.55 ± 0.10b	5.60 ± 0.07b	5.49 ± 0.09b	5.70 ± 0.08ab	5.87 ± 0.10a	NS	*	NS
Fruit diameter (cm)	4.33 ± 0.06c	4.66 ± 0.08b	4.80 ± 0.07ab	4.73 ± 0.07ab	4.79 ± 0.08ab	4.91 ± 0.09a	4.90 ± 0.08a	*	NS	NS
% dry pulp[a]	7.53 ± 0.05cd	7.58 ± 0.19c	7.04 ± 0.13cd	6.76 ± 0.18d	14.20 ± 0.33b	15.16 ± 0.17a	14.10 ± 0.32b	***	*	**
Glucose (g/kg FW[b])	13.57 ± 0.01d	20.79 ± 0.78a	16.55 ± 0.11c	18.74 ± 0.48b	13.21 ± 0.56d	15.32 ± 0.16c	15.55 ± 0.61c	***	*	***
Fructose (g/kg FW)	14.99 ± 0.01b	8.02 ± 1.03d	17.08 ± 0.18a	11.52 ± 0.97c	12.66 ± 0.75c	12.20 ± 0.28c	13.22 ± 0.53c	NS	***	***
Sucrose (g/kg FW)	–	–	–	–	–	–	–			
pH	4.30 ± 0.01b	4.41 ± 0.01a	4.14 ± 0.03c	4.42 ± 0.02a	4.22 ± 0.02b	4.24 ± 0.02b	4.28 ± 0.03 b	***	***	***
Titratable acids (%CA)	0.29 ± 0.01c	0.34 ± 0.01a	0.33 ± 0.01a	0.29 ± 0.01c	0.30 ± 0.01bc	0.30 ± 0.02bc	0.33 ± 0.01 ab	NS	NS	**
Nitrite (mg/kg FW)	–	–	–	–	–	–	–			
Nitrate (mg/kg FW)	10.16 ± 1.02b	8.24 ± 1.33b	15.30 ± 3.69a	4.70 ± 1.80c	19.91 ± 2.06a	9.64 ± 1.07b	16.39 ± 0.12a	**	NS	***
Glutamic acid (g/kg)	0.53 ± 0.05d	1.73 ± 0.12abc	2.03 ± 0.26ab	1.30 ± 0.11c	2.02 ± 0.29ab	1.56 ± 0.15bc	2.25 ± 0.16a	NS	NS	**
Tartaric acid (g/kg FW)	0.44 ± 0.05c	0.21 ± 0.05d	0.83 ± 0.10a	0.63 ± 0.04b	0.74 ± 0.04a	0.61 ± 0.04b	0.54 ± 0.03b	NS	***	***
Oxalic acid (g/kg FW)	0.07 ± 0.01ab	0.05 ± 0.01b	0.02 ± 0.002c	0.08 ± 0.01a	0.06 ± 0.01b	0.06 ± 0.01b	0.05 ± 0.01b	NS	**	***
Ascorbic acid (mg/100 g FW)	15.01 ± 0.08cd	19.42 ± 1.00b	23.77 ± 1.53a	12.86 ± 0.90d	15.76 ± 1.09c	19.36 ± 0.61b	12.64 ± 0.28d	**	***	NS
Malic acid (mg/100 g FW)	32.56 ± 0.23abc	28.43 ± 0.78d	31.40 ± 0.84bc	34.98 ± 1.22a	33.35 ± 1.20ab	34.48 ± 0.97a	30.65 ± 0.60cd	NS	NS	***
Citric acid (mg/100 g FW)	223.78 ± 1.55c	226.53 ± 5.24c	228.27 ± 2.13c	226.70 ± 5.79c	263.52 ± 2.95a	242.85 ± 7.79b	232.45 ± 3.94bc	***	**	**
Lycopene (μg/100 g FW)	2532.66 ± 13.13b	2648.90 ± 38.23a	2600.40 ± 19.43ab	2647.06 ± 39.37a	2670.06 ± 15.07a	2675.80 ± 25.66a	2657.41 ± 27.48a	NS	NS	NS
β-carotene (μg/100 g FW)	2.94 ± 0.05a	2.61 ± 0.08b	2.72 ± 0.03b	2.31 ± 0.04c	2.44 ± 0.05c	2.69 ± 0.06b	2.68 ± 0.06b	NS	***	***

The values presented in the table are means ± standard errors. The same letter in the row indicates not significantly different values based on one-way ANOVA and Fisher post hoc test ($P < 0.05$). Statistically significant differences based on two-way ANOVA are reported in columns F (fungus effect), B (bacterium effect) and F × B (interaction between fungus and bacterium).*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

NS, not significant, *CFD* Control 100, uninoculated plants with traditional fertilization, *CRD* Control 70, uninoculated plants with 70 % of the traditional fertilization, *19Fv1T* plants inoculated with *Pseudomonas* sp. 19 Fv1T with 70 % of the traditional fertilization, *C7* plants inoculated with *P. fluorescens* C7 with 70 % of the traditional fertilization, *Myc* plants inoculated with AMF and with 70 % of the traditional fertilization, *19Fv1T + Myc* plants inoculated with AMF and with *Pseudomonas* sp. 19 Fv1T with 70 % of the traditional fertilization, *C7 + Myc* plants inoculated with AMF and with *P. fluorescens* C7 with 70 % of the traditional fertilization

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

[a] %dry pulp represents the percentage of the mixture of tomato pulp with tomato skin dried in comparison with the fresh one

[b] FW is fresh weight

ANOVA showed the strong effect of both microorganisms (fungus and bacterium) on pH, but only the factor fungus and F×B are significant for titratable acidity modulation. The ratio sweetness index to titratable acidity is reported in Fig. 3. CRD tomato showed a significant reduction of the ratio compared to CFD; tomato inoculated with the two bacterial strains had a ratio values comparable to those of CFD treatment (two-way ANOVA for B was ***). AM inoculum is efficient in the modulation of this parameter only in combination with bacterial strains as confirmed by the two-way ANOVA (F was NS; FxB was **).

Nitrites were not detected in tomato fruits. Nitrate concentration in fruits of uninoculated plants, grown at the two levels of fertilization, was similar. AM fungi and the bacterial strain 19Fv1T inoculated alone and C7 + Myc boosted nitrate concentration in fruits of tomato plants. On the contrary, the nitrate level in fruits produced by C7 plants was lower than in all the other treatments.

The organic acids in tomato juice were identified by comparison of their retention times with those of standards and quantified by using their calibration curves and evaluating the UV absorbance spectrum. The retention times of the organic acids were stable: glutamic acid (9.71 min), tartaric acid (10.65 min), oxalic acid (11.40 min). The organic acid concentrations were affected by fertilization: while glutamic and ascorbic acid concentrations were higher in fruits of CRD than in CFD plants, the amount of malic, tartaric and oxalic acids decreased in CRD plants. The two bacterial strains differently affected the organic acid concentrations. The fruits produced by 19Fv1T plants contained more malic, tartaric, ascorbic and less oxalic acid than fruits of CRD plants while C7 plants showed a reduction in glutamic (even if not significantly) and ascorbic acid concentrations.

Myc plants produced fruits with a higher concentration of citric acid (Table 3). When inoculated in combination with AM fungi, both bacterial strains decreased the concentration of tartaric acid. The concentrations of malic and citric acids were enhanced in fruits of 19Fv1T + Myc plants; Myc and C7 + Myc plants showed a reduction of ascorbic acid compared to CRD plants.

None of the microorganism treatments affected lycopene concentration in fruits relative to CRD; only the reduction of fertilization induced an increase of lycopene concentration independently from the presence of soil microorganisms. Reduced fertilization negatively affected β-carotene concentration.

Discussion

In the present work, biofertilizers (AM fungi and PGPB) were used in the field, in a real industrial tomato farm. This approach, to our knowledge, is novel because it gives direct information about application of inocula in order to reduce chemical inputs and check the impact on tomato quality. Soil preparation and initial fertilization was according to the standard practice in an industrial tomato farm; our inocula (both AM fungi and PGPB) were used in the same way as could be used by any farmer.

The relatively low levels of AM colonization observed in the roots of tomato plants could depend on two factors. Firstly, high levels of N and P occurring in the soil before the transplanting are known to negatively affect the AM symbiosis establishment (Bonneau et al. 2013). Secondly, the extent of AM root colonization can be modulated according to the phenology; for instance, Johnson et al. (1982) reported that AM colonization is reduced during chrysanthemum flowering because fewer metabolites are available in the roots for the fungus. Also, fruit production is a major sink for carbon, and a similar decrease of the available carbohydrates for the fungal partner (resulting in decreased colonization) can be hypothesized.

Nevertheless, the relatively low levels of AM colonization affected both the yield and quality of tomato fruits, thus confirming the systemic effect of the fungal symbiosis as shown by Zouari et al. (2014) who used RNA-Seq to perform global transcriptome profiling just on tomato fruits.

Inoculation of tomato plants with a mixed mycorrhizal inoculum and two pseudomonad strains, alone or in combination, in a real tomato farm, resulted in the following three main effects, according to the different biological treatments: (i) increase of flowering, (ii) increase of the dimension and weight of tomato fruits and (iii) improved industrial (dry biomass, pH, nitrate and citrate concentrations) and nutritional (sugars, ascorbate and lycopene) features of fruits.

1. Flowering. Flowering is driven by a complex sequence of carbon and nutrient demand in plant organs which can be affected by AM fungi (Boldt et al. 2011). The higher flower and fruit number observed in mycorrhizal plants could be ascribed to an increased concentration of photosynthates and phytohormones that is modulated by microorganisms, as reported in Torelli et al. (2000) and Boldt et al. (2011). This is in agreement with the results reported by Poulton et al. (2002), showing that AM fungi colonization can increase the fitness of host species by influencing the reproductive functions.
2. Size of tomato fruits. Fruits of plants inoculated with 19Fv1T + Myc and C7 + Myc were heavier and larger in size than those of uninoculated plants. Our results are in agreement with various reports on tomato and other plants. As reported by Nzanza et al. (2012), tomato fruits produced by mycorrhizal plants were larger than those produced by uninoculated plants. Fruits produced by olive plants colonized by *G. intraradices* (alone or in combination with *G. mosseae*) were bigger and contained more oil than those produced by

control plants (Kapulnik et al. 2010). Similarly, cucumber fruit weight was increased by inoculation with *G. mosseae* or *G. versiforme* (1.4 and 1.3 times, respectively) (Wang et al. 2008). Nevertheless, a few authors have described negative effects on production in AM plants. For example, Barber et al. (2013) report that AM fungi from both organic and conventional farms decreased flower production relative to control treatments. Moreover, Michałojć et al. (2015) describe, in tomato, no effect of AM fungi on the total and marketable yield or on the number of fruits per plant. But, in the same study, fruits of tomato inoculated with AM fungi contain significantly more sugars as compared to plants growing without mycorrhizas. Large fruits in mycorrhizal plants, as hypothesized by Nzanza et al. (2012), could be associated with the triggering of molecules or enzymes (such as auxins and sugar synthases) responsible for modulating tomato fruit cell expansion. In particular, the presence of higher sugar concentration and auxins has been suggested to promote fruit cell expansion by causing an increase in cell wall extensibility (Nzanza et al. 2012).

The role of auxin in fresh fruit ripening is well known. Positive effects of bacteria on the growth of apricot, raspberry, tomatoes, sugar beet, apple, sweet cherry and barley were ascribed to the synthesis of bacterial auxin and cytokinin and to the capability to solubilize phosphate (Esitken et al. 2010). Increased amount of auxin in cabbage following inoculation with beneficial PGPB able to synthesize this hormone was reported by Turan et al. (2014).

3. Industrial and nutritional features of tomato fruits. One of the most important parameters for the industrial production of tomatoes is the percentage of dry biomass. The dry biomass of the fruits produced by Myc plants, alone or in combination with rhizospheric bacteria, was twice of that measured in fruits of uninoculated plants (whether CFD or CRD) and in those inoculated with the bacteria. Accordingly, Kapoor et al. (2004) reported in *Foeniculum vulgare* a higher dry biomass of fruits from mycorrhizal plants. Moreover, plants colonized by AM fungi produced fruits having a lower pH value and a higher titratable acidity compared to fruits produced by CFD plants. These data are relevant for industrial practices: the European community (Reg. CE 1764/86) ruled that, in tomato products, pH must be <4.5 and titratable acidity not higher than 10 %, thus fruits with a low pH do not require additional treatments. Organic acids are widely distributed in fruits and vegetables and play a critical role in maintaining the quality of a variety of foods; therefore, they are among the parameters most frequently assessed in order to determine fruit quality. They also are used extensively as food acidulating molecules in the manufacturing of beverages, fruit and vegetable drinks or juices (Shui and Leong 2002).

Organic acids contribute to the sensory properties of foods like flavour, which is an important quality characteristic (Marconi et al. 2007). The major organic acids in tomatoes are citric and malic acids, with citric predominating (Marconi et al. 2007). From this point of view, 19Fv1T + Myc plants have the highest fruit concentration of malic while Myc has the highest citric acid concentration.

Many compounds (sugars, acids and more than two hundred volatile constituents (Hart et al. 2015) determine the flavour and fragrance of tomato fruits. Sweetness is particularly appreciated in tomatoes for industrial use and is usually related to fructose and glucose concentration, mainly accumulated into the vacuole of fruit cells (Copetta et al. 2011). Our data showed a modulation of glucose concentration mediated by *Pseudomonas* sp. 19Fv1T and *P. fluorescens* C7 combined with mycorrhizal inoculum and consequent modulation of the sweetness index. Bacterial compounds could modulate photosynthesis and sugar concentration, in particular by affecting the regulation of plant abscisic acid as discussed in Bona et al. (2015) for strawberry fruits. The abscisic acid signalling pathway is related to sugar sensing (Rolland et al. 2006). Because sugars produced during photosynthesis act as signalling compounds in plant growth and development, the control of photosynthesis could constitute an interesting target for bacterial compounds (Bailly and Weisskopf 2012).

The nitrate concentration of tomato fruits is an important industrial parameter linked with the safety of the final product. In fact, the intake of nitrate in combination with an amine-rich diet could lead to the formation of the carcinogen N-nitrosodimethylamine in humans (Chung et al. 2002). Nitrate concentration in fruits produced by plants inoculated with *P. fluorescens* C7 was half of that measured in fruits produced by uninoculated plants. This might be explained by two effects: (a) *P. fluorescens* C7 reduced the uptake of nitrate and/or (b) it entered the root tissues (endophyte) and modulated the nitrate metabolism of the roots. In fact, interestingly, Mirleau et al. (2001) demonstrated that this bacterial strain is able to reduce nitrate in the rhizosphere. The opposite effect was observed in nitrate concentration in Myc, 19Fv1T and in C7 + Myc. This effect could be linked to the modulation of nitrate absorption induced by AM fungi. This result is in agreement with those obtained by Copetta et al. (2011). Nitrate uptake in plants is complex and involves different nitrate transporters in different plant organs. Hildebrandt et al. (2002) showed that nitrate transporters (both NADH and NADPH dependent) were up-regulated by AM symbiosis in roots and leaves of tomato plants. Nevertheless, the nitrate concentration found in our samples can be considered as low-risk concentration compared with the European Committee guidelines no. 1881/2006.

Considering tomato fruit's nutritional aspects, ascorbic acid (vitamin C) and carotenoids are important antioxidants. Vitamin C concentration was negatively modulated by AM fungi in respect to CRD fruits; this result is in agreement with those obtained by Copetta et al. (2011). Ascorbic acid was higher in fruits produced by plants treated with *Pseudomonas*

sp. 19Fv1T compared to fruits of the other treatments, while the other bacterial strain (C7) induced a decrease of ascorbic acid concentration. These results are in agreement with the literature; in fact, Esitken et al. (2010) reported a decrease of vitamin C concentration in plants treated with PGPB, while a significant increase of this compound was found in the study by Bona et al. (2015) in strawberry fruits of inoculated plants grown at reduced fertilization. The increased vitamin C concentration in the fruits of 19Fv1T plants could be linked with the greater availability of sugars, which are substrates for vitamin C biosynthesis (Cruz-Rus et al. 2011), and the different regulation of the citric acid cycle induced by the symbiosis.

The amount of carotenoids did not change following plant inoculation with the microorganisms versus the CRD treatment. Overall, the concentration of these antioxidant compounds were negatively affected by fertilization reduction and the presence of both AM fungi and PGPB did not ameliorate this effect. This effect induced by fertilization is in agreement with the results obtained by Copetta et al. (2011) in tomato fruits of plants inoculated with AM fungi in the presence or absence of different amounts of compost.

In conclusion, in this work, we showed that the use of biofertilizers (AM fungi and PGPB) in an open field trial in a real industrial tomato farm is useful for sustainable agriculture for two reasons: (a) it allows the reduction of chemical fertilization and (b) it enhances the industrial and nutritional quality of tomatoes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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