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A Composite Bioinoculant Based on the Combined Application of Beneficial Bacteria and Fungi

Henrietta Allaga ¹, Bettina Bóka ¹, Péter Poór ² , Viktor Dávid Nagy ¹, Attila Szűcs ¹, Istvánné Stankovics ³, Miklós Takó ¹ , László Manczinger ¹, Csaba Vágvölgyi ¹ , László Kredics ^{1,*} and Péter Körmöczi ⁴

¹ Department of Microbiology; Faculty of Science and Informatics; University of Szeged; Közép fasor 52, 6726 Szeged, Hungary; henrietta.allaga@gmail.com (H.A.); boka.tina@gmail.com (B.B.); viktor.david.nagy@gmail.com (V.D.N.); a.szunyi@windowslive.com (A.S.); tako78@bio.u-szeged.hu (M.T.); manczing@bio.u-szeged.hu (L.M.); csaba@bio.u-szeged.hu (C.V.)

² Department of Plant Biology, Faculty of Science and Informatics, University of Szeged, Közép fasor 52, 6726 Szeged, Hungary; poorpeti@bio.u-szeged.hu

³ Felső-Bácskai Agrolabor Ltd., Rákóczi Ferenc u. 19., 6430 Bácsalmás, Hungary; agrolabor@agrolabor.hu

⁴ Department of Field Crops Research, National Agricultural Research and Innovation Centre, Alsó kikötő sor 9, 6726 Szeged, Hungary; kormoczpeti@gmail.com

* Correspondence: kredics@bio.u-szeged.hu; Tel.: +36-62-544-516

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Abstract: A composite soil bioinoculant containing beneficial bacteria and fungi was developed for biocontrol of plant pathogens, phosphorous mobilization, stem degradation, humification, and nitrogen fixation. A *Trichoderma asperellum* isolate with outstanding in vitro antagonistic abilities toward a series of plant pathogenic fungi was included as a potential biocontrol component. The selected strain was also shown to promote growth and increase photosynthetic activity of tomato plants. For phosphorous mobilization and stem degradation, a *Trichoderma atrobrunneum* strain was selected, which produced cellulose-degrading enzymes even in the absence of stem residues, while this ability increased 10–15-fold in the presence of ground maize stem. The strain was also shown to produce large amounts of enzymes liberating organically bound phosphorous, as well as cellulase and xylanase activities in solid-state fermentation on various plant residues. A *Streptomyces albus* strain with excellent peroxidase-producing abilities was selected as a potential humus-producing component, while an *Azotobacter vinelandii* strain with the potential to provide excess nitrogen for crops was included for nitrogen fixation. The assembled soil bioinoculant had positive effect on the uptake of certain important macro- and microelements (potassium, sodium, and manganese) from the soil by field-grown tomato plants. The applied screening strategy proved to be applicable for the assembly of a composite soil bioinoculant with notable application potentials.

Keywords: biocontrol; plant growth promotion; soil inoculant; *Trichoderma*; *Azotobacter*; *Streptomyces*

1. Introduction

Chemical pesticides and fertilizers are applied world-wide in agricultural production. Pesticides are used for the prevention and control of plant pests and diseases in order to reduce or eliminate yield losses and maintain product quality. However, there are serious concerns regarding the risks resulting from occupational exposure to them, as well as from environmental pollution leading to the presence of their residues in the food-chain and drinking water [1]. Chemical fertilizers are used to supply plants with necessary elements (primarily phosphorous and nitrogen), thereby improving crop productivity; however, their application is resulting in pollution with phosphates and nitrates. The agricultural

run-off of phosphates deriving from fertilizers contributes to the eutrophication of fresh water bodies and also presents a serious threat to the biodiversity in terrestrial ecosystems [2], while the increased run-off of nitrogen fertilizers results in nitrate pollution of surface and groundwater [3]. Therefore, the need for alternative, environment-friendly, microbial soil treatment strategies with favorable effects on crop plants is emerging all over the world. Microbial abilities of biocontrol, plant growth promotion, stem degradation, phosphorous solubilization, humification, and nitrogen-fixation can be exploited for the development of microbial soil inoculants to be applied in sustainable agricultural production.

One of the main challenges in the agricultural use of beneficial microorganisms as plant growth promoters and/or biocontrol agents (BCAs) is their frequently inconsistent field performance [4,5], which may be due to a series of biotic and abiotic factors. Among the abiotic factors, physicochemical properties of the rhizosphere such as pH, temperature, water activity, and the chemical composition of the soil are varying in space and time, which substantially influences the performance of biocontrol and plant growth promoting microorganisms. Particular agents may exert different activities under different soil environmental conditions. Inconsistent field performance has long been identified as the major impediment to the wide-scale commercialization of beneficial microorganisms for agricultural applications [6]. A possible strategy to counteract inconsistencies due to varying environmental conditions is the development of consortial soil inoculants consisting of multiple beneficial organisms. The combination of efficient plant growth promoting microorganisms and BCAs may result in an increased consistency of field performance during different periods of the growing season, thereby enabling a more predictable increase in crop yields [7].

The aim of this study was to assemble a consortial soil bioinoculant based on the combined application of beneficial bacteria and fungi with the potential of increasing pathogen control, plant growth and crop yield, stem residue degradation, phosphorous mobilization, humification, and nitrogen fixation in treated agricultural soils.

2. Materials and Methods

2.1. Examined Strains

The microbial strains involved in this study derived from the Szeged Microbiology Collection, Szeged, Hungary (SZMC). The *Trichoderma* strains included in the study (*Trichoderma asperellum* SZMC 20866, and SZMC 20786; *Trichoderma harzianum* species complex (THSC) members SZMC 20761, SZMC 20762, and SZMC 20869; *Trichoderma atroviride* SZMC 20780 and SZMC 20781; *Trichoderma virens* SZMC 20779; *Trichoderma gamsii* SZMC 20783; and *Trichoderma hamatum* SZMC 20784) were isolated from Hungarian agricultural soil samples and initially identified by sequence analysis of the internal transcribed spacer (ITS) region [8]. However, as ITS sequence analysis is not able to discriminate between species belonging to THSC, the species level identity of strains SZMC 20761, SZMC 20762, and SZMC 20869 was determined during this study by the sequence analysis of a fragment of the *tef1* alpha gene [9] as *Trichoderma guizhouense*, *T. guizhouense* and *Trichoderma atrobrunneum* (GenBank accession numbers MN750371, MN750372, MN750373), respectively. Fungal isolates were maintained on yeast extract-glucose medium (5 g L⁻¹ glucose, 5 g L⁻¹ KH₂PO₄, 1 g L⁻¹ yeast extract, 20 g L⁻¹ agar).

Streptomyces sp. isolates (SZMC 0282, SZMC 0232, 00001, 00002, 00004, 00005, 00006, 00007, 00008, 00009, 00010, 00012, 00013, 00014, 00015, 00017, 00019, 00020, 00021, 00022, 00023, 00024, 00025, 00026, 00027, 00028, 00029, 00030, 00031, 00032, 00033, 00034, 00035, 00036, 00037, 00038, 00039, 00040, 00041, 00042, 00043, 00044, 00045, 00046, and 00047) and *Azotobacter vinelandii* SZMC 22195 were derived from soil samples. *Streptomyces microflavus* DSM 40561 was derived from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) strain collection and was included as control in the peroxidase-producing assays. Bacterial strains were maintained on glucose-yeast extract-malt extract *Streptomyces* medium (GYM-STR: 4 g L⁻¹ glucose, 4 g L⁻¹ yeast extract, 10 g L⁻¹ malt extract, 2 g L⁻¹ CaCO₃, and 20 g L⁻¹ agar).

2.2. Determination of Biocontrol Index (BCI) Values

Dual confrontation tests were performed in vitro in Petri dishes (90 mm in diameter) according to the method described by Szekeres et al. [10]. During the experiments, 11 different strains of plant pathogenic fungi were confronted with the *Trichoderma* strains, including 3 *Armillaria* species (*Armillaria mellea* SZMC 23638, *Armillaria ostoyae* SZMC 23080, and *Armillaria gallica* SZMC23076), 4 *Fusarium solani* species complex (FSSC) isolates (SZMC 11057F, SZMC 6241J, SZMC 11067F, and SZMC 11070F), as well as *Phoma cucurbitacearum* (SZMC 16088), *Alternaria alternata* (SZMC 16085), *Botrytis cinerea* (SZMC 6244J), and *Rhizoctonia solani* (SZMC 6252J). The tests were carried out in three replicates on malt extract agar (MEA) medium (10 g L⁻¹ glucose, 2.5 g L⁻¹ yeast extract, 20 mL L⁻¹ 20% malt extract, 20 g L⁻¹ agar). After the incubation period, digital photos were taken with a Nikon Coolpix P7700 camera (Nikon, Tokyo, Japan) from each Petri plate, and the area visibly covered by the *Trichoderma* strain as well as the area covered by *Trichoderma* and the pathogen together were calculated for each plate with the aid of the Image J software (<http://imagej.nih.gov/ij>). BCI values were calculated with Excel 2010 (Microsoft, Redmond, WA, USA) according to the formula: BCI = (area of *Trichoderma* colony/total area occupied by the colonies of both *Trichoderma* and the plant pathogenic fungus) × 100 [10].

2.3. Liquid and Solid-State Fermentations

For the investigation of cellulase and phosphatase activities, culturing was performed in 100 mL flasks containing 20 mL liquid minimal (10 g L⁻¹ glucose, 5 g L⁻¹ (NH₄)₂SO₄, 5 g L⁻¹ KH₂PO₄, 0.1 g L⁻¹ MgSO₄ × 7H₂O) or maize stem medium (2 g L⁻¹ dried maize stem ground with a coffee grinder (Bosch, Gerlingen, Germany), 1 g L⁻¹ NaNO₃). The minimal medium was inoculated with *Trichoderma* conidia to a concentration of 2 × 10⁵ mL⁻¹, while the maize stem medium was inoculated with the total amount of 5-days-old fungal mycelium pre-grown in 20 mL liquid minimal medium, filtered with the aid of filter paper and vacuum pump and washed with sterile distilled water. After 5 days of incubation at 25 °C in an IKA KS 4000 IC Control shaker (ProfiLab24, Berlin, Germany) at 180 rpm, samples were filtered, and the culture filtrates were used for extracellular enzyme activity measurements.

Extracellular enzyme activities of *T. atrobrunneum* SZMC 20869 from THSC and the industrially important *Trichoderma reesei* strain QM9414 (SZMC 22616) were also compared in solid-state fermentation (SSF) experiments using maize, wheat, sunflower, and canola stem residues as substrates. One gram amounts of ground plant residues were placed into 50 mL Erlenmeyers flask and moisturized with 5 mL distilled water. After sterilization, the substrates were inoculated with 2 × 10⁵ *Trichoderma* conidia. On the 8th day of fermentation, extractions were performed by adding 20 mL distilled water to the cultures and incubating for 3 h at 4 °C. The fluid phases were filtered through sterile gauze sheets into 15 mL centrifuge tubes and centrifuged at 4600 rpm for 10 min, 2 times by transferring the fluid phase to a new centrifuge tube. The 8× dilution of the fluid samples in distilled water were used for extracellular enzyme activity measurements.

2.4. Enzyme Activity Measurements

Cellobiohydrolase, β-glucosidase, β-xylosidase, and acidic phosphatase enzyme activities were measured with the chromogenic substrates p-nitrophenyl-β-D-cellobioside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-xylopyranoside, and p-nitrophenyl-phosphate (Sigma-Aldrich, Budapest, Hungary), respectively. Enzyme reactions were carried out in three replicates in the wells of 96-well microtiter plates (Sarstedt, Nümbrecht, Germany) by mixing 100 μL culture filtrate or SSF extract with 100 μL substrate solution (1 mg mL⁻¹ in distilled water). After 1 h of incubation at room temperature, enzyme reactions were stopped with 10% (w/v) Na₂CO₃ and the optical densities measured at 405 nm with a Spectrostar Nano microplate reader (BMG Labtech, Ortenberg, Germany).

Peroxidase assays of bacteria were carried out in liquid *Streptomyces* induction medium (STR-IND) 6 g L⁻¹ yeast extract, 8 g L⁻¹ xylan, 0.1 g L⁻¹ (NH₄)₂SO₄, 0.3 g L⁻¹ NaCl, 0.1 g L⁻¹ MgSO₄, 0.02 g

L^{-1} $CaCO_3$, $0.6 mL L^{-1}$ TE ($0.1 g L^{-1}$ $FeSO_4$, $0.002 g L^{-1}$ $MnSO_4 \times 7H_2O$, $0.09 g L^{-1}$ $ZnSO_4 \times 7H_2O$) inoculated with the examined *Streptomyces* strains. After 7 days of incubation ($28^\circ C$, 150 rpm), the samples were centrifuged at 7000 rpm for 10 min. The reaction mixture contained 0.2 mL phosphate buffer (100 mM, pH 7.2), 0.2 mL 2,4-dichlorophenoxy acetic acid (25 mM), 0.2 mL 4-aminoantipyrine (4AAP, 16 mM), 0.2 mL ferment broth, and 0.2 mL hydrogen peroxide (50 mM). The reaction mixtures were put into a $53^\circ C$ thermostat for 1 min, which was followed by the measurement of the optical density at 510 nm with a Spectrostar Nano microplate reader (BMG Labtech, Ortenberg, Germany). All measurements were carried out in three replicates.

In order to investigate the peroxidase enzyme production, dye decolorization assays were also performed. For this purpose, we used STR-IND medium supplemented with $20 g L^{-1}$ agar and Remazol Brilliant Blue (RBB), Methyl Orange (MO), or Neutral Red (NR) dyes at a concentration of $1 g L^{-1}$. The plates were inoculated with *Streptomyces* isolates with the aid of inoculation loop onto the middle of the Petri plates. Color changes were observed around the colonies after 1 week of incubation.

2.5. Growth Assay in Nitrogen-Free Medium

The growth kinetics of *A. vinelandii* strain SZMC 22195 were tested in nitrogen source-free liquid medium ($5 g L^{-1}$ glucose, $5 g L^{-1}$ mannitol, $0.1 g L^{-1}$ $CaCl_2 \times 2H_2O$, $0.1 g L^{-1}$ $MgSO_4 \times 7H_2O$, $0.005 g L^{-1}$ $Na_2MoO_4 \times 2H_2O$, $0.9 g L^{-1}$ K_2HPO_4 , $0.1 g L^{-1}$ KH_2PO_4 , $0.01 g L^{-1}$ $FeSO_4 \times 7H_2O$, pH 7.3). During an incubation period of 1 week ($30^\circ C$, 120 rpm), the optical densities of the liquid cultures were measured on days 1, 2, 4, and 7 at 620 nm with a Spectrostar Nano microplate reader.

2.6. Plant Material for Growth Chamber Experiments

Seeds of tomato (*Solanum lycopersicum* Mill. L. cvar. Ailsa craig) were germinated at $26^\circ C$ for 3 days in the dark, and the seedlings were subsequently transferred to 6×6 cm pots filled with vermiculite (Terracult GmbH, Siegburg, Germany) for 6 weeks. Plants were irrigated every third day with nutrient solution containing 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.5 mM KCl, 0.5 mM KH_2PO_4 , and 0.5 mM Na_2HPO_4 , pH 6.0. The concentrations of micronutrients were 0.001 mM $MnSO_4$, 0.005 mM $ZnSO_4$, 0.0001 mM $(NH_4)_6Mo_7O_{24}$, 0.01 mM H_3BO_4 , and 0.02 mM Fe(III)-EDTA. The plants were grown in a controlled environment under $300 \mu mol m^{-2} s^{-1}$ light intensity (emitted F36W/GRO lamps, Feilo Sylvania, Erlangen, Germany), with 12/12-h light/dark period, day/night temperatures of 24/22 $^\circ C$, and relative humidity of 55–60%. Plants were treated with 20 μL of *Trichoderma* suspension (1×10^6 conidia mL^{-1}) after the 3-days-long germination. Samples for measurements were prepared in each replicate from the second, fully expanded young leaves of tomato plants. After harvest, the plant height and root length as well as the biomass production were recorded in 5 replicates.

2.7. Measurement of Stomatal Conductance, CO_2 Assimilation, and Total Soluble Sugar Content

Stomatal conductance and CO_2 assimilation were measured in 3 replicate samples by a portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE, USA), as described by Poór et al. [11]. Data were recorded after 15 min light adaptation on $300 \mu mol m^{-2} s^{-1}$ light intensity and under constant conditions ($25^\circ C$, $65 \pm 10\%$ relative humidity, and controlled CO_2 supply of $400 \mu mol mol^{-1}$).

Total sugar contents were determined according to Dubois et al. [12]. One gram of leaf samples was homogenized in 10 mL distilled water and incubated in a $90^\circ C$ water bath for 45 min. Samples were centrifuged ($12,000 \times g$ for 15 min, at $4^\circ C$), and 40 μL of the supernatant was mixed with 400 μL of 1.8% phenol and 2 mL of concentrated sulfuric acid. The absorbance was measured from 5 replicate samples by a spectrophotometer at 490 nm.

2.8. Chlorophyll a Fluorescence Measurements

Chlorophyll a fluorescence was detected in 3 replicate samples with the portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, NE, USA) described above [11]. Leaves were dark-adapted for 15 min before the measurement of the minimal fluorescence (F_0) using weak measuring light. The

maximal fluorescence (F_m) was measured by applying a pulse (800 ms) of saturating light ($12,000 \mu\text{mol m}^{-2} \text{s}^{-1}$). The leaves were then illuminated continuously with actinic light ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 20 min, the light-adapted steady-state fluorescence (F_s) was recorded and the maximum fluorescence level (F_m') in the light-adapted state was determined with saturating pulses. The actinic light was next turned off and the minimum fluorescence level in the light-adapted state (F_0') was determined by illuminating the leaf with 3-s far-red light ($5 \mu\text{mol m}^{-2} \text{s}^{-1}$). The following chlorophyll fluorescence parameters were calculated: the maximal quantum yield of PSII photochemistry, $F_v/F_m = (F_m - F_0)/F_m$; the actual quantum yield of PSII electron transport in the light adapted-state, $\Phi_{\text{PSII}} = (F_m' - F_s)/F_m'$ [13]; the photochemical quenching coefficient, $q_P = (F_m' - F_s)/(F_m' - F_0')$ [14]; and the non-photochemical quenching $\text{NPQ} = F_m/F_m' - 1$ [15].

2.9. Pigment Analysis

For pigment analysis, a two-step extraction was applied. Fifty milligrams of leaf samples were homogenized in ice-cold 100% (v/v) acetone (1 mL) and extracted for 24 h. Samples were centrifuged ($12,000 \times g$ for 15 min at 4°C). The pellet was extracted again with 80% (v/v) acetone (1 mL) for 24 h. After spinning down ($12,000 \times g$, 15 min, 4°C), the supernatants were collected. The pigment composition was measured in 5 replicate samples as according to Lichtenthaler and Wellburn [16].

2.10. Field Experiment

A field study was performed in tomato culture (*Solanum lycopersicum* cvar. ACE-55) on sandy loam soil according to the yarn number of Arany (K_A), which is a humus-rich soil with good nutrient availability and water management. The GPS coordinates of the examined area are $46^\circ 05' 01.05'' \text{N}$, $19^\circ 26' 28.83'' \text{E}$. Three-week-old tomato seedlings were planted on 11 May 2019. A total of 220 seedlings were planted in the field with 40 cm row distance and 40 cm plant distance, with 20 seedlings in a row. At the beginning of the experiment, a bioinoculant preparation consisting of a mixture of two *Trichoderma* strains and two bacteria was prepared. The concentration of the bioinoculant was adjusted to 10^6 conidia mL^{-1} for both selected *Trichoderma* components, and 10^8 cells mL^{-1} for both selected bacteria. The application of the soil inoculant was performed after $100 \times$ dilution at a concentration of 100 mL L^{-1} , while the control area was not treated. Three control and eight treated rows were examined. No organic manure or chemical fertilizer was applied to the area during the soil preparation. Changes in the contents of soil macro- and microelements were measured three times during cultivation (I: 22 June, II: 6 July, and III: 3 August 2019) from rhizosphere samples taken from a depth of 15 cm. The total numbers of the control and treated plants were 60 and 160, respectively. During the experiment, the same plant protection measures were applied both in the control and treated area: Cuproxat FW (5 mL L^{-1}), Topaz (0.5 mL L^{-1}), Mospilan (200 mg L^{-1}), Wuxal (2 mL L^{-1}), Humusz (1 mL L^{-1}), as well as calcium (5 mL L^{-1}) and magnesium (5 mL L^{-1}) in the form of foliar fertilizer were used 3 times during the cultivation (20 May, 8 June, and 13 July 2019). Control and treated plants were examined separately.

2.11. Soil Examination Methods

Soil sampling and analysis were carried out according to the test methods prescribed by the Hungarian Standard [17,18]. The soil tests were carried out by the Felső-Bácskai Agrolabor Ltd., Bácsalmás, Hungary. Carbonate content was determined with a Scheibler's calcimetre (Bovimex, Székesfehérvár, Hungary). The total salt content was measured by the electric conductivity using a HI98311 conductivity meter (HANNA Instruments, Szeged, Hungary). These methods were based on the Hungarian Standard (MSZ) MSZ-08-0206-2:1978. The soil texture was determined by the yarn number of Arany (MSZ-08-0205:1978). The macro- and microelement content and the humus content were measured by a Lambda 25 UV/VIS Spectrophotometer (PerkinElmer, Waltham, MA, USA) according to the Hungarian standards MSZ 20135:1999 and MSZ-21470-52:1983, respectively. N-content was measured by the Kjeldahl method, K_2O with flame emission spectrophotometry (FES), while Mg and Ca content with flame atomic absorption spectroscopy (FAAS) after acidic ($\text{H}_2\text{SO}_4\text{-HClO}_4$)

digestion [19]. Samples were prepared with microwave digestion for microelement analysis (Cu, Mn, Zn, and Fe content) measured with FAAS [17,19,20].

2.12. Statistical Analysis

Data presented as average values resulted from at least 3 independent experiments. Statistical analyses were carried out for the measurement data with Sigma plot v11.0 software (Systat Software Inc., Erkrath, Germany) using Student's t test, and the differences were considered significant if $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***). The statistical analyses of the soil results and crop yield data were performed with the GraphPad Prism v8.3.0 software (GraphPad Software Inc., San Diego, CA, USA) applying two-way ANOVA, and the results were considered significant if $p < 0.05$ (*), $p = 0.02$ (**), $p = 0.001$ (***), and $p < 0.0001$ (****).

3. Results

3.1. Selection of the Components for the Soil Inoculant

The potential biocontrol component of the soil inoculant was selected based on the results of dual confrontation tests between *Trichoderma* strains and plant pathogenic fungi. As shown in Table 1 and Figure 1, the two examined *T. asperellum* strains were the most effective against many of the tested pathogens. The BCI values of *T. asperellum* SZMC 20786 were the highest against FSSC SZMC 11067F and SZMC 11070F and *Alternaria alternata* 16085, while against *Rizoctonia solani* and *Armillaria gallica* they even reached 100, which means that the *Trichoderma* could completely overgrow and inhibit these plant pathogens. Except for *T. atrobrunneum* SZMC 20869 and *T. virens* SZMC 20779, all other *Trichoderma* strains could completely inhibit the *R. solani* strain SZMC 6252J. Based on the results, strain *T. asperellum* SZMC 20786 was selected as the potential biocontrol component of the soil inoculant.

Eight *Trichoderma* strains were included in the screening for cellulose-degrading and phosphatase-producing abilities (Figure 2). High levels of β -glucosidase and cellobiohydrolase enzyme activities could be measured in the case of three *Trichoderma* strains, which included two isolates of THSC (*T. guizhouense* SZMC 20761, *T. atrobrunneum* SZMC 20869) and one isolate of *T. hamatum* (SZMC 20784). The cellulolytic activities of SZMC 20869 were inducible with maize stem powder (Figure 2A,B), while the other two strains possessed high enzyme activity values only in liquid minimal medium. Only low phosphatase enzyme activities could be detected for the examined *Trichoderma* strains, except for the above mentioned *T. atrobrunneum* SZMC 20869 strain (Figure 2C), for which increased enzyme activity levels could be observed in liquid medium supplemented with maize stem powder. Based on the results, strain *T. atrobrunneum* SZMC 20869 from THSC was selected as the potential stem-degrading and phosphate-mobilizing component of the soil inoculant.

In the dye decolorization assays performed with *Streptomyces* isolates, color changes could be observed only when RBB was applied. Only the isolates *S. albus* SZMC 0232 and SZMC 0282 and *S. microflavus* DSM 40561 gave positive reactions with the RBB dye. The results of peroxidase assays are shown in Figure 3, indicating that only the isolates *S. albus* SZMC 0232 and SZMC 0282 showed an increased peroxidase production. The activities of these two strains were about twice as high as those of the control strain *S. microflavus* DSM 40561. Based on the results, strain *S. albus* SZMC 0282 was selected as the potential humus-producing component of the soil inoculant.

Table 1. Biocontrol index values of the examined *Trichoderma* strains against plant pathogenic fungi.

Examined <i>Trichoderma</i> Strains	Plant Pathogens										
	FSSC SZMC 11057F	FSSC SZMC 6241J	FSSC SZMC 11067F	FSSC SZMC 11070F	<i>Armillaria mellea</i> MUCL 31056	<i>Armillaria ostoyae</i> SZMC 23080	<i>Armillaria gallica</i> SZMC 23076	<i>Alternaria alternata</i> SZMC 16085	<i>Phoma cucurbitacearum</i> SZMC 16088	<i>Botrytis cinerea</i> SZMC 6244J	<i>Rhizoctonia solani</i> SZMC 6252J
<i>T. asperellum</i> SZMC 20866	79.77 ± 1.22	83.71 ± 1.35	71.38 ± 3.04	75.61 ± 5.35	33.91 ± 6.67	100.00 ± 0.00	100.00 ± 0.00	64.32 ± 0.53	81.36 ± 1.26	100.00 ± 0.00	100.00 ± 0.00
<i>T. asperellum</i> SZMC 20786	77.76 ± 0.96	83.50 ± 6.97	88.84 ± 5.48	85.76 ± 2.12	47.68 ± 5.22	92.33 ± 3.51	100.00 ± 0.00	65.77 ± 1.42	80.63 ± 0.50	100.00 ± 0.00	100.00 ± 0.00
<i>T. atroviride</i> SZMC 20780	62.41 ± 2.90	63.39 ± 5.10	37.63 ± 2.15	51.19 ± 0.96	93.76 ± 2.89	100.00 ± 0.00	100.00 ± 0.00	51.43 ± 0.42	59.21 ± 1.37	100.00 ± 0.00	100.00 ± 0.00
<i>T. atroviride</i> SZMC 20781	63.91 ± 0.76	71.32 ± 4.40	39.93 ± 2.14	48.39 ± 2.53	97.43 ± 3.07	98.02 ± 2.00	100.00 ± 0.00	54.16 ± 0.27	56.82 ± 1.20	100.00 ± 0.00	100.00 ± 0.00
<i>T. gamsii</i> SZMC 20783	60.82 ± 1.42	67.42 ± 0.79	39.97 ± 1.74	59.36 ± 1.34	82.21 ± 9.56	100.00 ± 0.00	100.00 ± 0.00	49.22 ± 2.06	60.14 ± 0.75	50.70 ± 7.26	100.00 ± 0.00
<i>T. hamatum</i> SZMC 20784	68.22 ± 0.73	64.10 ± 0.67	47.88 ± 1.75	62.52 ± 0.63	96.71 ± 3.41	97.00 ± 0.00	92.75 ± 2.95	61.28 ± 1.38	67.96 ± 1.19	46.82 ± 1.06	100.00 ± 0.00
<i>T. guizhouense</i> SZMC 20761	63.02 ± 5.40	58.81 ± 1.72	63.39 ± 1.69	64.92 ± 0.87	96.70 ± 3.23	99.93 ± 0.11	100.00 ± 0.00	60.77 ± 1.06	61.19 ± 1.35	44.20 ± 4.21	100.00 ± 0.00
<i>T. atroviride</i> SZMC 20869	42.19 ± 2.53	47.46 ± 2.08	37.83 ± 0.17	38.40 ± 0.95	54.38 ± 1.98	100.00 ± 0.00	88.43 ± 4.27	18.02 ± 1.02	14.92 ± 1.89	42.26 ± 4.55	74.87 ± 7.34
<i>T. guizhouense</i> SZMC 20762	63.69 ± 1.23	58.47 ± 5.30	47.91 ± 0.37	59.77 ± 1.79	97.42 ± 2.4	100.00 ± 0.00	100.00 ± 0.00	59.33 ± 0.53	57.84 ± 1.95	49.43 ± 3.03	100.00 ± 0.00
<i>T. virens</i> SZMC 20779	40.66 ± 1.51	37.20 ± 1.38	53.43 ± 0.62	49.10 ± 2.91	53.48 ± 2.76	100.00 ± 0.00	86.19 ± 5.77	25.07 ± 2.33	35.59 ± 1.91	45.32 ± 8.37	62.37 ± 5.36

FSSC: *Fusarium solani* species complex; MUCL: Belgian Coordinated Collections of Microorganisms/MUCL Agro-food & Environmental Fungal Collection; SZMC: Szeged Microbiology Collection.

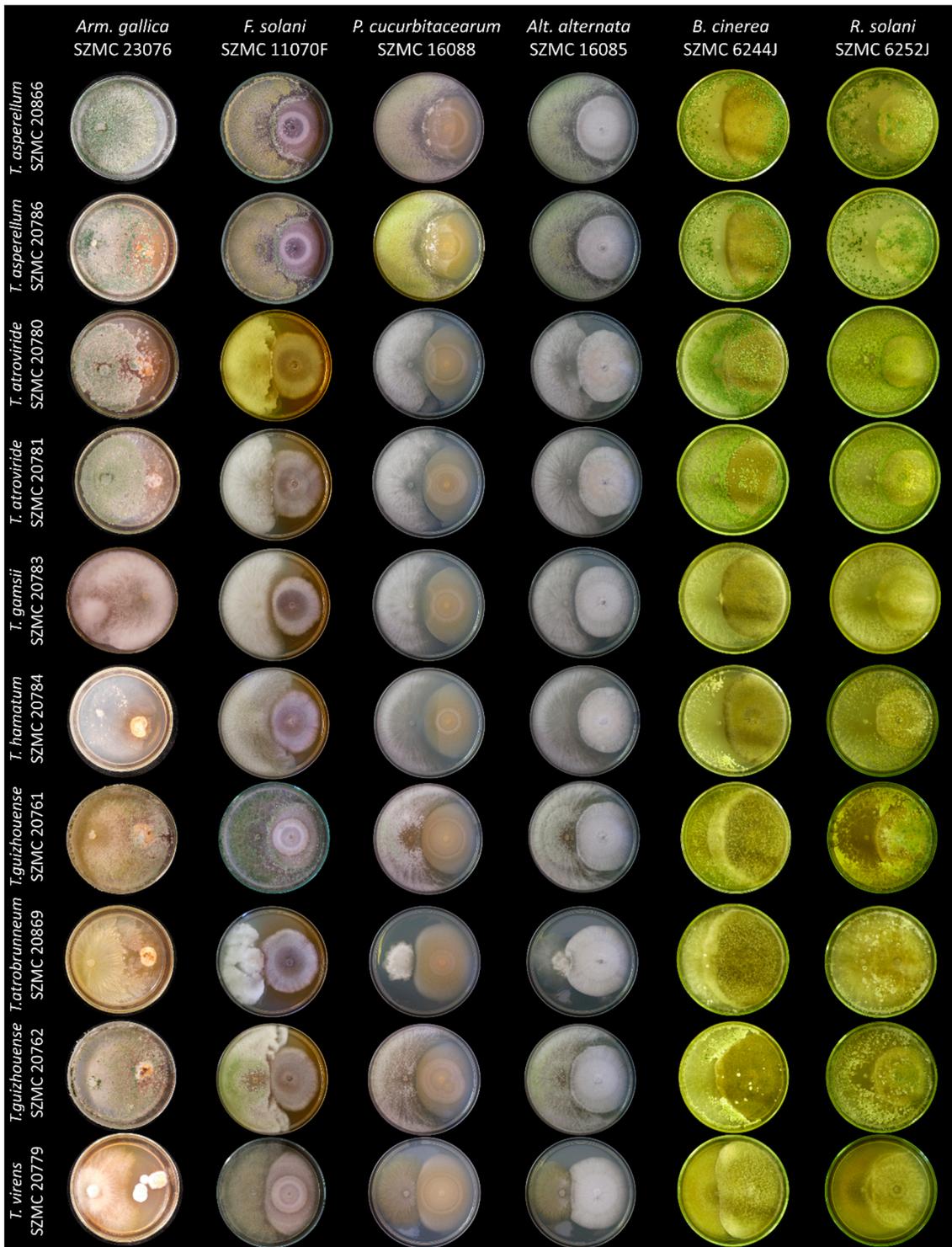


Figure 1. In vitro antagonism of *Trichoderma* strains against different plant pathogenic fungi examined in dual confrontation tests.

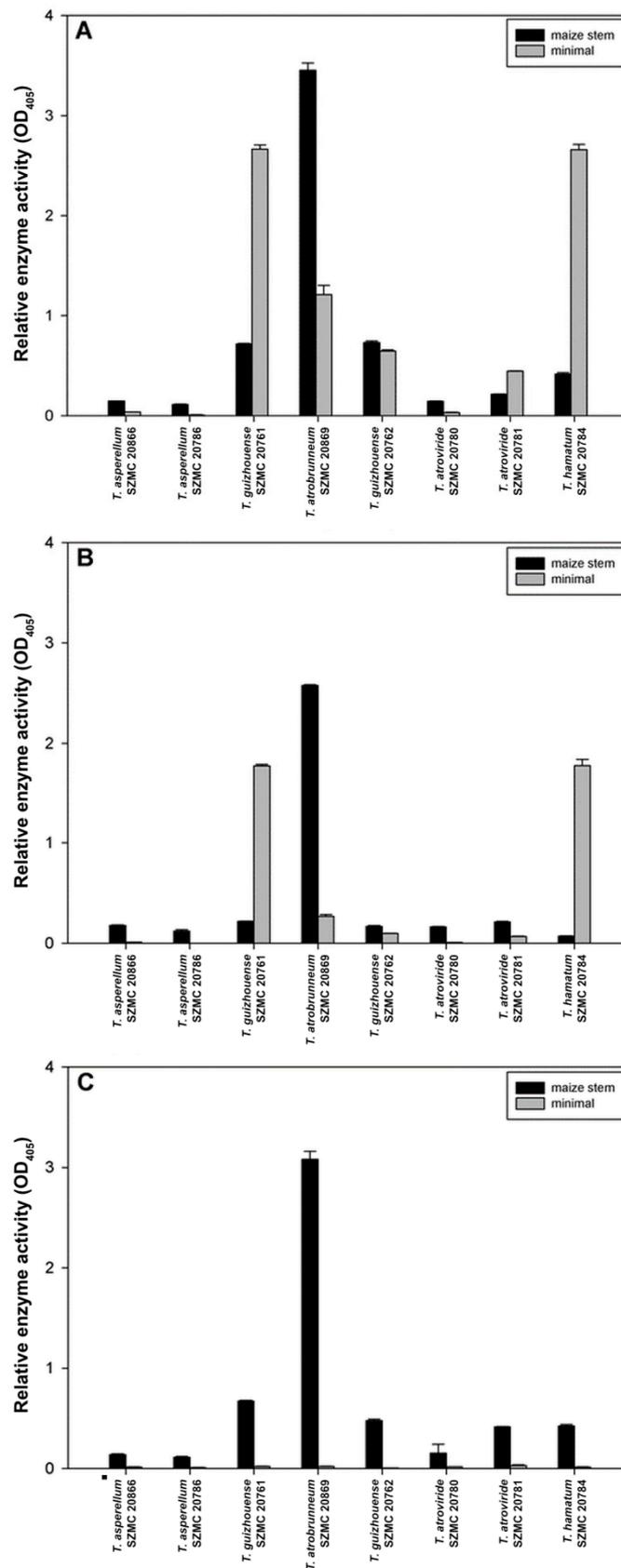


Figure 2. Extracellular enzyme activities of *Trichoderma* strains in liquid minimal and maize stem medium (mean \pm SE, $n = 3$). (A): β -glucosidase, (B): cellobiohydrolase, (C): phosphatase.

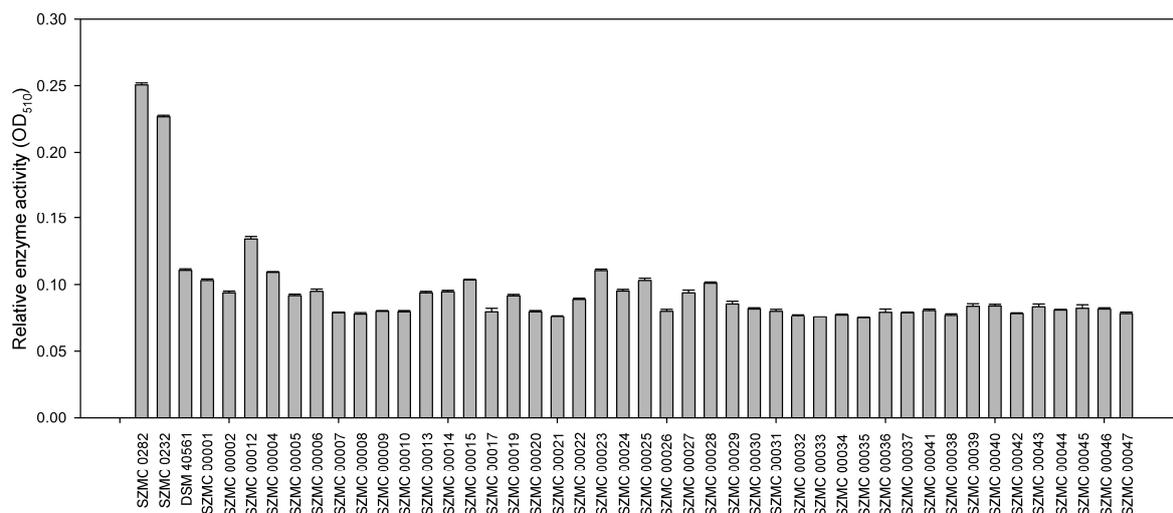


Figure 3. Peroxidase activities of *Streptomyces* isolates (mean \pm SE, $n = 3$).

As the growth kinetics of strain *A. vinelandii* SZMC 22195 revealed that it reached the concentration of 10^8 cells ml^{-1} after 4 days of incubation in nitrogen-free liquid medium and the cell concentration increased further until day 7 (Figure 4), this strain was selected as the nitrogen-fixing component of the soil inoculant.

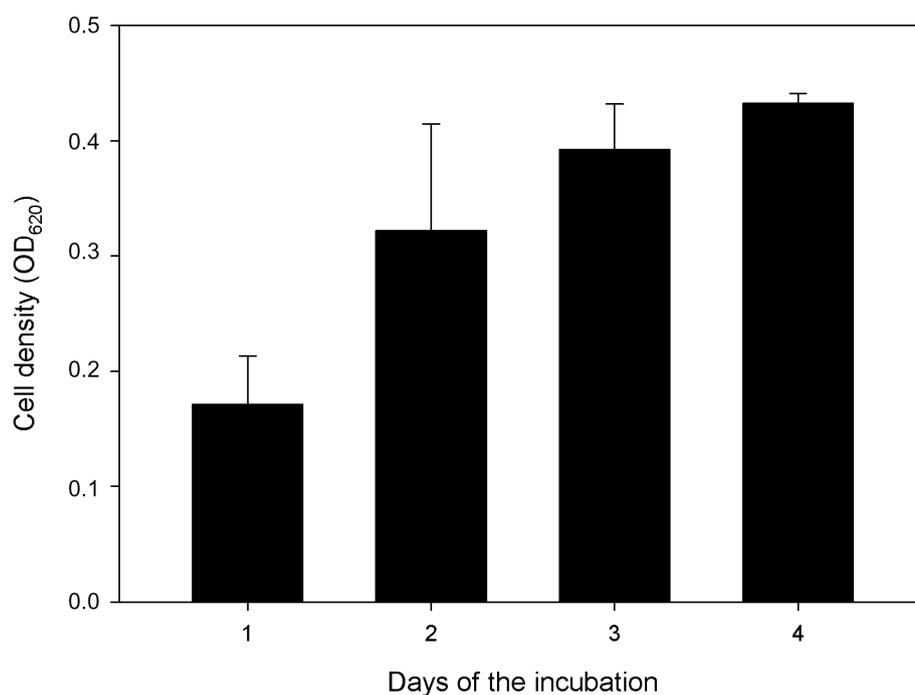


Figure 4. Growth kinetics of *Azotobacter vinelandii* SZMC 22195 in nitrogen-free liquid medium (mean \pm SE, $n = 3$).

3.2. Influence of *T. asperellum* Strain SZMC 20786 on the Shoot and Root Growth and Photosynthetic Activity of Tomato Plants

In the case of the *T. asperellum* strain SZMC 20786 selected for the composite bioinoculant, direct plant growth promotion and effects on photosynthetic activity were examined on tomato plants. Significant increases in the fresh weight of the roots and shoots could be recorded in comparison to the control plants (Figure 5). The results deriving from the measurements of stomatal conductance and CO_2 assimilation are shown in Figure 6. Treatment with strain SZMC 20786 resulted in a non-significant

increase in stomatal conductance (Figure 6A), coupled with a significant increase in both the CO₂ assimilation (Figure 6B) and the total sugar content (Figure 6C), indicating an increased photosynthetic activity in the plants treated with *T. asperellum*.

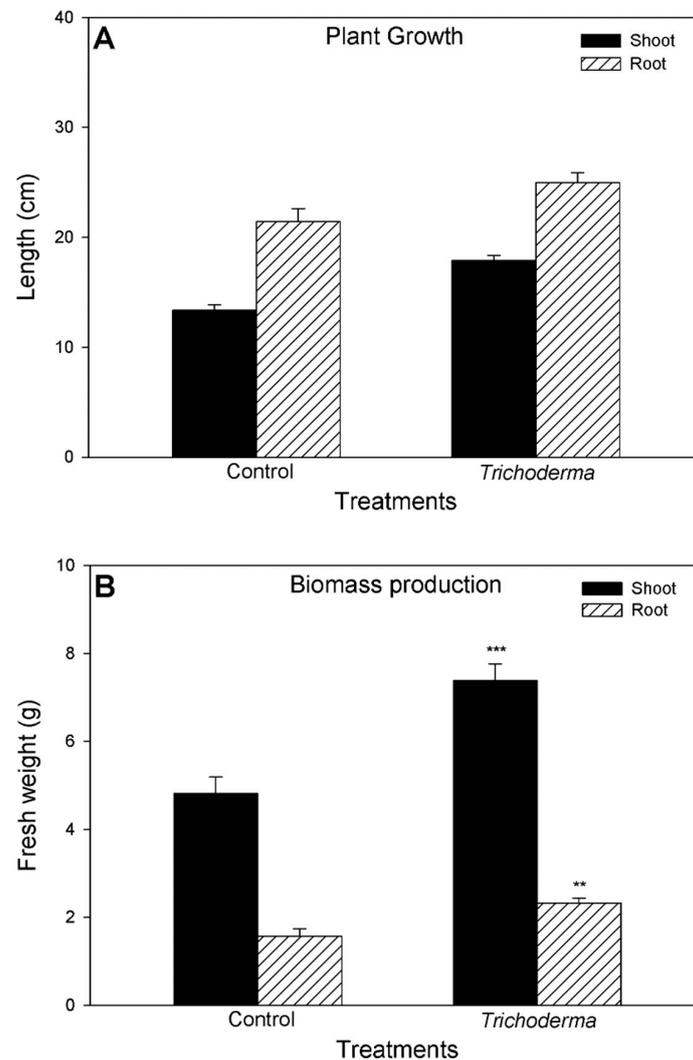


Figure 5. Effect of *T. asperellum* SZMC 20786 treatment on the growth parameters of tomato plants. (A) Root and shoot growth (mean \pm SE, $n = 10$), (B) root and shoot biomass (mean \pm SE, $n = 10$). Treated samples marked with asterisks are significantly different from the untreated control at $p \leq 0.01$ (**), or $p \leq 0.001$ (***)

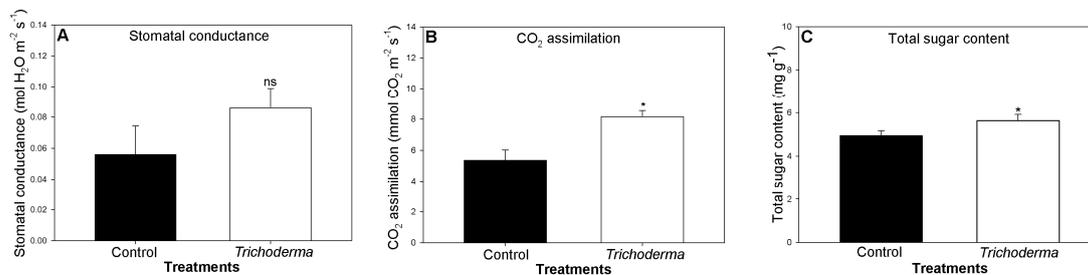


Figure 6. Changes in stomatal conductance (A), CO₂ assimilation (B), and total sugar content (C) in the leaves of tomato plants 6 weeks after treatment with *T. asperellum* SZMC 20786 (mean \pm SE, $n = 5$). Treated samples marked with asterisks are significantly different from the untreated control at $p \leq 0.05$ (*), ns: not significant.

This is also supported by the changes of the chlorophyll *a* fluorescence induction parameters: the maximal quantum yield of PSII photochemistry (Fv/Fm), the actual quantum yield of PSII electron transport in the light-adapted state (Φ PSII), the photochemical quenching coefficient (qP), and the non-photochemical quenching (NPQ) were increased in plants treated with *T. asperellum* strain SZMC 20786; however, a significant change could be measured only for qP (Figure 7). The *T. asperellum* treatment also resulted in non-significant increases in the levels of chlorophyll *a* + *b* and carotenoids (Figure 8).

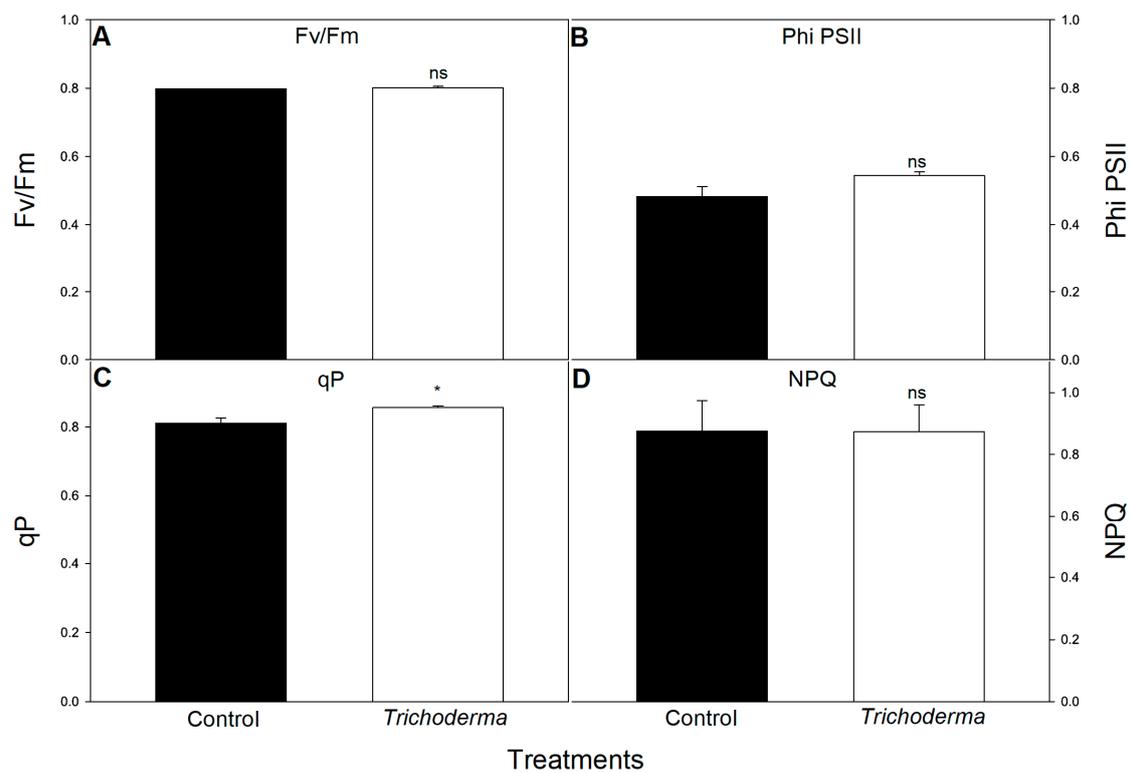


Figure 7. Changes of chlorophyll *a* fluorescence induction parameters ((A): Fv/Fm; (B): Φ PSII; (C): qP; (D): NPQ) in the leaves of tomato plants 6 weeks after treatment with *T. asperellum* SZMC 20786 (mean \pm SE, $n = 5$). Treated samples marked with asterisks are significantly different from the untreated control at $p \leq 0.05$ (*), ns: not significant.

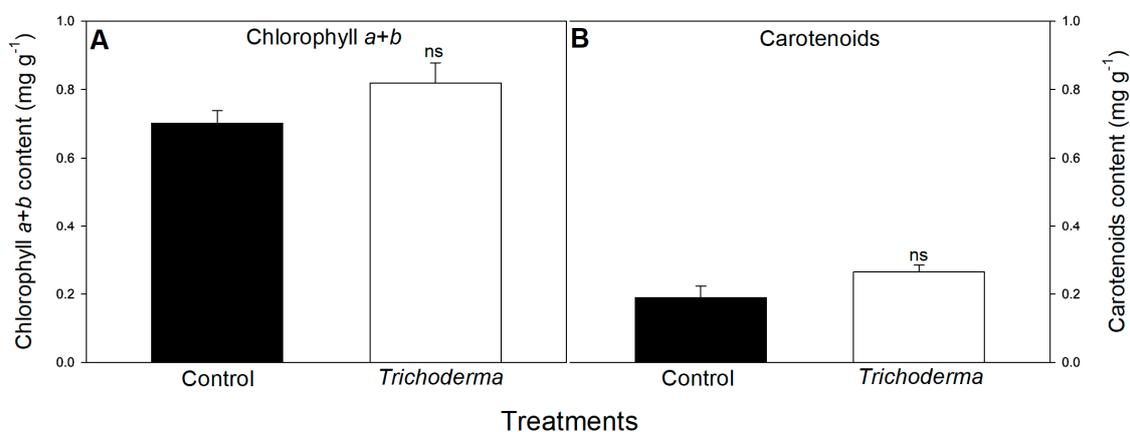


Figure 8. Changes in the content of chlorophyll *a* + *b* (A) and carotenoids (B) in the leaves of tomato plants 6 weeks after treatment with *T. asperellum* SZMC 20786 (mean \pm SE, $n = 5$). ns: not significant.

3.3. Solid State Fermentation of Plant Stem Residues with *T. atrobrunneum* in Comparison with *T. reesei*

The extracellular enzyme activities of *T. atrobrunneum* SZMC 20869 (THSC) selected for the composite bioinoculant were compared with those of the industrially important, hypercellulolytic *T. reesei* strain QM9414 (SZMC 22616) in SSF experiments performed on the residues of 4 different crop plants (wheat, maize, sunflower, and canola) as substrates (Figure 9). Strain SZMC 20869 was able to produce β -glucosidase, cellobiohydrolase, β -xylosidase, and phosphatase activities on all four examined stem residues. Although the industrial strain of *T. reesei* produced larger amounts of β -glucosidase, cellobiohydrolase, and β -xylosidase—the three examined plants' cell-wall-degrading enzymes (PCWDEs)—on maize stem and canola stem residues, and was also a better producer of the two cellulolytic enzyme activities on sunflower stem residues than *T. atrobrunneum* SZMC 20869; the selected *T. atrobrunneum* strain was more efficient in production of all examined PCWDEs on wheat straw as the substrate. Regarding phosphatase activities, strain SZMC 20869 had been proved to be better than *T. reesei* on sunflower stem residues and equal to it on maize stem residues.

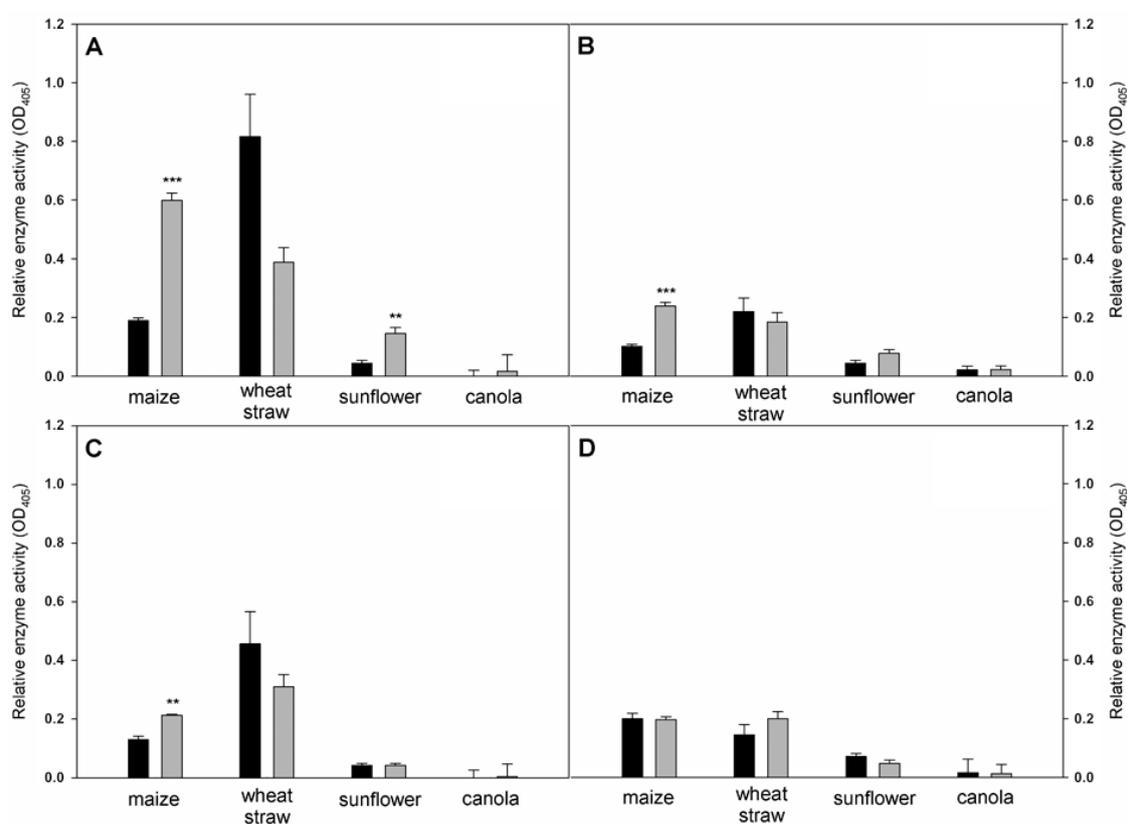


Figure 9. Extracellular enzyme activities of *T. atrobrunneum* SZMC 20869 and *Trichoderma reesei* SZMC 22616 after 8 days of solid-state fermentation on stem residues of wheat, maize, sunflower, and canola plants as substrates. (A) β -glucosidase, (B) cellobiohydrolase, (C) β -xylosidase, and (D) phosphatase (mean \pm SE, $n = 3$). ■: *T. atrobrunneum*; ▒: *T. reesei*. Columns of *T. reesei* marked with asterisks are significantly different at $p \leq 0.01$ (**), or $p \leq 0.001$ (***)

3.4. Field Experiment with the Combination of the Selected Bioinoculant Strains in Tomato Culture

Soil initial chemical and physical characteristics of the experimental area were as follows: pH: 7.6 (KCl), K_A : 35, carbonate: 10.6 m/m%, humus content: 1.84 m/m%, P_2O_5 : 2423 mg/kg, K_2O : 669 mg/kg, NOX-N: 11 mg/kg; SO_4 -S: 39 mg/kg, Cu: 4.4 mg/kg, Zn: 7.6 mg/kg, Mn: 13 mg/kg, Mg: 233 mg/kg, and Na: 99 mg/kg.

Changes in the soil macroelement, microelement, and humus content were monitored three times during the cultivation (Table 2). The initial potassium, sodium, and manganese contents of the total

area determined from soil samples taken on 11th May were 669 mg kg^{-1} , 99 mg kg^{-1} , and 13 mg kg^{-1} , respectively. According to the statistical analysis, the K_2O , Na, and Mn content of the soil changed significantly in the treated area compared to the control area. This could be the result of a more efficient uptake of K_2O , Na, and Mn in the case of the treated plants; however, it is also possible that the elements P, Na, and Mn could have been sequestered in the microbial biomass. The average tomato fruit crop yields per row were 22,470 g and 22,810 g for the control and treated rows, respectively; however, the increase in the crop size in the case of the treated rows did not prove to be significant. The average total green mass of control and treated rows were 11,613 g and 11,032 g respectively, with no significant difference.

Table 2. Changes in the macro- and microelement content of the soil during the cultivation period of tomato.

Parameter	Measure	Sampling Time	Mean \pm SD	
			Control Rows	Treated Rows
Humus	m/m %	I.	1.89 ± 0.04	1.98 ± 0.11
		II.	1.57 ± 0.09	1.90 ± 0.33
		III.	1.90 ± 0.10	1.93 ± 0.19
P_2O_5	mg kg^{-1}	I.	2478.33 ± 182.57	1895.88 ± 125.12
		II.	1677.67 ± 69.04	1546.88 ± 135.31
		III.	1694.00 ± 86.41	1145.25 ± 368.56
K_2O	mg kg^{-1}	I.	702.67 ± 23.70	558.00 ± 76.90 *
		II.	701.67 ± 26.66	451.75 ± 64.14 ***
		III.	821.33 ± 54.63	480.13 ± 85.53 ****
$\text{SO}_4\text{-S}$	mg kg^{-1}	I.	16.33 ± 0.47	17.00 ± 1.22
		II.	15.00 ± 0.82	16.88 ± 2.32
		III.	7.33 ± 0.94	7.25 ± 2.11
$\text{NO}_x\text{-N}$	mg kg^{-1}	I.	3.93 ± 0.54	4.68 ± 0.96
		II.	4.67 ± 1.96	4.64 ± 1.25
		III.	5.17 ± 0.66	5.24 ± 1.96
Na	mg kg^{-1}	I.	49.33 ± 0.94	45.00 ± 4.00
		II.	45.00 ± 12.75	25.88 ± 12.69 *
		III.	46.33 ± 4.11	33.88 ± 5.37
Mg	mg kg^{-1}	I.	209.67 ± 2.62	220.75 ± 2.59
		II.	194.33 ± 8.22	194.75 ± 12.16
		III.	229.00 ± 11.22	213.38 ± 25.70
Mn	mg kg^{-1}	I.	14.33 ± 2.05	10.75 ± 0.66
		II.	19.33 ± 1.25	17.63 ± 1.58
		III.	19.67 ± 1.25	15.88 ± 3.18 *
Zn	mg kg^{-1}	I.	6.20 ± 0.78	5.75 ± 0.72
		II.	5.70 ± 0.37	6.44 ± 0.74
		III.	4.73 ± 0.40	5.11 ± 1.16
Cu	mg kg^{-1}	I.	6.23 ± 1.11	4.36 ± 0.44
		II.	8.20 ± 0.33	7.76 ± 0.44
		III.	9.20 ± 1.55	9.15 ± 3.00

Soils samplings were performed on I: 22 June, II: 6 July, and III: 3 August 2019. Data marked with asterisks are significantly different from the untreated control at $p < 0.05$ (*), $p = 0.001$ (***), $p < 0.001$ (****).

4. Discussion

The soil inoculant developed during this study contains two *Trichoderma* strains (*T. asperellum* and *T. atroviride*) and two bacteria (*A. vinelandii* and *S. albus*) with potentially synergistic beneficial effects.

Members of the genus *Trichoderma* are geographically widespread filamentous ascomycetes from Hypocreales [21], which have long been known as agriculturally important, beneficial fungi with antagonistic abilities toward plant pathogenic fungi. *Trichoderma* antagonism is based on a series of different mechanisms including the competition for space and nutrients, antibiosis, mycoparasitism [22], plant growth promotion [23], enhancement of plant resistance to diseases [24,25], and relieving abiotic stress in plants [26]. These properties make many representatives of the genus *Trichoderma* (e.g., the THSC, *T. asperellum*, *T. atroviride*, or *T. virens*) to potential ingredients of soil inoculant and biocontrol preparations. However, when the practical application of a *Trichoderma* strain is planned, an exact, sequence-based, species-level identification is important to prevent the spread of species known as the causal agents of the green mold disease in mushroom cultivation [27–29] or of opportunistic infections in immunocompromised humans [30].

Strain *T. asperellum* SZMC 20786 was selected as a component of the composite soil bioinoculant due to its good in vitro antagonistic performance against different plant pathogenic fungi and its abilities to promote the growth of tomato plants and increase their photosynthetic activities. According to the literature, one of the mostly studied strains of “*T. asperellum*” for plant growth promotion is strain T203 [31], which, however, was recently reidentified as *T. asperelloides* [32]; thus, the number of studies about the plant growth promoting activities of *T. asperellum* sensu stricto is restricted. Qi and Zhao [33] demonstrated the plant growth promoting activities of *T. asperellum* strain Q1 on cucumber plants, and the positive effects were detected even when the plants were subjected to salt stress. In our study, *T. asperellum* strain SZMC 20786 showed positive effects on the CO₂ assimilation, total sugar content, and the photochemical quenching coefficient of tomato leaves. Similar results were obtained by Doni et al. [34], who found plant growth promotion as well as increased stomatal conductance and CO₂ assimilation in rice plants treated with *Trichoderma* sp. isolates. Other studies reported about the positive effects on the photosynthetic pigments exerted by *T. harzianum* strains on tomato [35] and wheat plants [36], as well as by *T. hamatum* on mungbean [37].

Another beneficial trait of many *Trichoderma* strains is their efficient ability to produce PCWDEs including cellulases and xylanases, which can be exploited both in the biotechnological industry and in the agriculture for the degradation of cellulose and xylan-containing materials, e.g., stem residues [38,39]. In accordance with these, another *Trichoderma* component, a *T. atroviride* isolate possessing good cellulase, xylanase, and phosphatase enzyme production capabilities has been included in the assembled soil inoculant.

The species *A. vinelandii* involves Gram-negative, aerobic, free-living soil-inhabiting gamma-proteobacteria from the *Pseudomonadaceae* family. This species is capable of direct nitrogen fixation from the atmosphere by three distinct nitrogenase systems under fully aerobic conditions, thereby providing plant roots with bioavailable nitrogen source [40]. This aerobic bacterium possesses various protection mechanisms for nitrogenase against oxygen, which include alginate formation [41]. Furthermore, phytohormone and siderophore synthesis as well as phosphate solubilization are also among the abilities of *Azotobacter* species. These properties were suggested to be directly involved in their plant growth promotion effect [42]. Considering the above facts, an *A. vinelandii* strain has also been included in the soil inoculant.

As peroxidases were shown to play an important role in the humification properties of *Streptomyces* species [43], the potential humus-producing component of the bioinoculant was selected from *Streptomyces* isolates with a peroxidase assay, which revealed a *S. albus* strain as the best peroxidase-producing isolate. The species *S. albus* is among the geographically most widely distributed members of the genus *Streptomyces*; it could be isolated from various habitats including sea sediments, sponges, and insects [44]. This species was shown to be able to biosynthesize heterologously diverse and important natural products and was suggested to encode important natural product gene

clusters [44]. Based on the genome sequence of *S. albus*, the secretion of a series of degradative enzymes (including amylases, chitinases, glucanases, proteinases/peptidases, and a cellulase) with supposed roles in breaking down heterogeneous alternative food sources in soil could be predicted [45]. The feather-degrading abilities of *S. albus* could be exploited during the development of an eco-friendly biofertilizer feather compost [46].

Several publications are available in the literature about the combined application of beneficial microorganisms for plant growth promotion and biological control. There are studies about the co-application of multiple bacteria including the combination of *Bacillus* and *Pseudomonas* for growth promotion and biological control of soil-borne diseases in pepper and tomato [47], as well as to increase rice yields [48], or the application of a mixture of fluorescent pseudomonads to suppress take-all disease of wheat [49]. There are also examples of co-application of fungi and bacteria, for instance the combination of *T. koningii* with fluorescent pseudomonads for the control of take-all disease of wheat [50], or the combination of *T. harzianum* with an *Alcaligenes* strain for the reduction of the incidence of rot disease caused by *Phytophthora capsici* in black pepper [51]. Other microbial combinations have been applied to promote the growth of tomato [52], to control tobacco diseases [53], or to improve the salinity tolerance of *Vicia faba* [54]. The combination of microorganisms could also increase the dry matter yield and nutrient uptake by wheat grown in a sandy soil [55].

5. Conclusions

The selection of the components for the composite soil inoculant assembled in this study was driven by the idea to combine various crop protective and plant growth promoting traits (biocontrol against plant pathogenic fungi, phosphorous mobilization, stem degradation, humification, and nitrogen fixation) of different microorganisms into a preparation, which may also have the potential to exert an increased consistency of field performance under various environmental conditions. The screening strategy performed during this study proved to be applicable for the assembly of a promising composite soil bioinoculant with notable application potentials.

Author Contributions: H.A., P.K., C.V., L.M. and L.K. contributed to the design and implementation of the research and evaluation of the results, and participated in the preparation of the manuscript; P.K. and L.K. performed the in vitro antagonism and liquid fermentation experiments; B.B. and L.M. contributed to the selection of the bacterial components of the bioinoculant; P.K. and P.P. designed and performed the experiments on tomato plants; V.D.N. and M.T. contributed to the solid-state fermentation experiments; H.A. performed and evaluated the field experiment; I.S. performed the measurements of the soil samples taken; and A.S. performed data curation and statistical analyses. The Tables and Figures were prepared by H.A., P.K., B.B., P.P., V.D.N. and C.V. All authors have read and agreed to the published version of the manuscript.

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