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ORIGINAL PAPER

Maize development and grain quality are differentially affected by mycorrhizal fungi and a growth-promoting pseudomonad in the field

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Abstract Arbuscular mycorrhizal (AM) fungi and plant growth-promoting bacteria (PGPB) can increase the growth and yield of major crops, and improve the quality of fruits and leaves. However, little is known about their impact on seed composition. Plants were inoculated with AM fungi and/or the bacterial strain *Pseudomonas fluorescens* Pf4 and harvested after 7 months of growth in open-field conditions. Plant growth parameters were measured (biomass, length and circumference of spikes, number of grains per cob, grain yield, and grain size) and protein, lipid, and starch content in grains were determined. Plant growth and yield were increased by inoculation with the microorganisms. Moreover, spikes and grains of inoculated plants were bigger than those produced by uninoculated plants. Regarding grain composition, the bacterial strain increased grain starch content, especially the digestible components, whereas AM fungi-enhanced protein, especially zein, content. Plant inoculation with the fluorescent pseudomonad and mycorrhizal fungi resulted in additive effects on grain composition. Overall, results showed that the bacterial strain and the AM fungi promoted maize growth cultivated in field conditions and differentially affected the grain nutritional content. Consequently, targeted plant inoculation with beneficial microorganisms can lead to commodities fulfilling consumer and industrial requirements.

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Introduction

The arbuscular mycorrhizal (AM) symbiosis established with most plant species (~80 %) (Smith and Read 2008) plays a key role in nutrient cycling (Gianinazzi et al. 2010) and contributes to an overall improvement of plant fitness as reflected by an increased plant biomass and a better protection against biotic (Lingua et al. 2002; Berta et al. 2005; Veresoglou and Rillig 2012; Sampò et al. 2012) and abiotic (Augé 2001; Volante et al. 2005; Daei et al. 2009; Bona et al. 2010; Aloui et al. 2011; Lingua et al. 2012) stresses. These positive effects on plant development, resulting partly from an improved nutrient supply, can be ascribed to the complex but not fully understood interactions between the plant and the fungus (Boldt et al. 2011; Bonfante and Requena 2011; Helber et al. 2011). The positive effects of AM fungi on plant growth have been mainly demonstrated by inoculating the plant host with one AM fungus. Obviously, this is not representative of the field situation, where several species of AM fungi can establish inside the root system and affect plant fitness (Jansa et al. 2008). Although some studies indicate that inoculation with more than one AM fungal isolate may not bring more benefit to the host plant (Daft and Hogarth 1983; Edathil et al. 1996), a mixture of AM fungi with complementary functions appears to be more beneficial to the plant than a single isolate (Koide 2000; Alkan et al. 2006; Gustafson and Casper 2006).

Plant growth-promoting bacteria (PGPB) can act either indirectly by soil-borne disease suppression or directly (Glick 1995) through a range of mechanisms including improvement of mineral nutrition (mainly through phosphate solubilization,

nitrogen fixation, IAA production, and siderophore synthesis), enhancement of plant tolerance to biotic or abiotic stresses, and modification of root development (Kloepper et al. 1989; Glick 1995; Gamalero et al. 2004, 2010; Glick et al. 2007). These bacterial activities may sustain and improve both plant growth and health under a wide variety of environmental conditions, including stressed ones. Although several bacterial formulations are currently available as commercial products for agricultural production, their efficiency in open field condition strictly rely on bacterial survival, viability, and activity along the root systems (Gamalero et al. 2005). Moreover, inoculation of mycorrhizal plants with bacterial strains can facilitate root colonization by AM fungi, which can further enhance plant performance (Frey-Klett et al. 2007). The mechanism by which bacteria stimulate the growth of AM fungi is still unknown, but there is evidence that synthesis of the enzyme 1-aminocyclopropane carboxylic acid (ACC) deaminase, lowering the levels of ethylene stress, is involved (Gamalero et al. 2008).

Increases in seed size, yield, and germination rate by AM fungal inoculation have been observed in several plant species (Lewis and Koide 1990; Koide and Lu 1995; Heppell et al. 1998; Allison 2002; Poulton et al. 2002), but their effect on seed composition is essentially still unexplored. Enhancement of oil, total triterpenoids, and azadirachtin contents in neem (*Azadirachta indica*) seeds following inoculation of plants with *Glomus fasciculatum* (Venkateswarlu et al. 2007) is one of the rare examples. In addition, few works have focused on the impact of PGPB on the composition of seeds. It has been reported that inoculation of canola plants with *Azospirillum brasilense* increases both seed protein and oil content while reducing the amount of glucosinolate and erucic acid in oil (Nosheen et al. 2011). Similarly, oil and protein content in sunflower seeds can be enhanced by plant inoculation with *Azospirillum* and *Azotobacter* (Akbari et al. 2011). However, no information is available about the effects of bacterial inoculation on the seed composition of major crops.

Maize is one of the most diffused crops worldwide. Its economic and nutritional value is mainly due to the high starch content that in mature seeds can represent about 75 % of the weight. Different uses of maize biomass and kernels other than human and animal nutrition, such as the production of biofilms and biofuels, have been recently explored. Besides being considered a source of energy, maize kernels also provide significant amounts (7–10 % of the kernel dry weight) of proteins. Zeins, belonging to the prolamin fraction, are rich in proline, glutamine, and asparagine residues and represent 50 % of the kernel proteins. Generally found as disulfide-bridged dimers, these proteins are involved in nitrogen storage for the developing seed (Momany et al. 2006). Zeins have been explored for polymer application, as their molecular properties offer several potential advantages as a raw material for film, coatings, and plastic applications. Renewed interest in zein as a polymeric material has been stimulated, in part, by the perceived negative impact of plastic on solid waste disposal (Lawton 2002). Lipids account for about 4 % of maize seed weight and are accumulated only in the germ. These compounds are of interest to the food industry, as a source of vegetable oil which is rich in unsaturated fatty acids such as oleic and linoleic acids (Goffman and Bohme 2001).

The efficacy of biofertilizers should be assessed under field conditions, where survival of the introduced microorganisms, as well as their activity on the host plant, is restricted by competing with the resident microflora. The aim of the present work was to characterize the effect of a consortium of beneficial microorganisms on maize (*Zea mays*, L.) cultivated in open-field conditions and watered by drip irrigation considering especially plant growth parameters, yield, and grain quality. For this purpose, maize plants were inoculated with a mixed AM fungal inoculum and/or the strain *Pseudomonas fluorescens* Pf4 that was previously shown to promote the growth of strawberry, cucumber, tomato, and maize under glasshouse conditions when inoculated on seeds or plantlets (unpublished data) and to modify anthocyanin concentration in strawberry fruits (Lingua et al. 2013).

Materials and methods

Bacterial strain isolation and identification

The bacterial strain was isolated from a wood soil located in Sassello (Savona, Italy). Fifty grams of fresh soil were added to 450 mL of sodium pyrophosphate (0.18 %), shaken at 25 °C, 180 rpm for 2 h and left to settle for 30 min. Serial dilutions were performed in sulphate buffer and 100 mL of each suspension was distributed in triplicate on King B Agar (TSA-Fluka) added with 100 mg mL^{-1} of cycloheximide (ICN, Irvine, CA, USA). The colonies showing fluorescence under UV light were selected, purified, and stored at −80 °C in 50 % glycerol.

The genomic DNA of the strain Pf4 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 rDNA was performed by using the primers fD1 (5′-ccgaattcgtcgacaac AGAGTTTGATCCTGGCTCAG-3′) and RP2 (5′-cccgg gatccaagcttACGGCTACCTTGTTACGACTT-3′) described by Weisburg et al. (1991). The PCR reaction was carried out as follows: initial denaturation at 94 °C for 5 min; 34 cycles at 94 °C for 1 min, 60 °C for 1 s, and 72 °C for 1 min and 30 s; and 72 °C for 10 min as the final extension time. The amplified PCR product was purified with Nucleo spin Extract II kit (Macherey-Nagel, M-Medical, Cornaredo, Milan) and sent to BMR Genomix (Padua, Italy) for sequencing.

The chromatograms were assembled using Sequencing Analysis Software version 5.1 (Applied Biosystems, Milan, Italy) and transformed in a text file for further bioinformatics analysis. 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/).

Physiological characterization of the bacterial strain Pf4

Qualitative siderophore production was assayed on universal Chrome Azurol S (CAS) agar (Schwyn and Neilands 1987). The bacterial strain was inoculated at the center of the plate and incubated at 28 °C for 3 days. Siderophore production was indicated by a color change from blue to orange on the CAS medium and measured as the ratio between the diameter of the halo (HD) and that of the colony (CD).

ACC deaminase activity was measured following the method described by Penrose and Glick (2003) with a standard curve of α-ketobutyrate between 0.05 and 0.5 μmol.

IAA was quantified according to Forni et al. (1992). The bacterial strain was inoculated in 20 mL of M9 salt minimal medium added to sucrose at 10 mM and L-tryptophan (400 mg mL^{-1}; Fluka) and incubated on a rotary shaker at 150 rpm in the dark at 28 °C for 4 days. The bacterial suspension was centrifuged at 4,500 rpm and 4 °C for 20 min. Two milliliters of the Salkowsky's reagent (Salkowski 1885) was added to 1 mL of the supernatant. After 30 min of incubation, the amount of IAA produced was evaluated at λ530 nm using an IAA solution (100 mg mL^{-1}) as standard. Acid phosphomonoesterase activity was measured as described by Eivazi and Tabatabai (1977) and modified by Gamalero et al. (2003) by measuring the amount of *p*-nitrophenol released by the substrate *p*-nitrophenylphosphate. Neutral and alkaline phosphomonoesterase activity was evaluated according Alef and Nannipieri (1995) modified by Gamalero et al. (2003) through the quantification of the phenol released by the enzymatic hydrolysis of the substrate disodium phenyl phosphate at pH 7 and 10, respectively.

AM fungal inoculum

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

Experimental field and plant treatment

The experiment was performed from March to September in open-field condition 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 %). Field soil was fertilized with potassium sulfate (400 kg/ha) and 18/46 N/P (350 kg/ha). Corn seeds (*Z. mays* var. Ostiglia) were sown on 14th March following double rows (about 35-cm-wide rows and 80-cm-wide double rows) with space of 25 cm between plants. Drip irrigation was carried out with a single plastic tube between two rows of the double rows. Three double lines (200 plants each) were treated with the bacterial strain (B), the mixed AM fungal inoculum (M), or the bacterial strain and the AM fungal inoculum (BM). An uninoculated double row was randomly selected as control (C). AM fungal inoculum was applied at sowing 6 cm under the drip irrigation tube as a soil-based inoculum (20 L/line and 120 mL/plant). To assess the hypothesis that *P. fluorescens* Pf4 can support the growth and modify the grain quality of older and more developed plants, bacterial cells were inoculated 2 and 3 months after sowing. Bacterial cells were produced on King's B agar (Fluka) plates at 28 °C for 36 h. Bacteria were scraped from the medium and suspended in 0.1 M $MgSO_4$, pelletted by centrifugation (4,500×*g*, 20 min), washed twice, and suspended in $MgSO_4$. The bacterial density of the suspension was determined by turbidity (λ=600 nm), adjusted to 10^{10} cfu mL^{-1} (1.5 L) and diluted with 300 L of water used for drip irrigation.

Sampling and plant growth

Flowering and kernel maturation was monitored after bacteria inoculation. Plants with only one kernel were harvested 4 September. The following parameters were determined on 15 plants per treatment: average shoot and internode length, shoot diameter, leaf number, fresh shoot, and root dry weight. Shoot dry matter was determined after grinding and drying at 70 °C for 7 days. Five root systems per treatment were randomly selected to evaluate the level of AM fungal colonization. The proportion (frequency) of root length colonized (*F*%), the intensity of AM fungal colonization in a root system (*M*%), and the abundance of arbuscules (*A*%), were determined according to Trouvelot et al. (1986).

Spike and grain parameters

Length and circumference of ten spikes per treatment and their fresh weight were recorded. All the grains were detached from the corncob, dried at 60 °C for 7 days, and dry weight of the grains and the corncob were measured. The number of grains and lines per cob, and the total and mean grain yield, were calculated. Moreover, 20 grains from the middle zone of each corncob sample were randomly selected and measured for length, width, and thickness.

Seed composition analysis

All reagent grade chemicals were from Sigma-Aldrich (Milano, Italy), if not otherwise indicated. Maize seeds were ground with a coffee grinder and the flour was sieved through a 60 μ mesh metal sieve. Fragments greater than 60 mesh were discarded. Moisture content was determined by heating one gram of sieved flour in a glass beaker using in an electric oven at 110 °C, until a constant weight was reached. Before each measurement, the samples were cooled to room temperature in a sealed jar containing silica gel as desiccant. Samples were analyzed in triplicate.

For lipid determination, 3 g of flour were extracted with 150 mL of *n*-pentane, at 37 °C for 8 h, using a Soxhlet apparatus. The defatted flour was then kept in an oven at 110 °C until a constant weight was reached. Samples were analyzed in triplicate. One gram of defatted flour was extracted with 20 mL of 50 mM sodium phosphate buffer, pH 7.5, containing 0.5 M NaCl. After stirring for 3 h at 4 °C, the suspension was centrifuged at 10,000×*g* for 30 min at 4 °C. The pellet was extracted again with the same buffer and the two supernatants pooled. This solution contained the soluble protein fraction. The insoluble pellet was extracted twice with 70 % ethanol containing 0.2 % 2-mercaptoethanol. After stirring for 3 h at 4 °C, the suspension was centrifuged at 10,000×*g* for 30 min at 4 °C. The pooled supernatants contained the prolamin fraction (zeins). Samples were analyzed in quadruplicate. All protein concentrations were determined according to Bradford (1976), using BSA as standard protein. Resistant, soluble, and total starches content was determined with a Megazyme (Wicklow, Ireland) assay kit (catalog number: K-RSTAR) according to the manufacturer's instructions.

Iron and zinc content

Maize seeds were ground finer than 60 μ mesh. Aliquots of about 0.2 g of flour samples were digested by a microwave digestor system (Anton Paar Multiwave-Eco) in Teflon tubes filled with 10 mL of 65 % HNO_3 by applying a two-step power ramp (step 1: 200 W in 10 min, maintained for 5 min; step 2: 650 W in 10 min, maintained for 15 min). After 20 min cooling time, the mineralized samples were diluted 1:40 with Milli-Q water and the concentration of Fe and Zn (as ^{57}Fe and ^{66}Zn) was measured by inductively coupled plasma (ICP) mass spectroscopy (Bruker AURORA M90 ICP-MS). An aliquot of an internal standard solution (^{45}Sc, ^{89}Y, and ^{159}Tb) was added both to samples and calibration standards to a final concentration of 20 μg/L. Typical analysis interferences were removed using collision–reaction–interface with an H_2 flow of 65 mL/min through the skimmer cone.

Statistical analysis

Data were statistically analyzed by one-way ANOVA using Statview version 5.0 statistics package (SAS Institute Inc, Cary NC, 27513) and expressed as mean value±standard error. The significance of the data was evaluated by Fisher's probable least-squares difference test with cut-off significance at $P=0.05$ based on the comparison among all the treatments. Data on mycorrhizal colonization, expressed as percentage, were submitted to arcsin transformation before the analysis.

Results

Mycorrhizal root colonization

Maize plant roots were naturally colonized by autochthonous AM fungi. However, the frequency and the intensity of the mycorrhizal colonization, as well as arbuscule abundance, were significantly higher in plants treated with the AM fungal inoculum than in control plants. Inoculation of maize plants with the bacterial strain did not affect the root colonization by natural or introduced AM fungi (Table 1).

Bacterial strain identification and characterization of plant beneficial physiological traits

The strain Pf4 was a Gram-negative, rod-shaped bacterium producing a fluorescent yellow-green pigment when irradiated with UV light on King's B agar. It was cytochrome oxidase positive and able to oxidize, but not to ferment, glucose. The results of the nearest neighbors search against all bacterial 16S rDNA reference sequences available at the NCBI World-Wide Web database showed that the strain belongs to the *P. fluorescens* species (max identity 100 %). *P. fluorescens* Pf4 synthesizes siderophores, is able to solubilized phosphates at neutral, acid, and alkaline pH and produce the phytohormone IAA (Table 2). The strain does not produce ACC deaminase.

Table 1 Frequency of mycorrhiza (*F*%), AM colonization intensity (*M*%), and arbuscule abundance (*A*%) in the root systems of corn plants inoculated (*Pseudomonas fluorescens* Pf4 (B), AM inoculums (M), and *P. fluorescens* Pf4 and AM inoculums (BM)) or not (C, control) with beneficial microorganisms and cultivated under field conditions

	F%	*M*%	*A*%
C	55.3±6.4 a	6.9±0.9 a	2.5±0.9 a
B	56.0±7.1 a	9.9±1.2 a	5.8±1.3 a
M	98.0±4.5 b	27.7±4.6 b	17.2±4.0 b
BM	94.7±2.7 b	26.4±1.9 b	15.7±2.0 b

Values (mean±standard error) in each column designated with the same letters are not significantly different ($P \leq 0.05$) according to Fisher's least significant test

Table 2 Analysis of functional traits of *Pseudomonas fluorescens* Pf4

Physiological traits	Qualitative assay (enzymatic activities±SE)
Siderophore synthesis (cm, halo diameter/colony diameter)	3.8±0.2
ACC deaminase (nmol α-ketobutyrrate mg^{-1} protein h^{-1})	0.0±0.0
IAA (μg IAA mL^{-1} h^{-1})	103.2±1.9
Acid phosphomonoesterase (μg *p*-nitrophenol mL^{-1} h^{-1})	1,005.1±4.6
Neutral phosphomonoesterase (μg phenol mL^{-1} h^{-1})	2,746.7±1.6
Alkaline phosphomonoesterase (μg phenol mL^{-1} h^{-1})	3,982.2±2.8

Values are the mean of five replicates±standard error

Plant growth

The effects of AM fungal inoculum and *P. fluorescens* Pf4 on plant growth were evaluated by a number of morphometric and ponderal parameters. Shoot dry weight was higher in plants treated with bacteria and/or AM fungi. The shoot lengths of AM plants were lower (−12.1 %, $P<0.0001$), with shorter distance between internodes (−15.5 %, $P<0.0001$) and larger biomass (+16.6 %, $P<0.0001$) than those of the uninoculated plants. *P. fluorescens* Pf4, alone or combined with AM fungi, increased the number of leaves. The main effect of the bacterial strain was to enhance root development (Fig. 1); dry weight of root systems of plants inoculated with the strain Pf4 was increased by 68 % compared with uninoculated plants ($P=0.0124$) (Table 3).

Spike and grain parameters

Spike dry weight was increased by inoculation with the bacterial strain and/or the AM fungi (+34.5 % B plants, $P=0.0053$; +51.2 %, M plants, $P<0.0001$; and +53.7 %, BM plants, $P<0.0001$). In addition, spikes produced by maize plants treated with the AM inoculum were longer (+18 %, $P<0.0001$) and larger (+6.2 %, $P=0.0027$), compared with those produced by controls (Table 4; Fig. 2). The number (+24.5 %, B plants, $P=0.0043$; +31.2 %, M plants, $P=0.0004$; +27.6 % BM plants, $P=0.0015$) and the dry weight (+36.1 %, B plants, $P=0.0042$; +52.8 M plants, $P<0.0001$; +54.4 %, BM plants, $P<0.0001$) of grains per spike were higher in maize plants inoculated with the beneficial microorganisms compared with those produced by uninoculated plants. Inoculation with the microorganisms affected the size of the grains. Grains produced by maize plants inoculated with the bacterial strain or the AM fungi were longer ($P=0.0322$ and 0.0014, respectively) and larger ($P=0.0415$ and 0.0420, respectively) than those produced by uninoculated plants, whereas grains from plants inoculated with both *P. fluorescens* Pf4 and AM fungi were longer ($P=0.0057$) and thicker ($P=0.0188$) than those produced by control plants (Table 4; Fig. 3). Cobs and grains shown in Figs. 2 and 3 were representative of the real situation.

Seed composition

The microorganisms inoculated onto maize plants affected the accumulation of kernel components relevant to nutritional issues. The results are summarized in Table 5. Plant inoculation with the bacterial strain influenced the total starch content in grains ($P<0.0001$). The amount of digestible starch, compared with untreated control plants, significantly increased ($P<0.0001$), whereas that of resistant starch slightly decreased ($P=0.0031$). The total protein content was reduced ($P<0.0001$) by the bacterial strain both for the soluble ($P<0.0001$) and the ethanol-soluble ones (zeins; $P=0.0226$) protein fractions. By contrast, the treatment with AM fungi increased only the protein content of kernels ($P<0.0001$) and reduced the amount of digestible starch ($P<0.0001$).

Fig. 1 Root systems of maize plants cultivated under field conditions: non-inoculated (*C*), inoculated with *P. fluorescens* Pf4 (*B*), inoculated with a mixed mycorrhizal inoculum (*M*), and treated with the bacterial strain and the AM inoculum (*BM*)

Table 3 Growth parameters of corn plants inoculated or not (control (C)) with beneficial microorganisms (*Pseudomonas fluorescens* Pf4 (B), AM inoculums (M), and *P. fluorescens* Pf4 and AM inoculums (BM)) and cultivated under field conditions

	C	B	M	BM
Shoot dry weight (g)	334.5±27.3 a	400.9±22.7 b	398.8±13.9 b	407.4±19.7 b
Shoot length (cm)	251.3±3.0 a, b	254.4±2.0 b	220.9±4.7 c	242.6±3.0 a
Internode distance (cm)	20.0±0.4 a	18.9±0.3 b	16.9±0.3 c	18.3±0.3 b
Shoot diameter (cm)	24.7 ±1.0 a	28.8±0.5 b	29.9±0.7 b	28.4 ±0.5 b
Leaf number	12.6±0.3 a	13.5±0.2 b	13.1±0.3 a, b	13.3±0.3 b
Root dry weight (g)	68.0±5.9 a	114.7±16.4 b	58.0±8.7 a	88.4±13.0 a, b

Values (mean±standard error) in each row designated with the same letters are not significantly different ($P \leq 0.05$) according to Fisher's least significant test

Interestingly, in this case a rebalancing of the relative amount of protein fractions was evident. Indeed, the concentration of zeins was boosted ($P < 0.0001$), whereas that of the water-soluble protein fraction decreased ($P < 0.0001$). As far as lipid content is concerned, no significant variation were observed among the treatments. Inoculation of the maize plants with the AM fungi and the bacterial strain did not lead to synergistic, but to additive, effects on seed composition compared with plants inoculated with one of the two microorganisms. However, the resistant starch content and the amount of water-soluble proteins in grains produced by plants inoculated with AM fungi and the strain Pf4 was similar to that of grains produced by plants treated with the bacterial strain alone ($P = 0.0720$ and 0.2779, respectively).

Plant inoculation with AM fungi and the strain Pf4 increased the amount of Fe and Zn, two nutritionally relevant metal elements, accumulating in the kernels. The results of ICP-MS determinations are reported in Table 5. Plant treatment with the fluorescent pseudomonad and the AM fungal inoculum led to significant differences from the control (Pf4, $P = 0.0231$ and $P = 0.0169$ for Fe and Zn, respectively; AM, $P = 0.0380$ and $P = 0.0125$ for Fe and Zn, respectively). As far as Fe was concerned, about a 37 % increase was observed following bacterial inoculation and about a 34 % increase in the AM fungal treatment. Zn accumulated equally with both kinds of inoculation (about +42 % compared with control). Co-inoculation of the microorganisms appeared to have no mutual benefit as the amounts of the deposited metals were similar to that observed following a single inoculation. No significant differences ($P < 0.05$) were observed among single microorganism treatments (BM vs. B, $P = 0.1087$ and BM vs. M, $P < 0.9639$, for Fe; BM vs. B, $P = 0.1051$ and BM vs. M, $P = 0.0830$, for Zn).

Discussion

Data from the presented work clearly show the effects induced by *P. fluorescens* Pf4, alone or in combination with a mixed AM fungal inoculum, on the growth of maize plants in open field conditions. In addition, the amount of protein, starch, and lipids of the kernels are affected. These storage compounds are relevant when considering the possible use of maize as food, feed for livestock or raw material for industry. *P. fluorescens* Pf4 has been found to increase growth of the reed *Phragmites australis,* of tomato and maize plantlets, and the number of fruits in strawberry cultivated under greenhouse conditions (unpublished data). Physiological traits such as the synthesis of phytohormones and iron chelators, as well as the capability to solubilize phosphate, may be involved in the plant growth

Table 4 Morphometric parameters of the spike and the grains produced by maize plants inoculated or not (control (C)) with beneficial microorganisms (*Pseudomonas fluorescens* Pf4 (B), AM inoculums (M), and *P. fluorescens* Pf4 and AM inoculums (BM)) and cultivated under field conditions

	C	B	M	BM
Spike dry weight (g)	141.5±12.5 a	190.3±10.9 b	214.0±10.9 b	217.5±11.6 b
Spike length (cm)	15.0±0.5 a	16.2±0.4 a	18.3±0.5 b	18.0±0.5 b
Spike circumference (cm)	16.6±0.3 a	17.2±0.2 a, b	17.7±0.2 b	17.4±0.2 b
Total grain number per spike	497±44 a	619±22 b	652±19 b	634±23 b
Grain dry weight per spike (g)	119.8±10.8 a	163.1±9.4 b	183.1±9.5 b	185.0±9.7 b
Mean grain length (mm)	11.6±0.2 a	12.4±0.3 b	12.8±0.2 b	12.6±0.2 b
Mean grain width (mm)	7.8±0.2 a	7.4±0.2 b	8.2±0.1 c	7.7±0.1 a, b
Mean grain thickness (mm)	3.3±0.1 a	3.3±0.1 a	3.4±0.1 a	3.6±0.1 b

Values (mean±standard error) in each row designated with the same letters are not significantly different ($P \leq 0.05$) according to Fisher's least significant test

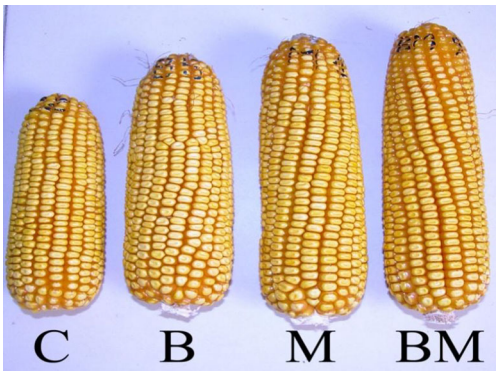

Fig. 2 Cobs of produced by maize plants cultivated under field conditions: non-inoculated (*C*), inoculated with *P. fluorescens* Pf4 (*B*), inoculated with a mixed mycorrhizal inoculum (*M*), and treated with the bacterial strain and the AM inoculum (*BM*)

promotion activity. However, the plant beneficial effects observed under controlled conditions are often not repeatable under open field conditions where stressful environmental factors and the autochthonous microflora, can hamper the efficacy of the introduced bacterial strain (Dutta and Podile 2010).

Under field conditions, both *P. fluorescens* Pf4 and the AM fungal inoculum increased maize plant biomass. This growth enhancement was evident for both shoots and roots of plants inoculated with *P. fluorescens* Pf4, but only for shoots of plants treated with the AM fungal inoculum. In particular, the shoots of plants inoculated with AM fungi were shorter and thicker; these are desirable traits leading to improved plant stability and increased resistance to wind and stem breaking. Co-inoculation of *P. fluorescens* Pf4 and the AM fungal inoculum did not however have synergistic effects on plant growth compared with single inoculated plants. This is consistent with the lack of positive effects induced by the bacterial strain on the establishment of the AM symbiosis involving either autochtonous or inoculated AM fungi. Moreover, the bacterial strain did not show ACC deaminase activity that is involved in facilitating AM colonization of plant roots (Gamalero et al. 2008). Besides

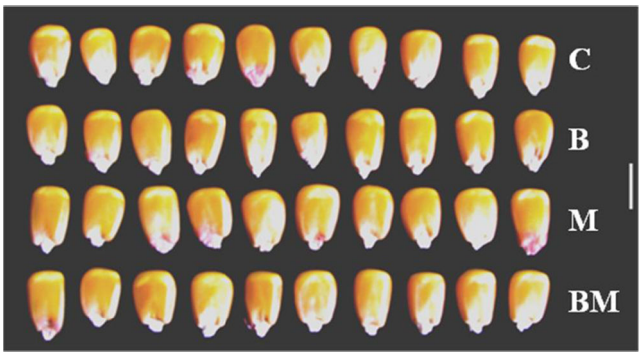

Fig. 3 Grains produced by maize plants cultivated under field conditions: non-inoculated (*C*), inoculated with *P. fluorescens* Pf4 (*B*), inoculated with a mixed mycorrhizal inoculum (*M*), and treated with the bacterial strain and the AM inoculum (*BM*)

Table 5 Maize seed composition expressed as g/100 g of grain

	C	B	M	BM
Starch	68.7±0.3 a	74.3±0.2 b	66.1±0.3 c	73.5±0.1 d
Resistant starch	1.4±0.0 a, b	1.2±0.0 c	1.5±0.0 a	1.3±0.1 b, c
Digestible starch	67.3±0.3 a	73.1±0.2 b	64.6±0.3 c	72.1±0.1 d
Lipids	4.7±0.3 a	4.1±0.3 a	4.9±0.3 a	4.4±0.3 a
Proteins	9.5±0.0 a	9.2±0.0 b	10.1±0.0 c	10.0±0.0 c
Water-soluble proteins	5.9±0.0 a	5.5±0.0 b	5.2±0.0 c	5.5±0.0 b
Prolamins (Zeins)	3.7±0.0 a	3.6±0.0 b	4.8±0.0 c	4.5±0.0 d
Moisture	8.0±0.1 a	7.9±0.1 a	8.8±0.2 b	8.0±0.1 a
Iron	26.3±1.5 a	36.9±1.7 b	33.9±1.5 b	34.0±1.4 b
Zinc	33.5±1.1 a	42.8±1.3 b	42.6±0.9 b	39.5±1.0 b

Iron and zinc are expressed as micrograms per gram of grain. Values (mean±standard error) in each row indicated with the same letters are not significantly different ($P \leq 0.05$) according to Fisher's least significant test

C control, *B Pseudomonas fluorescens* Pf4, *M* AM inoculums, *BM P. fluorescens* Pf4 and AM inoculum

improving plant growth, both the bacterial strain and the AM inoculum increased the biomass of the spikes. Interestingly, only the spikes harvested by plants treated with AM fungi, being longer and larger than those from untreated plants, showed a different size compared with control. Moreover, the abundance and the biomass of the grain per spike were enhanced by beneficial microorganisms inoculated alone or in combination. This is consistent with results obtained in other works performed on maize plants inoculated with AM fungi (Subramanian et al. 1997; Subramanian et al. 2008) and PGPB (Javed et al. 1998; Zahir et al. 1998). Similarly to the spikes, also the grains from plants inoculated with AM fungi or/and the strain Pf4 were longer and larger than those collected by untreated control plants. While AM colonization can increase seed size in addition to seed yield (Lu and Koide 1991; Koide and Lu 1992), a recent work indicated that, because of a compensating mechanism, the increase of the seed size in canola induced by *A. brasilense* is associated with a decrease of the seed number (Nosheen et al. 2011).

AM colonization can affect the amount and the quality of sugars, organic acids, and secondary metabolites in fruits, such as tomato (Ordookhani et al. 2010; Copetta et al. 2011; Giovannetti et al. 2012; Nzanza et al. 2012), papaya (Vázquez-Hernandez 2011), and chile ancho (Mena-Violante et al. 2006). Similarly, plant beneficial bacteria increased the content of vitamin C in strawberry (Ertuk et al. 2012), improve fruit quality in sweet cherry (Akca and Sezai 2010) and modulates the antioxydant content in functional foods (Nautiyal et al. 2008). However, very few works deal with the modification of the seeds induced by beneficial soil microorganisms (Venkateswarlu et al. 2007; Nosheen et al. 2011; Kumar et al. 2011; Subramanian et al. 2013).

Our results showed that the microorganisms inoculated on the root differentially affected the seed composition. The main effect of *P. fluorescens* Pf4 on seed composition was the increase of the starch content, especially of the digestible fraction. On the opposite, the concentration of proteins, both the water soluble and the zeins, was reduced by the bacterial strain. This finding is of importance, since the main maize allergens belong to the water soluble protein group, being for example chitinase an albumin and lipid transfer protein a globulin (Pastorello et al. 2009). Conversely, the main effect of the AM fungi was the increase of the proteins, especially of the zeins, associated to the reduction of digestible starch content. These data on maize seed protein increase are supported by new evidence on enhanced nitrogen nutrition in AM plants (Smith and Read 2008) and in agreement with the results obtained by Kumar et al. (2011) who showed increased protein content in wheat grains of AMF inoculated plants. Moreover, protein contents are highly correlated with P nutrition, generally ameliorated in mycorrhizal plants (Smith and Smith 2011), but even more improved when different fungi are involved: it has recently been shown that different AMF species show functional diversity in terms of growth, P uptake and P transporter gene expression in maize, and that co-inoculation of these fungi results in the highest expression level of a P transporter (Tian et al. 2013). Nutritional quality assessment of maize grains included the determination of Fe and Zn concentration. Inoculation with AM fungi and the bacterial strain increased the amount of Fe and Zn, two nutritionally relevant metal elements, accumulated in the kernels. Mycorrhizal symbiosis has been already shown to influence positively the accumulation of these two minerals that are relevant, for example, to livestock and poultry industry (Subramanian et al. 2008; Balakrishan and Subramanian 2013). Our results prove that the bacterial inoculation treatment also enhanced the storage of Fe and Zn. In addition, mycorrhizal plants appeared to be more effective in accumulating Zn, rather than Fe. Interestingly, the effects of co-inoculation are not additional. Moreover, hormonal responses are triggered by AM plants (Ludwig-Muller 2010). It has been demonstrated that regulatory networks of cellular differentiation and function in barley grains is coordinated by hormones such as gibberellins (Thiel et al. 2008). Barea and Azcon-Aguilar showed long time ago (Barea and Azcon-Aguilar 1982) that the AM fungus *Glomus mosseae* is able to produce gibberellins. More recently, Song et al. (2012) have shown that AMF dramatically increase the content of gibberellins in *Amorpha fruticosa*. Anyway, the physiological traits at the base of the effects induced by AMF on maize seed quality are still unclear. Interestingly, an additive effect, though not synergistic, was induced by co-inoculation of both bacterial and AM inoculum on grain composition.

From an applicative point of view, it is worth noting that targeted inoculation interventions may lead to the production of commodities which best fit the requirements of specific industrial uses, in the context of a sustainable agriculture.

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