Research Article

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Co-inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops

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Abstract

BACKGROUND: The application of beneficial microorganisms at transplanting can promote rapid transplant establishment (starter effect) for achieving early and high yields. The aim of this study was to evaluate the biostimulant effects of *Glomus intraradices* BEG72 (G) and *Trichoderma atroviride* MUCL 45632 (T) alone or in combination on plant growth parameters, yield, chlorophyll index (SPAD), chlorophyll fluorescence and mineral composition of several vegetable crops.

RESULTS: The *T. atroviride* strain was capable of producing siderophores and auxin-like compounds under a wide range of substrate pH conditions (5.5–8.0). The highest shoot, root dry weight, SPAD and chlorophyll fluorescence in lettuce, tomato and zucchini was observed in the G + T combination, followed by a single inoculation of G or T, whereas the lowest values were recorded in the uninoculated plants. Under greenhouse conditions, the shoot dry weight was significantly increased by 167%, 56%, 115%, 68% and 58% in lettuce, melon, pepper, tomato and zucchini, respectively, when supplied with both beneficial microorganisms in comparison with the control. This increase in root and shoot weight was associated with an increased level of nutrient uptake (e.g. P, Mg, Fe, Zn and B). Under open field conditions, the lettuce shoot and root dry weight increased by 61% and 57%, respectively, with biostimulant microorganism application in field conditions. For zucchini, early and total yields were significantly increased by 59% and 15%, respectively, when plants were inoculated with both microorganisms.

CONCLUSION: The application of the biostimulant tablet containing both G and T can promote transplant establishment and vegetable crop productivity in a sustainable way.

Keywords: arbuscular mycorrhiza fungi; inoculation; mineral composition; SPAD index; *Trichoderma atroviride*; vegetables

INTRODUCTION

The environmental impact of cropping systems, low cost of production and, as a consequence, the necessity of reducing soil chemical applications have become important objectives in agriculture systems that were previously dominated by productivity.[1] To avoid nutrient pollution and to preserve the economic margin, farmers must optimize the application of fertilizer,[2] which has been a powerful tool for increasing yields in the last three decades. This is especially true for vegetable cropping systems where fertilizer application has often been associated with an increase in environmental pollution due to the limited nutrient uptake by the shallow root apparatus and adverse soil conditions.[3] A wide spectrum of compounds and microorganisms have been considered capable of enhancing plant nutrient uptake and crop growth, especially under adverse soil conditions. These are referred to as metabolic enhancers and biostimulants.[4]

As defined by the European Biostimulant Industry Council, plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress and crop quality.[5]

Among the beneficial microorganisms, arbuscular mycorrhizal (AM) fungi are the most widespread root fungal symbionts and are associated with the vast majority of higher plants.[6] Artificial plant

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inoculation with appropriate formulation of AM fungi improves plant adsorption of water and nutrients, with a reduction in the use of chemical fertilizers and pesticides, resulting in higher crop sustainability.[7] The association of AM fungi with plant roots alters plant–soil interactions and enhances plant growth under stressful edaphic conditions.[8] AM fungi have been shown to enhance plant nutrient acquisition (P, N, K),[9] overcome the detrimental effects of salinity[10] and alkalinity,[11] improve drought tolerance[12] through greater effective root area and penetration of the substrate, and activate and excrete various enzymes of infected AM fungi roots and/or hyphae.[8]

Trichoderma is a genus of saprotrophic fungi that have also been reported to promote plant growth, in addition to their biocontrol activities, acting by either the production of antimicrobial compounds or the parasitism of fungal plant pathogens.[13] Moreover, *Trichoderma* spp. have been found capable of improving the solubility of soil micronutrients such as Zn, Cu, Fe and Mn.[14] *Trichoderma* spp. can also produce metabolites with hormone activities like indole-3-acetic acid or auxin analogues.[15]

The combined use of AM fungi and *Trichoderma* spp. has been reported in several studies, but with contrasting results. Several studies have demonstrated a positive effect of the dual inoculation on plant performance in the presence and absence of plant pathogens,[16] whereas others reported a reduction in plant shoot and root dry weights.[17,18] The above findings are related to the antagonistic non-target action of several *Trichoderma* spp. via mycoparasitism on the AM fungal mycelium.[19] For example, De Jaeger *et al.*[20] reported a reduction in P acquisition in mycorrhizal plants because of the disruption of the external and internal hyphae continuity of *Glomus intraradices* by *T. harzianum*. Therefore, the compatibility of AM fungi with *Trichoderma* spp. should first be evaluated to guarantee the successful application of the inoculum. The application of beneficial microorganisms is particularly useful in transplanting when it is important to promote rapid transplant establishment (starter effect) for achieving early and high yields. Moreover, the benefits of microorganism application are maximized in fumigated soils under greenhouse conditions due to the destruction of desirable soil microflora.

We hypothesize that the combined application of AM fungi *G. intraradices* BEG72 and *T. atroviride* MUCL 45632 at transplanting could enhance the performance of several vegetable crops such as lettuce, melon, pepper, tomato and zucchini squash that are widely grown under greenhouse and open field conditions. *Glomus intraradices* BEG72 was selected because of its ability to improve the growth and yield of different vegetable crops, mainly due to a better uptake of P, N, K and several micronutrients.[13–15] *Trichoderma atroviride* MUCL 45632 was chosen because of its compatibility with *G. intraradices* BEG72 in preliminary experiments (data not shown). Based on the above consideration, *in vitro* laboratory trials (Experiment 1) were conducted to verify the capability of *T. atroviride* MUCL 45632 to promote plant growth through production of indole-3-acetic acid and siderophores under different growing conditions. Moreover, three experiments were conducted in the following order to evaluate the effects of *G. intraradices* BEG72 and *T. atroviride* MUCL 45632 (alone or in combination) on: (i) growth, SPAD index and chlorophyll fluorescence of lettuce, tomato and zucchini grown at an early stage in a growth chamber; (ii) plant growth parameters, SPAD index and mineral composition of lettuce, melon, pepper, tomato and zucchini transplants grown under greenhouse conditions; (iii) crop performance (growth, yield and quality), physiological parameters (SPAD index and chlorophyll fluorescence) and mineral composition of lettuce, and zucchini under open field conditions.

MATERIALS AND METHODS

Experiment 1

Identification of Trichoderma plant growth promoting traits

Trichoderma atroviride strain MUCL45632, kindly provided by Itapollina SpA (Rivoli Veronese, Italy), was maintained on potato dextrose agar (PDA; Oxoid, Cambridge, UK) slants at 4 °C. The fungal strain was evaluated *in vitro* for various plant growth-promoting phenotypic traits such as indole acetic acid (IAA) and/or analogue detection and siderophore production.

The production of IAA and IAA analogues by *T. atroviride* strain MUCL 45632 was determined spectrophotometrically as previously reported. [21] Liquid cultures were grown in 100 mL potato dextrose broth medium (PDB), buffered at pH 5.5, 6.7 and 8.0, and were supplemented or not with 0.250 μg mL^{-1} of L-tryptophan (Sigma-Aldrich Srl, Milan, Italy) as an IAA precursor. Moreover, other cultures of *T. atroviride* MUCL 45632 were grown in 100 mL half-strength tryptic soy broth (TSB; Sigma-Aldrich), with or without L-tryptophan (2.5 mmol L^{-1}). For *Trichoderma* spp., each flask (500 mL capacity) was inoculated with two plugs collected from the margin of fungal actively growing culture in PDA. After 7 days of incubation in a rotary shaker (140 rev. min^{-1} at 27 °C), the mycelium and conidia were removed from 10 mL of culture by centrifugation and filtration on cellulose acetate sterile filter units (0.22 μm Millipore, Cork, Ireland).

IAA production was determined in 1 mL filtered culture that was added to 1 mL Salkowski reagent.[21] The mixture was incubated at room temperature for 25 min and the absorbance was measured at 530 nm. The concentration of auxin-like compounds was determined by comparison with a standard curve specifically prepared with IAA (Sigma-Aldrich). The determination of IAA was assessed from four independent cultures in each treatment.

Siderophore production was assessed according to Milagres *et al.*[22] Modified chrome azurol S (CAS) methods were applied to overcome the high toxicity of the CAS-blue agar medium caused by the detergent addition. Petri dishes (10 cm in diameter) were prepared with potato dextrose agar (PDA) and the medium, after becoming solid, was cut into halves, one of which was replaced by CAS-blue agar. The plates were inoculated individually on the PDA side with a fungal mycelial plug (5 mm) of active culture and then incubated at 27 °C for 10 days. Siderophore production rate was determined daily by measuring the advance of the colour change front on the side containing the CAS-blue indicator. Uninoculated control plates of CAS agar were also assessed as described above and no colour change was observed. The determination of the CAS reaction rate was determined from ten replicates.

Experiment 2

Plant material, growth conditions and experimental design

The second experiment was conducted in a growth chamber at Tuscia University, Central Italy. The growth chamber was programmed to maintain a 12 h photoperiod with corresponding 23 °C light/18 °C night and 65% relative humidity. The average photosynthetic photon flux at the canopy level was 450 μmol m^{-2} s^{-1}. Seeds of lettuce (*Lactuca sativa* L. cv. Bionda Degli Ortolani), tomato (*Solanum lycopersicum* L. cv. San Marzano) and

zucchini squash (*Cucurbita pepo* L. cv. Grezini) were surface sterilized with a solution containing 80% ethanol. Seeds were sold by SAIS seed company (Cesena, Italy). After sterilization (10 min), the seeds were washed twice with sterile distilled water. Immediately after sterilization, seeds were sown in Petri dishes (10 cm diameter) on 10 April 2013. Seedlings at the cotyledonary stage were transplanted to pots filled with 98 cm^3 quartziferous sand. Treatments were arranged in a randomized block design with four replicates. Each experimental unit consisted of ten plants. The experiments included the following treatments: 1.5 g organic matter (OM), 250 spores of *G. intraradices* BEG72 mixed with 1.5 g organic matter (OM + G), 1×10^7 colony-forming units (CFU) of *T. atroviride* MUCL 45632 mixed with 1.5 g organic matter (OM + T), 250 spores of *G. intraradices* BEG72 plus 1×10^7 CFU *T. atroviride* MUCL 45632 mixed with 1.5 g organic matter (OM + G + T), and non-treated or control treatment. The *G. intraradices* BEG72 was kindly provided by Itapollina SpA (Rivoli Veronese, Italy). Spore production of *G. intraradices* was made on leek plants (*Allium porrum* L.) grown under greenhouse conditions in benches filled with sterile vermiculite as described by Calvet *et al.*[23] Conidia production of *T. atroviride* was carried out in a solid-state fermentation bioreactor filled with a sterile wheat bran-based substrate. Spore production process of both *G. intraradices* and *T. atroviride* was carried out at Italpollina Co. The organic matter (41% organic carbon) was mainly composed of cellulosic residues of cereal crops used for mass production of *T. atroviride*. It was ground (particle size < 3 mm), dried in a ventilated oven at 80 °C and sterilized in an autoclave at 120 °C for 20 min before use. Nutrient solution was supplied to all treatments.

Nutrient solution management

The basic nutrient solution was a modified Hoagland and Arnon formulation. All chemicals used were analytical grade, and the composition of the nutrient solution was: 7.0 mmol L^{-1} $N-NO_3^-$, 1.5 mmol L^{-1} S, 0.2 mmol L^{-1} P, 2.7 mmol L^{-1} K, 5.5 mmol L^{-1} Ca, 1.5 mmol L^{-1} Mg, 20 μmol L^{-1} Fe, 9 μmol L^{-1} Mn, 0.3 μmol L^{-1} Cu, 1.6 μmol L^{-1} Zn, 20 μmol L^{-1} B and 0.3 μmol L^{-1} Mo. The nutrient solution was prepared using demineralized water. Electrical conductivity (EC) and the pH of the nutrient solution were 1.8 dS m^{-1} and 6.0, respectively. Plants were manually fertirrigated once a day.

Data collection and analysis

At the end of the second experiment (21 days after transplanting), a chlorophyll meter (SPAD-502; Minolta Corp. Ltd, Osaka, Japan) was used to take readings from the fully expanded functional leaves (fourth from the apex). Measurements were made at a central point on the leaf between the midrib and leaf margin. The meter was shielded from direct sunlight by the operator during each measurement. Fifteen leaves were measured randomly per plot and averaged to a single SPAD value for each treatment. Modulated chlorophyll fluorescence was measured in dark-adapted (for at least 15 min) leaves in the same leaf leaflet in four plants per experimental unit using a chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd, King's Lynn, UK) with an excitation source intensity higher than 3000 μmol m^{-2} s^{-1} at the sample surface. The minimal fluorescence intensity (F_0) in a dark-adapted state was measured in the presence of a background far-red light to favour rapid oxidation of intersystem electron carriers. The maximal fluorescence intensities in the dark-adapted state (F_m) were measured by 0.8 s saturating pulses (3000 μmol m^{-2} s^{-1}). The maximum quantum yield of open photosystem II (PSII) (F_v/F_m) was calculated as $(F_m - F_0)/F_m$.[24]

On the same date, plants were separated into shoots and roots. All plant tissues were dried in a forced-air oven at 80 °C for 72 h for biomass determination.

Experiment 3

Plant material, growth conditions and experimental design

The third experiment was conducted in a 300 m^2 polyethylene greenhouse located at the Tuscia University experimental farm, central Italy (42° 25′ N, 12° 08′ E) in spring 2013. Daily temperature was maintained between 20 and 30 °C. Night temperature was always greater than 16 °C and relative humidity ranged from 55% to 85%.

Seeds of lettuce cv. Bionda Degli Ortolani, melon (*Cucumis melo* L. cv. Retato Degli Ortolani), pepper (*Capsicum annuum* L. cv. Rosso), tomato cv. San Marzano and zucchini squash cv. Grezini were germinated in polystyrene trays filled with vermiculite (120 holes per tray for lettuce, pepper and tomato; 60 holes per tray for melon and zucchini squash). The seedlings were transplanted on 17 May into pots (diameter 14 cm, height 12 cm) containing 1.5 L fluvial sand. The pots were placed on 16 cm wide and 5 m long troughs, with 30 cm between pots and 30 cm between troughs, resulting in a plant density of 11 plants m^{-2}.

Treatments were arranged in a randomized complete block design with four replicates. Each experimental unit consisted of ten plants. The experiments consisted of two treatments: compressed biostimulant tablet and the non-treated or control treatment. In control plots, plants received nutrient solution only, without any addition of the biostimulant. The biotimulant tablets were obtained by mixing 250 spores of *G. intraradices* from leek culture and 4.5×10^5 CFU of *T. atroviride* from the bioreactor culture with the organic matter. Organic matter, mixed with microorganism propagules, was compressed as a tablet ('Click', Italpollina S.p.A.) by a compression machine available at the Italpollina Co. The biostimulant tablets were applied before transplantation by placing one tablet per pot under the transplant roots.

Nutrient solution management

The basic nutrient solution was a modified Hoagland and Arnon formulation having the same composition as that used in Experiment 2. The nutrient solution was prepared using demineralized water. The electrical conductivity (EC) and pH of the nutrient solution were 1.8 dS m^{-1} and 6.0, respectively.

The nutrient solution was pumped from independent tanks and administered through one emitter per plant (flow rate 2 L h^{-1}). Irrigation scheduling was performed using electronic low-tension tensiometers (LT Irrometer, Riverside, CA, USA), which control irrigation based on substrate matric potential. Tensiometers were placed at the approximate midpoint of the pots (~6 cm depth). In each treatment, three tensiometers were installed and were located in different pots to provide a representative reading of the moisture tension. Tensiometers were connected to an electronic programmer that controlled the beginning (−5 kPa) and end of irrigation (−1 kPa), which correspond to high and low tension set points for the major part of media.[25] The timing varied from three to seven fertirrigations of 1–3 min per day. Nutrient solution supply ended when the leachate was equal to 30% of the applied nutrient solution; the 30% excess of the applied solution was collected for recycling.[26] Typically, leaching fractions of 20–30% are needed to maintain the EC in the substrate at the recommended level.

Data collection and analysis

At the end of the third experiment (35 days after transplanting) a chlorophyll meter (SPAD-502) was used to record readings from 15 fully expanded functional leaves (fourth from the apex) per treatment. On the same date, the plant height and number of leaves of vegetable transplants were recorded for eight plants per plot. The transplants of lettuce, melon, pepper, tomato and zucchini squash were then separated into stems, leaves and roots. Roots were rinsed to remove sand and subsamples were saved for assessment of AM fungi root colonization. All plant tissues were dried in a forced-air oven at 80 °C for 72 h for biomass determination. Shoot biomass was equal to the sum of aerial vegetative plant parts (leaves + stems).

Root colonization by AM fungi was determined on the same plants sampled for shoot and root measurements. Root samples were cleared with 10% KOH, stained with 0.05% trypan blue in lactophenol, as described by Phillips and Hayman,[27] and microscopically examined for AM fungi colonisation by determining the percentage of root segments containing arbuscules + vesicles using a gridline intercept method.[28]

Detection and quantification of *Trichoderma* was conducted at the end of Experiment 3 using serial plating soil dilution on to a *Trichoderma*-selective agar medium (TSM) as described by Elad *et al.*[29] Ten grams of each root/substrate sample was suspended in sterilized distilled water to give a dilution of 1:10. Serial dilutions were made to 10^{-8}. Aliquots (10 μL) of each dilution and replicates (four) were spread on to TSM medium in Petri plates. The plates were then incubated at 28 °C for 2–4 days. At the end of the incubation period, fungal colonies of *Trichoderma* were counted, and the number of CFU per gram of dry soil was calculated.[29]

Dried leaf tissue was ground in a Wiley mill to pass through a 20-mesh screen and 0.5 g of the dried tissue was analysed for the following content: N, P, K, Ca, Mg, Fe, Mn, Zn, Cu and B. The N concentration of leaf tissue was determined after mineralization with sulfuric acid using the Kjeldahl method,[30] whereas the other macronutrient and micronutrient concentrations were determined by dry ashing at 400 °C for 24 h, dissolving the ash in HNO_3 (1:20, v/v) and assaying the solution obtained by an inductively coupled plasma emission spectrophotometer (ICP Iris; Thermo Optek, Milan, Italy).[31]

Experiment 4

Plant material, crop management and experimental design

The fourth experiment was conducted in the summer growing season of 2013 at the Experimental Farm of Tuscia University. The soil was a sandy loam soil of volcanic origin (bulk density, 1.1 g cm^{-3}; pH 7.1; organic matter, 1.8%; available P, 21 mg kg^{-1}; exchangeable K, 3380 mg kg^{-1}; textural analysis: 67% sand, 18% silt and 15% clay). The soil had not been cultivated during the previous 3 years.

Lettuce (cv. Bionda Degli Ortolani) and zucchini (cv. Grezini) plantlets were transplanted at the three-to-four true leaf stage in the open field on 9 August. For both crops, treatments were arranged in a randomized complete block design with four replicates. For lettuce, the experimental unit consisted of four rows, 2.5 m in length, with 0.25 m row spacing and 0.25 in-row spacing containing 40 plants, thus resulting in a theoretical plant population of 160 000 plants ha^{-1}. For zucchini the unit consisted of three rows, 6.4 m in length, with 1.2 m row spacing and 0.8 in-row spacing containing 24 plants, thus resulting in a theoretical plant population of 10 417 plants ha^{-1}.

Experiment 4 consisted of two treatments: a treatment with the compressed biostimulant tablet (as reported in Experiment 3) and the non-treated or control treatment. In control plots, the plants received only fertiliser, without any addition of biostimulant. The compressed tablet biostimulants were applied before transplantation by placing one tablet per hole under the transplant roots.

Pre-plant fertilizer was broadcast (18 kg ha^{-1} N and 46 kg ha^{-1} P_2O_5 for lettuce; 36 kg ha^{-1} N and 92 kg ha^{-1} P_2O_5 for zucchini) and incorporated into the soil. Additional fertilizer (80 kg ha^{-1} N and 100 kg ha^{-1} K_2O for lettuce; 120 kg ha^{-1} N and 100 kg ha^{-1} K_2O for zucchini) was applied weekly using the drip system for zucchini and biweekly for lettuce using NH_4NO_3 and KNO_3 as sources of N and K, respectively. Fertirrigation was performed using a drip irrigation system with in-line emitters located 0.30 m apart and an emitter flow rate of 3.4 L h^{-1}. Water supply was based on actual water requirements (*I*), which was calculated as follows:

$$I = (ETo \times Kc) \quad (1)$$

where ETo is the potential ET from micrometeorological data using the FAO Penman–Monteith method[32] and Kc is the crop coefficient, which was estimated by the percentage ground cover of foliage development measured with a digital camera pointed straight upward beneath the canopy. All other management procedures during the cropping period were performed according to the best farming management practices.

Measurements and analysis

Thirteen days after transplanting, for both lettuce and zucchini plants, SPAD measurements were recorded using the same procedure as in Experiment 2. Modulated chlorophyll fluorescence was measured in dark-adapted (for at least 15 min) leaves as in Experiment 2.

The number of lettuce and zucchini leaves was recorded for six plants per plot. Shoot tissues were dried in a forced-air oven at 80 °C for 72 h for biomass determination. Leaf area (LA) was measured with an electronic area meter (Delta-T Devices Ltd, Cambridge, UK). Lettuce was harvested 32 days after transplanting. Fifteen plants per plot were separated into shoots to determine marketable fresh yield and roots and their tissues were dried in a forced-air oven at 80 °C for 72 h for biomass determination. Four lettuce leaves per experimental unit were sampled, frozen and stored in liquid nitrogen for quality analysis. The frozen samples were reduced to a fine powder with a mortar and pestle under liquid nitrogen. Spare powder was freeze-dried and used for measurements of nitrate content. To measure nitrate content, 50 mg lettuce samples were extracted by placing the samples in 1 mL water at 70 °C for 40 min. After centrifugation at 12 000 × *g* for 5 min, the supernatant was used for the determination of nitrate content by an enzymatic assay[33] performed with a Helios dual-wavelength (340–400 nm) spectrophotometer (Thermo Spectronic, Cambridge, UK).

For the zucchini experiments, the number of fruit, mean fruit weight, and early and total yields were recorded three times per week for six plants. Fruits were harvested when they reached marketable quality (over 12 cm). Fruits that were deformed, badly misshapen or measured less than 12 cm were considered unmarketable. [34]

Six representative fruits from each plot were analysed for quality parameters. Immediately after harvest, fruit firmness (N cm^{-2}) was determined by removing three discs from the skin surface in the equatorial area using a penetrometer (Bertuzzi FT 011; Brugherio, Milan, Italy) fitted with an 8 mm diameter round-head probe.

The liquid extract, obtained from liquefying and filtering the mesocarp of each fruit, was analysed for total soluble solids (TSS) content by using a refractometer (model N1, Atago Co. Ltd, Tokyo, Japan) and expressed as °Brix at 20 °C. Acidity was determined by potentiometric titration with 0.1 mol L^{-1} NaOH up to pH 8.1 using 10 mL juice. The results were expressed as percentage citric acid in the juice. The pH of the fruit juice was measured with a pH meter (HI-9023; Hanna Instruments, Padua, Italy). Fruits were dried in a forced-air oven at 80 °C for 72 h and weighed to determine the fruit dry matter (DM). The nitrate content of zucchini fruit was determined using the same procedure as in the lettuce experiment. The zucchini experiment ended 61 days after transplanting.

At the end of the experiment, ten zucchini plants per plot were separated into leaves, stems and roots. Roots were rinsed to remove soil, and subsamples were saved for the assessment of AM fungi root colonization. All plant tissues were dried in a forced-air oven at 80 °C for 72 h for biomass determination. Root colonization by AM fungi, and the macronutrient (N, P, K, Ca and Mg) and micronutrient content (Fe, Mn, Zn, Cu, and B) of lettuce and zucchini leaves at the end of the trials were determined using the same procedures as in Experiment 3.

Statistical analyses

In all experiments, ANOVA tests were conducted using the SPSS 10 software package for Windows 2001 (www.ibm.com/software/analytics/spss). Duncan's multiple range test was performed at $P = 0.05$ on each of the significant variables measured.

RESULTS

Experiment 1: evaluation of *Trichoderma* capability to produce IAA and siderophores

In half-strength tryptic soy broth, IAA was detected at the following levels: 2.9 ± 0.2 and 4.4 ± 0.38 mg L^{-1} in the absence and presence of L-tryptophan, respectively (Fig. 1). Moreover, the production of auxin-like compounds after 8 days of growth by fungal strain MUCL45632 was lower on potato dextrose broth (PDB) than in half-strength tryptic soy broth and only a slight stimulation of IAA production was detected after indole derivative additions in PDB (Fig. 1). In the PDB substrate, IAA and IAA-related compounds were detected at similar levels under acid, neutral and alkaline growing conditions.

An active production of siderophores was observed by differential responses of colour change in the CAS reaction applied to strain MUCL45632, grown on PDA (Fig. 2). A good rate of colour change of approximately 1–4 mm d^{-1} was observed during incubation. Moreover, a purple colour change was assessed in the medium, together with yellow-orange colour, indicating the capability of *T. atroviride* to produce siderophore compounds (Fig. 2).

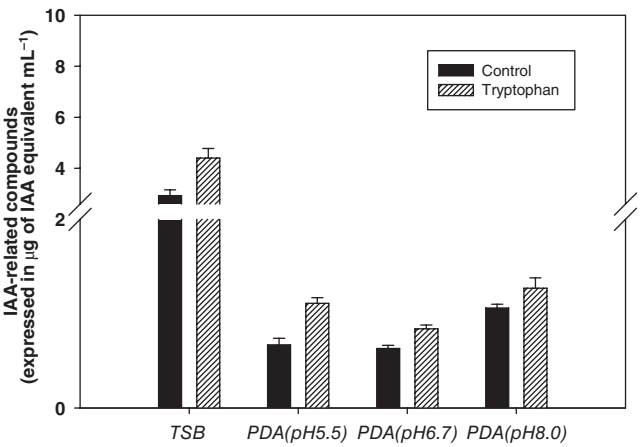

Figure 1. Production of IAA and IAA-related compounds by *T. atroviride* MUCL45632 in liquid cultures with tryptic soy broth (TSB) and potato dextrose broth (PDB) buffered at pH 5.5, 6.7 and 8.0. Control cultures were grown in the absence of tryptophan as an IAA precursor. Each value represents a mean of four replicates ± SE.

Experiment 2: effects of *G. intraradices* and *T. atroviride* alone or in combination on transplant performance

In the second experiment, we found that lettuce, tomato and zucchini growth parameters (shoot and root dry weight) were significantly affected by OM, G and T treatments alone or in combination. It is evident from Table 1 that inoculated or treated plants showed a significant increase in the morphological parameters in comparison with the control. The highest shoot and root dry weight in lettuce, tomato and zucchini was observed in the OM + G + T combination, followed by a single inoculation of *G. intraradices* or *T. atroviride* with OM, whereas the lowest values were recorded in the uninoculated plants. Moreover, inoculation with *G. intraradices* or *T. atroviride* alone or in combination increased the SPAD index by 10%, 8% and 11% for lettuce, tomato and zucchini, respectively, when compared with the uninoculated plants (Table 1). Finally, the lowest efficiency of PSII in dark-adapted leaves for the three vegetable crops, measured as the F_v/F_m ratio, was recorded in the control in comparison with OM + G, OM + T, and especially, OM + G + T treatments (Table 1).

Experiment 3: effect of the biostimulant tablet on vegetable crop performance under greenhouse conditions at the early development stage

The percentage of AM root colonization at the end of Experiment 3 was significantly affected by the biostimulant tablet application (Table 2). No AM fungi colonization was recorded in the roots of control plants, whereas in plants supplied with the biostimulant tablet the percentage of AM infection increased significantly among vegetable species. The percentage root colonization was 35%, 32.1%, 31.7%, 30.2% and 27.3% for tomato, melon, pepper, lettuce and zucchini squash, respectively (Table 2). The number of

Figure 2. CAS-blue assay performed on fungal strain *T. atroviride* MUCL45632. Changes in the colour of the indicator side can be observed.

Table 1. Effect of organic matter (OM), *T. atroviride* (T) and *G. intraradices* (G) alone or in combination on shoot dry weight, root dry weight, SPAD index and maximum quantum use efficiency of PSII in the dark-adapted state (F_v/F_m) of lettuce, tomato and zucchini plants 21 days after sowing (Experiment 2)

	Shoot dry weight (g per plant)			Root dry weight (g per plant)			SPAD index			F_v/F_m		
Treatment	Lettuce	Tomato	Zucchini	Lettuce	Tomato	Zucchini	Lettuce	Tomato	Zucchini	Lettuce	Tomato	Zucchini
Control	0.43d	1.11c	0.96d	0.09c	0.29c	0.15c	24.9b	32.3b	29.1b	0.65d	0.78c	0.80c
OM	0.64c	1.30bc	1.14c	0.11bc	0.35b	0.20b	25.6b	34.2ab	29.5ab	0.70c	0.80bc	0.81bc
OM + G	1.09b	1.55b	1.42b	0.12bc	0.47a	0.24ab	27.1ab	34.8a	32.4a	0.72b	0.81b	0.82b
OM + T	0.99b	1.48b	1.31b	0.14ab	0.31b	0.23b	26.0ab	35.0a	32.4a	0.73b	0.82b	0.83b
OM + G + T	1.21a	1.80a	1.63a	0.17a	0.55a	0.27a	29.5a	35.1a	32.6a	0.75a	0.85a	0.85a

Means within columns separated using Duncan's multiple range test, $P = 0.05$.

Trichoderma colonies recovered from the substrates and roots in the biostimulant treatment ranged between 4.5 and 6.0 log CFU g^{-1} for the five vegetable crops tested.

The final plant height and number of leaves per plant in lettuce, melon, pepper, tomato and zucchini were significantly affected by biostimulant application (Table 2). Moreover, the SPAD index was significantly higher in pepper, tomato and zucchini transplants supplied with live inoculum of *G. intraradices* and *T. atroviride*, in comparison with uninoculated transplants, whereas no significant differences were observed for lettuce and melon (Table 2). Similar to plant height and leaf number, the shoot and root dry weights were significantly affected by biostimulant application. The shoot dry weight was significantly increased by 167%, 56%, 115%, 68%, and 58% in lettuce, melon, pepper, tomato and zucchini, respectively, when supplied with the biostimulant in comparison with the control treatment (Table 2).

The major and trace element concentrations of lettuce, melon, pepper, tomato and zucchini leaves as a function of biostimulant application are displayed in Table 3. The concentration of N in leaves of lettuce was significantly increased by the biostimulant application, whereas no significant difference was recorded among the remaining crops (Table 3). The concentration of P significantly increased by 92%, 59%, 6%, 100% and 233% in lettuce, melon, pepper, tomato and zucchini, respectively, when supplied with the biostimulant tablet in comparison with the control treatment (Table 3).

The concentration of K in the leaves of pepper and zucchini was significantly increased by 16% and 9%, respectively, in the inoculated compared to uninoculated transplants, whereas no significant difference was observed among the biostimulant treatments in lettuce, melon and tomato (Table 3). The concentration of Ca in leaves was only affected in pepper transplants, with the highest values recorded with the biostimulant application. The concentration of Mg was significantly increased by 15%, 9%, 43% and 13%, in lettuce, melon, pepper and tomato, respectively, when supplied with the biostimulant in comparison with the control treatment (Table 3). The highest concentration of micronutrients, particularly Fe, Zn and B, were observed in the inoculated treatments, whereas

Table 2. Effect of the biostimulant tablet containing both *T. atroviride* and *G. intraradices* on root mycorrhization, plant height, leaf number, SPAD index, shoot dry weight and root dry weight of lettuce, melon, pepper, tomato and zucchini plants 35 days after transplanting (Experiment 3)

Treatment	Root mycorrhization (%)	Plant height (cm)	Leaf number (no. per plant)	SPAD index	Shoot dry weight (g per plant)	Root dry weight (g per plant)
Lettuce						
Control	0.0b	14.8b	16.5b	25.6a	1.8b	0.4b
Tablet	30.2a	19.5a	24.8a	26.2a	4.8a	0.9a
Melon						
Control	0.0b	35.0b	10.2b	31.6a	7.5b	1.3b
Tablet	32.1a	54.8a	14.2a	32.9a	11.7a	1.6a
Pepper						
Control	0.0b	49.0b	14.5b	47.9b	2.7b	0.6b
Tablet	31.7a	65.7a	20.8a	49.2a	5.8a	1.4a
Tomato						
Control	0.0b	46.8b	11.8b	46.4b	7.5b	1.3b
Tablet	35.0a	48.5a	12.3a	48.3a	12.6a	2.7a
Zucchini						
Control	0.0b	24.3b	9.0b	36.5b	4.5b	0.7b
Tablet	27.3a	39.3a	10.0a	39.3a	7.1a	1.0a

Means within columns separated using Duncan's multiple range test, $P = 0.05$.

Table 3. Effect of the biostimulant tablet containing both *T. atroviride* and *G. intraradices* on mineral composition of lettuce, melon, pepper, tomato and zucchini leaves (Experiment 3)

	Macronutrients (g kg^{-1})					Micronutrients (mg kg^{-1})				
Treatment	N	P	K	Ca	Mg	Fe	Mn	Zn	Cu	B
Lettuce										
Control	15.8b	1.3b	30.4a	7.2a	2.0b	22.8b	51.1a	7.7b	1.6a	18.0b
Tablet	22.1a	2.5a	30.5a	6.9a	2.3a	28.9a	62.2a	10.0a	1.2a	26.7a
Melon										
Control	13.3a	1.7b	16.4a	22.8a	3.2b	15.5b	17.9b	13.0a	2.8a	19.9b
Tablet	13.5a	2.7a	18.0a	24.6a	3.5a	27.8a	21.0a	14.7a	1.7a	24.5a
Pepper										
Control	17.2a	3.5b	24.6b	8.3b	2.1b	24.2b	24.8b	28.7b	2.8a	33.3b
Tablet	18.8a	3.7a	28.6a	10.8a	3.0a	26.8a	41.0a	38.1a	3.1a	42.5a
Tomato										
Control	11.2a	1.4b	15.3a	14.5a	2.3b	13.6b	26.8a	8.6b	2.0a	19.3b
Tablet	11.9a	2.8a	15.9a	15.0a	2.6a	15.3a	25.2a	10.9a	1.8a	24.6a
Zucchini										
Control	12.9a	1.2b	21.6b	20.1a	3.2a	17.9b	21.6b	13.0b	2.2a	27.7b
Tablet	14.3a	4.0a	23.6a	19.8a	3.3a	26.3a	26.1a	18.1a	2.6a	33.2a

Means within columns separated using Duncan's multiple range test, $P = 0.05$.

no significant difference among treatments was observed for Cu (Table 3).

Experiment 4: evaluation of the biostimulant tablet under field conditions

Thirteen days after transplanting, the leaf number, leaf area and shoot dry weight were highly influenced by biostimulant application. Application of the biostimulant tablet increased the leaf number, leaf area and shoot dry weight by 6%, 168% and 169% for lettuce and by 12%, 43% and 52% for zucchini (Table 4). Moreover, the lowest SPAD index and the lowest efficiency of the PSII in dark-adapted leaves were recorded in the control in comparison with the biostimulant treatment (Table 4).

The percentage of AM root colonization at the end of Experiment 4 was significantly affected by biostimulant application (Table 5). The percentage of AM infection was significantly increased by 87% and 112% in lettuce and zucchini, respectively, in comparison with control plants (Table 5). Moreover, at the end of the experiment, lettuce shoot and root dry weight increased by 61% and 57%, respectively, whereas no significant difference was recorded for the nitrate content (average 694 mg kg^{-1} fresh weight) (Table 5).

Early and total yields were significantly increased by 59% and 15%, respectively, when zucchini was supplied with live inoculum of *G. intraradices* and *T. atroviride*, in comparison with uninoculated plants (Table 5). The lowest total yield of the control treatment was due to a reduction in both the average fruit weight and number of fruit per plant (Table 5). Moreover, no significant differences among treatments were observed for fruit firmness (average 1.8 N cm^{-2}), juice pH (average 6.7), titratable acidity (average 0.67%) and nitrate content (average 295 mg kg^{-1} fresh weight), whereas fruit dry matter and TSS content were significantly higher with biostimulant tablet treatment (5.5% and 4.9 °Brix) compared with non-treated plants (5.0% and 4.1 °Brix).

Macronutrient concentrations, particularly N and P, were significantly increased by 13% and 27%, respectively, when the biostimulant was supplied to lettuce (Table 6). A similar trend was recorded for P concentration in zucchini leaves, with an increase of 16% (Table 6). Finally, the micronutrient concentrations, particularly Fe, Zn and B, were significantly increased by 24%, 34% and 38% in lettuce and by 18%, 12% and 24% in zucchini, respectively, when supplied with the biostimulant in comparison with the control treatment (Table 6).

Table 4. Effect of the biostimulant tablet containing both *T. atroviride* and *G. intraradices* on leaf number, leaf area, shoot dry weight, leaf SPAD index and maximum quantum use efficiency of PSII in the dark-adapted state (F_v/F_m) of lettuce and zucchini plants 13 days after transplanting (Experiment 4)

Treatment	Leaf number (no. per plant)	Leaf area (cm^2 per plant)	Shoot dry weight (g per plant)	SPAD index	F_v/F_m
Lettuce					
Control	6.8b	164.4b	0.55b	24.7b	0.68b
Tablet	7.2a	442.2a	1.48a	36.7a	0.71a
Zucchini					
Control	5.0b	523.6b	4.8b	44.4b	0.75b
Tablet	5.6a	749.9a	7.3a	46.4a	0.79a

Means within columns separated using Duncan's multiple range test, $P = 0.05$.

Table 5. Effect of the biostimulant tablet containing both *T. atroviride* and *G. intraradices* on root mycorrhization (M), shoot and dry fresh weight, root dry weight and leaf nitrate content of lettuce plants, and on root mycorrhization (M), early yield, total yield, total fruit number and fruit mean weight of zucchini plants at the end of the trial (Experiment 4)

	Lettuce					Zucchini				
Treatment	M(%)	Shoot fresh weight (g per plant)	Shoot dry weight (g per plant)	Root dry weight (g per plant)	Nitrate (mg kg^{-1} fresh weight)	M(%)	Early yield (kg per plant)	Total yield (kg per plant)	Total fruit number (no. per plant)	Fruit mean weight (g per fruit)
Control	15b	369b	17.5b	2.1b	678a	17b	0.34b	2.57b	15.6b	165.1b
Tablet	28a	628a	28.1a	3.3a	710a	36a	0.54a	2.96a	17.2a	172.1a

Means within columns separated using Duncan's multiple range test, $P = 0.05$.

DISCUSSION

At present, the application of beneficial microorganisms is encouraged in agriculture because of their potential to increase crop production in an environmentally friendly way. The interactions between mycorrhizal fungi and other soil organisms are complex. Although beneficial interactions have frequently been mentioned,[35] several authors have also reported antagonistic interactions with bacteria and fungi that may affect the functioning of AM symbioses.[36] The results of Experiment 2 demonstrated that the co-inoculation of *G. intraradices* BEG72 and *T. atroviride* MUCL 45632 synergistically increased SPAD index, maximum quantum use efficiency of PSII, shoot and root dry weight of lettuce, tomato and zucchini transplants compared with the inoculation of *G. intraradices* or *T. atroviride* alone, although OM + G and OM + T treatments enhanced the plant growth parameters when compared with the control treatment (Table 1). Co-inoculation of *Trichoderma* spp. and AM inoculations have been found to promote growth and plant development of numerous crops.[14,21,37] Chandanie *et al.*[16] observed that combined inoculation of *G. mosseae* and *T. harzianum* synergistically increased the plant biomass of cucumber compared with the inoculation of *T. harzianum* or AM fungi alone. Camprubi *et al.*[38] found a synergetic effect on growth of *Citrus reshni* by the interaction between *G. intraradices* and *T. aureoviride*. Similarly, Haggag and Abd-El Latif[39] and Calvet *et al.*[40] showed that the co-inoculation of *G. mosseae* and *T. harzianum* had a synergetic effect on the growth of geranium and marigold plants. Moreover, our results contrast markedly with the absence of effects observed in other studies, such as Fracchia *et al.*[41] Our results also contrast with the antagonistic effect observed by McAllister *et al.*[17] and Martinez *et al.*[18] It is worth noting that the effects of the interaction between AM fungi and *Trichoderma* may vary depending on the AM fungi and *Trichoderma* strains.

The results of the third and fourth experiments conducted under greenhouse and open field conditions indicate that the application of *G. intraradices* BEG72 and *T. atroviride* MUCL 45632, mixed with OM and compressed as a tablet, markedly improved the growth parameters (e.g. leaf height, leaf number, shoot and root dry weight) of the five vegetable transplants at early stages of development, and enhanced the crop performance of lettuce and zucchini squash 13 days after transplanting (e.g. leaf number and shoot dry weight; Table 4) and also at the end of the experiments (e.g. yield and yield components; Table 5). Our results are in line with those of Nzanza *et al.*,[14,42] who observed an increase in biomass production during the first stages of crop growth (starter effect) and also an enhancement in yield and yield components of tomato inoculated with *T. harzianum* and *G. mosseae*. Moreover, inoculated lettuce and zucchini plants (Experiment 4) were capable of maintaining higher chlorophyll content (SPAD index) and higher maximum quantum use efficiency of PSII, especially as (F_v/F_m) is frequently used as an indicator of photoinhibition or stress damage to the PSII.[43]

Presumed mechanisms involved in the stimulation of plant growth by *G. intraradices* and/or *T. atroviride* include interactions with plant roots, in which the two fungi penetrate and colonize root tissues without eliciting specific defense responses against the colonizing strain.[15] The production of plant growth hormones or analogues is one of the mechanisms by which strains of *Trichoderma* can enhance plant growth and consequent yield. In total, 162 species of *Trichoderma* fungi have been reported to produce auxins, which are key hormones affecting plant growth and development that can be produced by fungi in both symbiotic and pathogenic interactions with plants.[21] This was the

Table 6. Effect of the biostimulant tablet containing both *T. atroviride* and *G. intraradices* on mineral composition of lettuce and zucchini leaves (Experiment 4)

	Macronutrients (g kg^{-1})					Micronutrients (mg kg^{-1})				
Treatment	N	P	K	Ca	Mg	Fe	Mn	Zn	Cu	B
Lettuce										
Control	25.6b	2.6b	27.1a	7.8a	2.5a	20.1b	43.4b	9.3b	2.0a	20.0b
Tablet	29.0a	3.3a	30.0a	8.1a	2.6a	25.0a	50.9a	12.5a	1.9a	27.5a
Zucchini										
Control	38.3a	5.0b	44.7a	23.3a	7.1a	23.5b	43.8a	18.3b	2.0a	21.4b
Tablet	38.6a	5.8a	44.2a	24.8a	6.7a	27.8a	45.7a	20.6a	2.5a	26.5a

Means within columns separated using Duncan's multiple range test, $P = 0.05$.

case of *T. atroviride* MUCL 45632, which was capable of producing auxin-like compounds under a wide range of substrate pH conditions (5.5–8.0), especially in the presence of L-tryptophan (Fig. 1). It is well known that auxin-like compounds increase plant rooting: primary root elongation, lateral root development and root hair formation. The above findings explain the increase of root growth in plants inoculated with *T. atroviride* MUCL 45632. Similarly, Harman[44] found that a strain of *T. harzianum* induced approximately twice as many deep roots in maize and beans compared with the uninoculated control. *Trichoderma harzianum* strains were also reported to have increased the height, shoot and root dry weight in tomato seedlings transplanted into pots in the greenhouse.[45] Moreover, Altomare *et al.*[37] noted that *T. harzianum* increases the solubility of phosphate and micronutrients such as iron and zinc ions, which have slow solubility, and stimulates the secretion of exogenous enzymes, siderophores[46] and vitamins. This was the case because siderophore production was also recorded by *T. atroviride* MUCL 45632 grown *in vitro* on plates (Fig. 1). Although it has been reported that *Trichoderma* spp. were able to produce only hydroxamate type siderophores, it is worth noting that for several fungal strains, at neutral pH (pH of CAS-blue solid indicator), the hydroxamate complexes are reddish-orange, whereas catechol complexes are purple in colour at the same pH.[47] Considering the colour changes observed for strain MUCL45632 in the CAS-blue assay (Fig. 2, Experiment 1), we concluded that the current *Trichoderma* strain was able to produce both siderophores: hydroxamate and catechol compounds. Several studies have shown that plant growth enhancement by AM fungi is related to the colonization level and to the extent of external mycelium, the latter being important for nutrient uptake, P in particular.[48] Inoculation with *G. intraradices* BEG72 enhanced root growth especially in tomato and zucchini (Experiment 2), leading to an increase in root surface. A stimulation of root auxin production after mycorrhizal inoculation may explain the increase of root growth observed in mycorrhized plants, as reported for maize.[49] The increase in root growth and the development of an external mycelium enhanced the nutrient uptake of the tested crops. Our results are in line with those of Rouphael *et al.*[11] and Cardarelli *et al.*,[50] who observed an improvement in nutrient uptake when plants were inoculated with *G. intraradices* BEG72. The better plant nutritional status due to greater absorption of nutrients in the greenhouse (e.g. P, Mg, Fe, Zn and B; Table 3) and open field experiments (e.g. P, Fe, Zn and B; Table 6) via an increase in root surface can be considered an indirect effect of the beneficial microorganisms. Finally, the increase in crop performance after co-inoculation of *G. intraradices* BEG72 and *T. atroviride* MUCL 45632 was due to the production of stimulatory compounds (auxin-like compounds), and the improvement in mineral nutrient availability and uptake (higher root uptake surface and release of siderophores by *T. atroviride* in the root zone).

CONCLUSIONS

It can be concluded that the *T. atroviride* MUCL 45632 used in Experiment 1 was capable of producing siderophores (hydroxamate and catechol-type siderophores), as well as auxin-like compounds under a wide range of substrate pH conditions (5.5–8.0). With respect to the enhancement of vegetable plant growth, SPAD index and chlorophyll fluorescence, co-inoculation with *G. intraradices* and *T. atroviride* were more effective than any other combination. Based on the results of the short-term experiment (Experiment 3), application of the biostimulant tablet containing *G. intraradices* and *T. atroviride* markedly improved the growth parameters of lettuce, melon, pepper, tomato and zucchini transplants at early stages of development due to greater absorption of major and trace elements (e.g. P, Mg, Fe, Zn and B) via an increase in root surface. Our results also demonstrated the beneficial effect of inoculating lettuce and zucchini with the biostimulant tablet containing both microorganisms on yield and yield components. The improved crop performance of inoculated lettuce and zucchini plants was attributed to the phytostimulation effect (starter effect) and to an improved plant nutritional status (higher N, P, Fe, Mn, Zn and B concentration for lettuce and higher P, Fe, Zn and B concentration for leaf zucchini) of crops.

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