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Abstract

Wheat is one of the most important crops in the world. Its production can be influenced by a diversity of beneficial and pathogenic rhizospheric microbes, including fungi. Amongst them, beneficial *Trichoderma spp*. can be used as alternatives to chemical fertilizers, as they are cheaper and harmless to the environment. Our study aimed to isolate, identify, and characterize *Trichoderma spp.* from Lebanon associated to wheat. Two *Trichoderma* strains belonging to *T. afroharzianum,* and *T. guizhouense* species, were isolated and found to be endophytes enhancing root growth and producing

IAA. Inoculation also improved seedling development, and boosted general production. These *Trichoderma spp.* have thus the capacity to be used as organic fertilizers for wheat.

Keywords: *Trichoderma spp*., Endophyte, Auxins, 3-indole-acetic acid, Plant root growth promotion, Wheat yield.

Introduction

Wheat (*Triticum* sp. L.) is one of the most important crops in the world, ranking third behind corn and rice, while providing the basic nutrients (carbohydrates, proteins, vitamins, minerals, and fibers) to humans (Asseng et al., 2011). The world population is projected to exceed 8 billion by the end of 2022, and the demand for wheat is expected to exceed 880 million metric tons by 2050 (https://www.fao.org/worldfoodsituation/csdb/en/). Wheat culture has thus to be widened for overcoming world increasing demand. In addition, recent challenging crisis like Covid-19 (coronavirus disease 2019) pandemic and the world conflicts negatively impact wheat production and will probably threaten wheat markets in the near future (Glauben et al. 2022; ASCAME, 2022). On the other hand, communities are requesting for sustainable, cleaner, and harmless production approaches to avoid more climate change development. In this context, alternative strategies have to be followed, and finding new safer solutions is necessary in order for example to reduce the widespread use of chemical fertilizers which negatively affect earth's natural resources. As a result, recent studies aimed at improving biofertilizers and plant bio stimulants using beneficial bacteria, yeasts, or fungi. Among fungi, *Trichoderma* spp. are ubiquitous free-living fungi (Almi & Dehimat, 2016; Esposito & da Silva, 1998) of the *Hypocreaceae* family, occurring widely in all soils (Ghazanfar et al., 2018) and representing major components of the soil mycoflora (Kubicek et al., 2003; Widden & Abitbol, 1980). These ascomycete fungi, which inhabit root ecosystems, grow and proliferate using carbohydrates secreted by plant roots. They also behave as endophytes, penetrating the epidermis of the root tissue (rhizodermis), and few cell layers below (Topolovec-Pintarić, 2019). They proliferate better at mesophilic temperatures (15–35 ° C) (Carro-Huerga et al., 2021) and tolerate a wide pH range (Topolovec-Pintarić, 2019). These fungi improve plant photosynthetic capacity, and induce defenses (Vargas et al., 2009, 2011) and many stress-sensitive genes (Alfano et al., 2007; Brotman et al., 2012; Morán-Diez et al., 2012). *Trichoderma* are among the best studied biological control microbes, and are marketed as active ingredients in biofertilizers (plant growth promoters), biopesticides, bioremediates,

and natural resistance stimulants. Consequently, their application reduces production costs, and negative environmental impact (Dennis and Webster 1971a; Dennis and Webster 1971b; Harman and Kubicek 2002; Ghazanfar et al. 2018). *Trichoderma* fungi improve plant growth through the solubilization of nutrients, and therefore have better effects under nutritional and abiotic stress conditions (Mastouri et al., 2010, 2012; Shoresh et al., 2010). The use of *Trichoderma* in seed coating also improves physiological stresses such as aging, and seed dormancy (Delgado-Sánchez *et al*. 2010; 2011; Mastouri *et al*., 2010). The plant hormone auxin controls many aspects of development including plant tissue growth, cell division, cell differentiation, and protein synthesis. The plant naturally produced auxin IAA that can be also produced by microorganisms like fungi including Trichoderma.

In this study we identified and characterize two *Trichoderma* strains from Lebanon and tested their ability to promote wheat growth.

Materials and methods

Plant material:

The wheat cultivar 'Apogee' was used for fungal characterization (G. Li *et al*. 2017; Gatti *et al.* 2018). Apogee has a relatively brief life cycle under long days, and without vernalization, it flowers 25 days after planting.

Soil and plant sampling:

Samples in this study were collected on May 2020 (late spring and early summer) from three different sites in Lebanon namely: 1) The international center for agricultural research in the dry areas (ICARDA) -Terbol; 33.81263953421374, 35.99166470541601, 2) Lebanese agricultural research institute (LARI) – Tal Amara; 33.86500666714277, 35.984715713142634, and 3) Doueir; 33.39043029381532, 35.417987723093724. For every location, five samples from soil, together with five plant samples were screened for *Trichoderma spp*. .

Soil samples were taken from the soil section called "horizon", using a 0 to 15 cm top layer shovel, representing the surface area of the profile, where nutrients and most of the roots are present, and where nutrient absorption takes place. This also corresponds to the area where *Trichoderma* spp. are generally found. Sampling was carried out within a radius of 10 to 30 cm from the base of the plant depending on the case. Each sample was transferred in an appropriate plastic bag with its reference (place, date,

sample number, etc.). Five samples were collected, and homogenized before the final packaging of approximately 1 kg of the sample, placed in plastic bags (to avoid contamination) was kept frozen (-20°C).

For plant sampling, 5-6 independent plant samples (root and stem) spaced at least 10 m apart were placed in plastic bags and kept frozen (-20°C).

Fungus isolation from soil:

1g of soil was homogenized in 10 ml sterile distilled water by thorough vortexing, then filtrated onto sterile Miracloth®. The filtrate was then plated onto 9 cm diameter petri dishes (100 ul filtrate plated per dish), containing potato dextrose agar (PDA) amended with rose Bengal (0.15 g/L), pentachloronitro-benzene (0.15 g/L), chloramphenicol (34 µg/ml), and ampicillin (100 µg/ml). One petri dish was plated per soil mixture.

Fungus isolation from plants:

The internal colonization of roots or stems by potential *Trichoderma* sp. was detected following surface sterilization of approximately 2 cm root fragments for 10 sec in 95% ethanol, rinse in sterile water for 10 sec, submersion for 20 sec in 2.5% sodium hypochlorite, then washed three times (2 times for 20 sec, and a third time for 60 sec) in sterile water. Fragments were then let dry under laminar flow on sterile filter. 0.5 cm fragments were placed on PDA amended with rose Bengal, pentachloronitro-Benzene, chloramphenicol and ampicillin. Plates were incubated at 24°C in the dark for 4-6 days, until fungal development. After 4 days a large number of fungal strains appeared and were transplanted (1st transplanting incubate at 26°C). Later, 10 days old, pre-incubated Petri dish (26°C) was selected, in order to make the 2nd transplant on PDA complemented with Rose Bengal (for Soil and Plant samples). A week after, purification was done by scraping half a colony, depending on the growth rate. Purification was done by plating 1/10, 1/100 or 1/1000 dilutions on PDA complemented with Rose Bengal. After 7 days, the fungal strains were transplanted on PDA and incubated at 26°C.

DNA isolation, sequencing and phylogenetic analyses:

DNA isolation: Liquid culture were done in potato dextrose broth (PDB) medium (50ml), with 5 plugs (agar + fungi) for 3 days (under agitation) according to (Atoui et al., 2012), with modifications as follows: for approximately 200 mg of powder (ball or mortar grinding). 1 mL of extraction buffer (200 mM Tris – HCl, pH 7.5; 288 mM NaCl; 25 mM EDTA, pH8.0; 0.5% SDS) was added and vortexed vigourously till homogenization. Tubes were centrifuged at 12,000 rpm for 5 minutes (4°C). 750 µL

were then transferred to a new tube, and 215 µL of 3M potassium acetate were added, mixed and incubated on ice for 30 min, centrifuged at 12,000 rpm for 15 min at 4°C. 700 µL of the supernatant were collected, transferred to a new tube and 500 µL of cold isopropanol (-20°C) were added. Tubes were centrifuged at 12,000 rpm for 15 min at 4 ° C. The supernatant was removed, and the pellet washed with 500 µL of cold 70% ethanol (-20°C) for 5 min (4°C). The tubes were vortexed, and centrifuged at 12,000 rpm for 5 min at 4 ° C. The supernatant was removed, the pellet was briefly dried, and resuspended in 200 µL of sterile milliQ H2O + 1 mg/mL RNase A (1/10 stock solution at 10 mg/mL). The sample was incubated for 1h at 37 ° C, and DNA concentration was done using NanoDrop. As control, 2 to 5 µL of each sample was run on 0.8% agarose gel.

DNA sequencing: Further *Trichoderma* identification was done by partial sequencing of the transcribed spacer sites (ITS) (550 bp), the Calmoduline gene (CAL) (458 bp) and the Elongation Factor (EF) gene (350 bp). The oligonucleotides used for amplification and sequencing are: for the calmoduline (CAL-228F: GAGTTCAAGGAGGCCTTCTCCC / CAL-737R: CATCTTTCTGGCCATCATGG), for the elongation factor gene (EF1-728F: CATCGAGAAGTTCGAGAAGG/ EF1-986R: TACTTGAAGGAACCCTTACC), and for the internal transcribed space (ITS1: TCCGTAGGTGAACCTGCGG/ ITS4: TCCTCCGCTTATTGATATTGC). The nucleotide sequences were compared with those deposited in GenBank using the BLAST program on the NCBI website.

The phylogenetic tree was constructed using the MEGA-X program (Kumar et al., 2018). The sequences used correspond to a concatemer of the CAL, EF and ITS1 sequences. Supplementary Table (1) shows detailed supplementary information for the strains used in the phylogenetic analyses, with their corresponding geographic origin, substrate, voucher number, and GenBank accession numbers. Briefly, the evolutionary history was inferred by using the Maximum Likelihood method, and Tamura-Nei model (Tamura & Nei, 1993). The bootstrap consensus tree, inferred from 1000 replicates (Felsenstein, 1985), is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions, reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985). Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join, and BioNJ algorithms, to a matrix of pairwise distances, and were estimated using the Tamura-Nei model, and then the topology, with superior log likelihood value was selected. This analysis involved 26 nucleotide sequences.

Morphological characterization of the *Trichoderma* strains:

The fungal strains were maintained on PDA with glucose at 26°C for 7 days. Microscopic visualization of *Trichoderma* conidiophores was done using fungi grown for 3 days on a PDA plate complemented with glucose (Glc).

Endophytism tests:

The endophytic capacity was tested using the Apogee spring wheat variety. The endophytism behavior of the 2 *Trichoderma* strains was examined using confocal microscopy. Two methods of inoculation were used and described below as the PDA inoculation method and the spore inoculation method. This experiment was repeated twice, and 5 samples were taken for each condition (with and without *Trichoderma*) for analysis.

For the PDA inoculation method, the seed sterilization protocol was modified from Jaber (2018). Seeds were soaked for 5 min with bleach (0.6% sodium hypochlorite) with stirring (150-200 rpm), then rinsed three times for 10 min with sterile milliQ water. They were then stratified for 3 to 5 days in the dark at 4°C, in distilled sterilized water. The inoculation was done on a PDA with 1% Ampicillin (100 mg/ml)/1% Chloramphenicol (34 mg/ml) dish. Five wheat seeds were spread over a circle of 3 cm diameter and cultured at 26 °C and in the dark. 24 hours later, a *Trichoderma* plug was added (7 days of culture on PDA) in the center of the petri dish and incubated at 26°C. Finally, roots were harvested after 5 days of culture.

For the spore inoculation method, the seed sterilization was carried out as mentioned above. The coating of the seeds with *Trichoderma spores* was then done as follow: the seeds and a spore solution (three 7 days old Trichoderma petri dishes were used, where germinated spores were rubbed off by distilled sterilized water, then filtered to collect a free-hyphae spore solution, and finally measure the concentration using Thoma's-cell slide to get a 10^7 spores / 10ml final concentration. The solution was then shacked for 20-30 min, then dried for a few minutes in the hood, before sowing. Seeds were then sown in glass tubes in ½ MS (2.2g / L) with phytagel (2g / L), about 5mm deep, to prevent the roots from remaining on the surface.

Root samples treatment for imaging: After 5 days of incubation, roots were collected, gently cleaned from the excess of external fungal colonization, cut in 1.5 / 2.0 cm length, and washed with water or PBS 1X: 20 min. Root fragments were fixed overnight in a mixture of 95% Ethanol - 100% acetic acid (3: 1) (v: v). Samples were then stabilized1h stabilization 1X PBS.

Roots were included in 1X PBS with 3% agarose for 5 min ("Seaken LE Agarose for electrophoresis") (0.6 g of agarose in 20 mL). For staining, cross-sections were immersed in the staining solution (20 µg/mL propidium iodide; 10µg/mL WGA-Alexa Fluor 488, 0.02% Tween 20 made up in 1X PBS) for 2 min, immersed in water for few seconds to remove the staining solution excess. Cross sections of wheat roots were visualized to check endophytism using Confocal microscope (Zeiss LSM880).

In planta wheat yield evaluation:

To evaluate wheat yield under controlled conditions, 10 days old plantlets, from seeds, pre-cultured on both, PDA (PDA approach), and phytagel ½ MS media (tube approach), were used. 32 plantlets from each treatment (with, and without *Trichoderma*) were transplanted to sterilized peatmoss soil (www.jiffygroup.com- Substrates for the professional horticulture) with perlite (3:1). Wheat yield and yield components, including number of spikes/plants, number of grains/plants, grain weight/plant (g), whole plant dry weight (g), hundred grain weight (g), and harvesting index (HI%). were then measured.

All plants were kept in a growth chamber, under 16h: 8h light to dark period, with 265 µE m−2 s−1, at 22 ^{o}C, and 20 ^{o}C, day to night temperatures, and 60% relative humidity (RH). Regular irrigation was practiced, in parallel with fertilization practices at 14:12:32 N:P:K, plus 41 g Hortrilon engrais micronutrients (B 0.5%, Cu 2.5%, Fe 5.0%, Mn 2.5%, Mo 0.5%, and Zn 0.6%) (https://www.fertil.fr/).

Statistical analysis:

Statistical analysis was conducted for the treatments, on a completely randomized design (CRD), and a factorial completely randomized design, with 32 replicates. The R software (R 3.3.4; R development core team, 2017) was used to analyze experimental data, calculate means, standard deviations, bar charts, and interaction, using the R package "ggplot2" (Wickham, 2009). Analysis of variance (ANOVA) was performed using the "lmerTest" R package (Kuznetsova et al., 2017).

To test the effect of the *Trichoderma* strains on wheat root development, *Trichoderma* spores (10^7 spores/10 ml) were incubated with wheat seeds in a tube containing 0.5X MS medium (Kapusi & Stoger, 2022) with phytagel without sugar. 10 days after germination, we measured the primary root growth.

Trichoderma auxin production:

Auxin production was measured using the Gusmiaty method (Gusmiaty et al., 2019) with some modifications in presence or absence of tryptophan.

Briefly, five plugs of each *Trichoderma* strain with, and without tryptophan were incubated in 0.5X MS liquid medium (2151,45 mg/L), 2-(N-morpholino) ethanesulfonic acid (MES) (0.25 g/L), glucose 2%, pH = 5.78 adjusted with 1M KOH (3 biological repeats).

To determine the auxin concentration (IAA in our case), Salkowski reagent, and IAA standard curve were done, according to (Gusmiaty et al., 2019). A standard IAA solution curve showing the relationship between the standard IAA solution (x), and its absorbance (y), was made, and allowed the quantification of IAA production by *Trichoderma* using the following equation: Y= a+ bX (a = Intersep, b = Slope (Regression Coefficient), Y = Absorbance, X = Concentration).

Preparation of Total Protein Extracts and SDS-PAGE:

Total protein extracts were prepared from wheat grains grounded in liquid nitrogen using mortar and pestle. From 50 mg of flour, total soluble proteins were extracted at room temperature in 960 µl thiourea/urea lysis buffer (Harder et al., 1999) containing 7 M urea, 2 M thiourea, 6 mM Tris-HCl, 4.2 mM Trizma® base (Sigma-Aldrich, Lyon, France), 4% (w/v) CHAPS) supplemented with 162 µl of the protease inhibitor cocktail Complete Mini (Roche Diagnostics France, Meylan, France). Then, 22,5 µl of dithiothreitol (DTT, 1 M, Sigma-Aldrich), 4 µl of DNase I (Roche Diagnostics) and 10 µl of RNase A (Sigma-Aldrich) were added to the sample. For each sample, the protein extract was stirred for 30 min at 4°C and then centrifuged (35,000g, 15 min) at 4°C. The supernatant was submitted to a second clarifying centrifugation as above. The final supernatant corresponded to the total protein extract. Protein concentration in each extract was measured according to Bradford (1976). Bovine serum albumin was used as a standard. Separation of proteins was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% (w/v) polyacrylamide stacking gel and a 12% (w/v) polyacrylamide resolving gel as described by Laemmli (1970). Electrophoresis was carried out at a constant current of 110 volts (10 mA) for 2 h using a Tris-glycine running buffer with of 0.2%. After electrophoresis, proteins were stained with GelCode® Blue Stain Reagent (Thermo Scientific, Rockford, IL).

Results

Characterization of two Lebanese *Trichoderma* strains isolated from wheat

In order to isolate *Trichoderma* fungi associated to wheat in Lebanon, 40 fungal isolates were isolated in total from three sampling sites. 21 were isolated from soil and 19 were isolated from plants. DNA was prepared from them and the ITS sequences amplified and sequenced. Based on the ITS sequences from these 40 colonies, 2 fungal isolates of the genus *Trichoderma* were identified (Supplementary Table 2). The two isolates were derived from site 3 (Doueir) and were named S3PA (isolated from stem base), and S3SB (isolated from soil). The other isolates included fungi from the genus: *Alternaria, Aspergillus, Boeremia, Cladorrhinum, Fusarium, Macrophomina, Microdochium, Mucor, Penicillium, Stromatinia,* and *Talaromyces* (Supplementary Table 2).

A more precise molecular description of these two *Trichoderma* fungi was done by characterizing the partial sequences of their Elongation factor (EF-1) and Calmodulin (Cal) gene sequences. The three partial sequences (ITS-EF-1 and Cal) were then concatemerized (550, 350, and 489 bp, respectively) and compared to the same concatemerized sequences of 14 *Trichoderma* species (Supplementary Table 1) from the public database and representing the *Trichoderma* diversity. The result of this analysis (Figure 1) showed that the two isolates probably belong to the two species *T. afroharzianum* and *T. guizhouense* belonging to the *Harzianum* species complex.

Morphological characterization of the two Lebanese *Trichoderma* strains

After 48h at 26°C on PDA, the *T. guizhouense* formed an abundant aerial mycelium, cottony and radiating a white color. At 72 h, the formation of a greenish ring was observed, which increased by time of incubation, and diffused in the media, until covering almost the whole petri dish at 168 h. The conidia were visualized from 48h in broad concentric bands (green), in the aerial hyphae (Figure 2A). After 72h of growth on PDA (26°C), *T. afroharzianum* produced an abundant aerial mycelium, cottony, radiating a white color (Figure 2B). A dense hyphae growth in the petri dish center was observed after transplantation of *Trichoderma*, while 96 h later, the formation of a greenish ring was observed, which increased with the time of incubation, and diffused in the media, until covering almost the whole petri dish at 168 h.

We then morphologically characterized the two newly isolated strains. After 3 days, pyramidal conidiophores were repeatedly branched, irregularly arranged in whorls, and showed clusters of divergent, usually unevenly folded, barrel shaped, to nearly sub-globose phialides. The ellipsoidal to globose conidia tend to be greenish to light turquoise, sometimes hyaline to cluster in aggregates at the

terminal of the phialides (Zhu & Zhuang, 2015). Conidiophores of *T. guizhouense*, showed opposite branches. The main axis and each branch terminated at a cruciate whorl of 3 ampulliform phialides (Figure 2C). Conidia, appearing within 48–72 h, were typically abundant, and disposed in one, or three concentric rings, around the point of inoculation. Green pigment sometimes diffused in the medium (Figure 2B). After 3 days of incubation on PDA, the pyramidal conidiophores of *T. afroharzianum* showed opposite branches. The main axis, and each branch terminated at a cruciate whorl of 3 ampulliform phialides green to dark green when aging (Figure 2D).

The two isolated strains thus grow rapidly on PDA medium with phenotypic characteristics of other *Trichoderma* stains (Chaverri et al., 2015).

The Lebanese *Trichoderma* strains are wheat root endophytes

Trichoderma fungi were previously described as endophytes (Tseng et al., 2020). In order to know if the two Lebanese strains isolated from wheat behave also as endophytes, two modes of inoculation were tested, either by inoculating the seeds by co-cultivation on PDA medium (PDA inoculation method), either by spore coating of the seeds and germination of the seeds *in vitro* (spore inoculation method, see Material and Method section). In both cases the root sections of the plants were stained with propidium iodide and WGA-Alexa Fluor 488, staining the fungus in green. These sections were observed using confocal microscopy. Using both inoculation methods, fungal hyphae were observed inside the root for the two *Trichoderma* strains (Figure 3). The infection level observed using the PDA inoculation method seemed to be higher than the spore inoculation method that had lower concentration of the spore solution. In addition, *T. guizhouense* infected rhizodermis and cortex cells using the first methods (Figure 3A, B) and mostly rhizodermis using spore coating (Figure 3 E, F). The *T. afroharzianum* strain appears less efficient in root colonization, invading mostly the rhizodermis cell layer using the PDA inoculation (Figure 3.C, D) and colonizing only the root surface using spore coating (Figure 3 G, H). Using leaves of 4 weeks old plants, we were unable to reisolate the inoculated fungi, suggesting that these *Trichoderma* strains are not able to colonize the aerial part of the plant.

Altogether this experiment shows that the two strains can behave as fungal wheat root endophytes but that *T. guizhouense* can invade more efficiently root tissue as compared to *T. afroharzianum* and that the inoculation method can influence the level of colonization.

The two *Trichoderma* strains promote wheat root growth and wheat aerial development and produce auxin

Trichoderma strains have been often used as plant growth promoting microorganisms (Tseng et al., 2020). In order to know if the two strains studied here can also behave as plant growth promoting fungi (PGPF), we tested their growth promotion effect on young seedlings *in vitro*, using the spore coating method and ½ MS medium without sucrose.

This experiment shows that the presence of the two *Trichoderma* strain resulted in a significant increase of the primary, and secondary root growth, as well as the aerial part of the plant (Figure 4A). Total root, and aerial length (cm) of inoculated wheat plants, with and without *T. guizhouense* and *T. afroharzianum* respectively, is shown in Figure 4B. The results indicated that plants which were incubated with either *T. guizhouense* or *T. afroharzianum* had increased root growth and aerial development as compared to non-inoculated plants. Root length was increased by 37.6% and 37.7% for plants inoculated by *T. afroharzianum* and *T. guizhouense* respectively. For the aerial part we observed a 5.2% increase in length with both fungi. In this experiment, the inoculation by the *Trichoderma* strains enhanced principal root length (55%), total root length (70%), and aerial part lengths (35%) as well as total aerial plant weight (Figure 4B and Supplementary Figure 1A).

Total root, and aerial mass of wheat plants, with and without *T. guizhouense* and *T. afroharzianum* respectively are shown in Supplementary Figure 1C. The inoculation with *T. afroharzianum* resulted in an increased of 61% for the total root mass and 41%, for total aerial mass, while the treatment with *T. guizhouense* resulted in an increase of 63% for total root mass and 43%, for the aerial mass.

We further measured the principal root length of inoculated wheat plants over a 10 days period (kinetics), with and without *T. guizhouense* or *T. afroharzianum*. The root growth promoting effect of the two strains was already detectable 2 days after sawing and continued to increase over the 10 days period (Supplementary Figure 1A).

The increased root growth observed in presence of the two strains might result from fungal auxin production. We thus measured auxin production by the two *Trichoderma* strains in culture, in presence and absence of tryptophan, an auxin precursor. This experiment shows that the two strains can produce auxin in culture and that addition of tryptophan enhances IAA production by 2.5%, and 1.23%, for *T. guizhouense* and *T. afroharzianum* respectively.

These experiments show that wheat inoculation by the *Trichoderma* Lebanese strains enhances root growth and this might result from auxin production by the fungi.

Inoculation by the *Trichoderma* Lebanese strains enhances wheat yield

As shown above, *Trichoderma* inoculation by the two *Trichoderma* strains enhanced wheat seedling growth. We thus tested their effect on the complete development of the wheat plants grown in soil. The two inoculation methods described above were used before plant transplantation in pots but no significant difference could be observed as a result of the inoculation method. Plants were grown to maturity and the different wheat yield components measured (Figure 5, Supplementary Figure 2). This analysis showed that the plant weight (Figure 5A), the number of spikes per plant (Figure 5B), the total grain weight per plant (Figure 5C) and the grain number per plant (Figure 5D) were increased following inoculation by the two fungi. In contrast the 100 grains weight remained unchanged. Altogether, yield harvest index (HI%) was increased around 6% (PDA inoculation method) and 10% (spore inoculation method) by the fungal inoculations (Supplementary Figure 2B).

In order to know whether fungal inoculation could change seed protein quantity as well as seed protein pattern of the grains we analyzed them in plants inoculated by the two strains and following the two inoculation methods. As shown on Supplementary Figure 2C neither the protein contents nor the protein profiles were modified in the wheat grains by the *Trichoderma* inoculation.

Thus, inoculation of plants, independently of the method used increased wheat yield including number of spikes/plants, number of grains/plants, grain weight/plant, whole plant dry weight, and harvesting index (HI%), but did not modify the seed protein content

Discussion

Plants interact with many microorganisms in nature and it is believed that a better understanding of these interactions can help develop a sustainable agriculture less dependent from pesticides and polluting fertilizers. Among interacting microorganisms some are deleterious (pathogens) but others are beneficial (Singh et al., 2018). The fungus *Trichoderma* is widely studied as a beneficial microorganism, promoting plant growth but also protecting plants against pathogens (Mukhopadhyay & Kumar, 2020). *Trichoderma* can be used as PGPF with dicot or monocot crops like tomato and wheat for example (Sood et al., 2020; Stewart & Hill, 2014).

The beneficial natural endophyte characteristic of most of the *T. harzianum* species members, on a wide number of plant species, resulted in their used as bio stimulants improving plant development,

nutrient absorption, as well as resistance to various biological and environmental stresses (Kakabouki et al., 2021). During endophytic interaction, *Trichoderma* colonizes the root surface and produces various changes in plant morphological traits when the hyphae begin to penetrate the root epidermis. The production of hydrolytic enzymes such as cellulases and proteases typically aids root penetration (Enshasy et al., 2020) but also prime the plant defenses, enhancing protection against pathogens (Mukhopadhyay & Kumar, 2020). *Trichoderma spp.* can also produce elicitors that may manipulate hormone signal transduction, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), frequently involved in systemic resistance in plants (Nawrocka & Małolepsza, 2013; Zhao et al., 2021). *Trichoderma* species can also improve plant growth through a variety of other mechanisms, including mycoparasitism, antibiosis, toxin degradation, inactivation of pathogenic enzyme pathways, resistance against pathogens, enhanced nutrient solubilization and uptake, inorganic nutrient sequestration, together with enhanced root hair development (Shahriar et al., 2022).

Among crops, wheat is one of the major staple foods around the world. Previous studies have shown that it can benefit from interaction with *Trichoderma* fungi for growth promotion or pathogen protection (TariqJaveed et al., 2021). In this study we have isolated two *Trichoderma* strains from Lebanese soils, characterized them, and evaluated their growth promotion effect on wheat. Molecular and taxonomic characterization showed that the two strains are *T. guizhouense*, and *T. afroharzianum* isolates belonging to the Harzianum complex (Chaverri et al., 2015; Stummer et al., 2020). The morphology of the two strains is in agreement with *Trichoderma* morphology (Zhu & Zhuang, 2015). In addition, these two isolates were also able to colonize the root rhizoderm, and external cortex, while markedly enhancing seedling root growth. In contrast our study suggested that the two Lebanese isolates lack the ability to colonize wheat aerial tissues under the conditions used. Growth conditions might influence colonization as the *T. afroharzanium* strain was isolated from the base of the aerial part of a field grown plant.

The influence of the two *Trichoderma* strains inoculation on wheat root development suggested that they may produce auxin (Nieto-Jacobo et al., 2017). IAA is the main auxin promoting root system development, and able to modify root architecture in the *Trichoderma* plant interaction (Illescas et al., 2021). Additional *Trichoderma* metabolites, and proteins (effectors) are also known to significantly modulate IAA production in plants, resulting in root hair growth and enhanced root mass development (Illescas et al., 2021). These results are in agreement with previous studies showing that auxin-generating *Trichoderma* strains interact with wheat and other plants (Illescas et al., 2021; Pelagio-Flores et al., 2017). After root colonization, *Trichoderma* fungi stimulate root development and modify

plant metabolism, by releasing numerous secondary compounds (Kakabouki et al., 2021). For example, harzianic acid is one of *T. harzianum* metabolites that is responsible for promoting plant growth (Vinale et al., 2014; Xie et al., 2021) and acting as biocontrol agent.

Recent studies caried out on model plants like *Arabidopsis thaliana* inoculated by *T. atroviride, and T. guizhouense* strains showed that *Trichoderma* volatiles increased endogenous sugar contents in shoots, roots and root exudates, and could enhance *Arabidopsis* root development, root architecture, and promote the symbiosis efficiency. These effects were suggested to be related to auxin transport and signaling (Esparza-Reynoso et al., 2021; Y. Li et al., 2022). In the meantime, approaches of inoculating *Trichoderma* to promote plant growth varied between seed/seedling treatments, soil processing, and foliar spray (Bhandari et al., 2021).

IAA biosynthesis in fungi is tryptophan-dependent, and supplying this amino acid to the medium, increases IAA production (Nieto-Jacobo et al., 2017). We indeed could enhance IAA production in culture of *T. afroharzianum*, and *T. guizhouense* by adding tryptophan to the medium. Further studies have to be carried out to indicate the optimum IAA concentration, required to reach the maximum root growth, and/or yield, and good agronomic traits, with precise determination of *Trichoderma* inoculums (Harman, 2000).

In this work we tested the ability of *T. afroharzianum* and *T. guizhouense* to enhance wheat growth (plant growth promotion) using two seed inoculation methods: 1) PDA mediated inoculation, and 2) spore inoculation. Our resulted showed a notable increased in seedling development during the first 10 days following seed inoculation by the *T. afroharzianum*, and *T. guizhouense* fungi (Figure 4. A,B). This result is in agreement with previous studies (Kucuk, 2014) showing that root and shoot growth of wheat could be improved by *T. harzianum, isolated* from Turkey.

Similar effects were observed when inoculating seedlings with other *Trichoderma spp*. (Oliveira *et al.* 2018; Kthiri et al. 2020; Mahato, Bhuju, et Shrestha 2018; Anjum et al. 2020). These studies indicated that many growth or yield parameters like the percentage of germination (PG), root length (RL), shoot length (SL), total length (TL), fresh root mass (FRM), fresh shoot mass (FSM), total fresh mass (TFM), dry root mass (DRM), dry shoot mass (DSM), total biomass (BIO), root mass ratio (RMR), shoot mass ratio (SMR) and aerial part/root system ratio (AP/RS), were augmented. Similarly, *T. yunnanense*, and *T. afroharzianum* isolates produced IAA under salt stress (200 mM), and could stimulate photosynthesis, water utilization, and growth of wheat in saline conditions (Oljira et al. 2020; Zhang, Gan, et Xu 2018).

Our study is also in agreement with previous studies (Colla *et al.* 2015; Silletti et al. 2021), where seed coating could encourage seed germination, and the quality of yield and grain, including protein and mineral composition in durum wheat. Xue *et al.* (2017) inoculated six different *Trichoderma* strains on wheat seedlings and found that the six strains had the ability of increasing plant dry weight, and total yield, over a three years experiment. This is in line with our data showing that treatment with the two Lebanese *Trichoderma* strains could significantly affect plant yield. The HI% indicated an increased yield, as a response to treatments with both *T. guizhouense* and *T. afroharzianum* strains but *T. afroharzianum* showed higher impact on yield and its components. The fungal growth promotion effects are not completely described but it is known that *Trichoderma* can stimulate plant food scanning by enhancing root elongation in order to invade more, either shallow surface, or even deeper underneath areas, seeking for nutrients uptake. This capacity probably essentially relies on auxin production (Zin & Badaluddin, 2020). In agreement with this, in our study *T. afroharzianum* and *T. guizhouense* isolated strains effectively strongly promoted primary and secondary root elongation.

In addition, *Trichoderma spp.* are known to increase the plant nutrient availability needs by solubilizing minerals such as Fe, Mn, Zn, and P, and providing extra nitrogen to the plant. As a result, after *Trichoderma* colonization, secondary metabolites production is also increased (Kakabouki et al., 2021).

Protein content of grains is an important agronomic character for wheat (Illescas et al., 2022). Protein analysis of the grains revealed that in our experiments the inoculation by *Trichoderma spp.* did not modify their protein nature and content. This shows that *Trichoderma* can promote wheat growth and enhance grain production, without changing the grain protein composition. This observation might be correlated with the other part of our study indicating that the weight of hundred grains is not changed despite yield increase.

In conclusion, with the increased global demand, during a chain of serious crisis, and environmental impacts, expanding wheat production is considered an urgent request. Unconventional strategies are necessary for guaranteeing food security and providing sustainability with safer applications. In this study two *Trichoderma* strains were isolated from the Lebanese lands, identified, characterized, and proved to be endophytes. We proposed that IAA production by the two strains may explain their growth promoting effect.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

NM, PR, MD, FA and NB conceived and designed the experiments. NM, CM, PR, MD, GA, AP, BC and LR conducted the experiments and analyses. PR, NM, GA, and FA drafted the manuscript. All authors have read and approved the final version of the manuscript.

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674

675

676 **Figure legends**

677 **Figure 1.** Phylogenetic tree of CAL-EF-ITS branch includes only species in the *T. harzianum* complex.
678 Two species are identified from Lebanon and are placed in this tree: *T. afroharzianum* (T. S3PA) and
679 *T. guizhouense* (T. S3SB). Strains used in this phylogenetic analysis are described in supplementary

680 Table 1 with their corresponding geographic origin, substrate, voucher number and GenBank accession
681 numbers. *T. agressivum* is used as an outgroup in this analysis.

682

683 **Figure 2**. Growth of *T. guizhouense* (A) and *T. afroharzianum* (B) for one week on PDA
684 supplemented with glucose. The age of the culture is indicated below the picture in hours.
685 Microscopic visualization of conidiophores of *T. guizhouense* (C) *T. afroharzianum* (D) (x1000). (I)
686 hyphae, (II) conidiophores, (III) Phialides and (IV) conidia (the scale bar corresponds to 10μm).

687

688 **Figure 3**. A-D: cross section of wheat root seedlings inoculated with *T. guizhouense* (A, B) or *T.*
689 *afroharzianum* (C, D) using the PDA inoculation method. E-H: cross section of wheat root seedlings
690 inoculated with *T. guizhouense* (E, F), *with T. afroharzianum* (G, H) using the spore inoculation
691 method. (Ep) epidermis, (C) cortex (Cc) central cylinder, (Hy) fungal hyphae. The fungal hyphae are
692 stained green using WGA-Alexa Fluor 488, and the root cell wall are stained red using propidium
693 iodide, (BDFH correspond to the boxes of ACEG respectively).

694

695 **Figure 4.** Growth of roots and aerial parts after 10 days of culture, with and without *Trichoderma*
696 (A); I) not inoculated , II) a representative seedling inoculated with *T. guizhouense*, III) a
697 representative seedling inoculated with *T. afroharzianum*, (B) Total root, and aerial length (cm) of
698 non-inoculated (N.I.) or inoculated wheat plants with *T. guizhouense* (*T.G.*) and *T. afroharzianum*
699 (*T.A.*), respectively, (C) IAA production capability of the two *Trichoderma* isolates (T.G. and T.A.)
700 with and without tryptophan.

701

702 **Figure 5.** Yield parameters: A) Total dry plant weight (g)/plant; B) Number of spikes/plant; C) Total
703 grains weight (g)/plant; D) Number of grains/plant, as influenced by PDA and spore inoculation
704 methods. N.I.: non-inoculated; T.G.: wheat plants inoculated with *T. guizhouense*; T.A.: wheat plants
705 inoculated with *T. afroharzianum*. Values in the same graph having different letters are significantly
706 different, at $P \leq 0.01$ (ANOVA test).

707

Supplementary material

Supplementary Table 1: Strains used in the phylogenetic analyses, with their corresponding geographic origin, substrate, voucher number and GenBank accession numbers

Supplementary Table 2: Fungal strains isolated from Lebanon from different ecological site.

Supplementary Figure 1. (A) principal root length (cm) over a 10 days period. (B) total root, and aerial length (cm). (C) total root, and aerial mass (g). N.I.: non-inoculated; T.G.: wheat plants inoculated with *T. guizhouense*; T.A.: wheat plants inoculated with *T. afroharzianum*. Values in the same column having different letters are significantly different, at $P \leq 0.01$ (ANOVA test).

Supplementary Figure 2. (A) Harvest index (%) and (B) 100 grain weight (g). N.I.: non-inoculated; T.G.: wheat plants inoculated with *T. guizhouense*; T.A.: wheat plants inoculated with *T. afroharzianum*. (C) protein analysis (composition and content) of wheat plants non-inoculated (cont) or inoculated with *T. guizhouense* (TGUI) or *T. afroharzianum* (Taf) as influenced by PDA inoculation method (PDA) or the spore inoculation method (Tubes). The protein content of 100 grains in each condition is indicated below the gel pictures. Values in in each graph having different letters are significantly different, at $P \leq 0.01$ (ANOVA test).

Supplementary Material

Supplementary Tables

Supplementary Table 1. Strains used in the phylogenetic analyses, with their corresponding geographic origin, substrate, voucher number and GenBank accession numbers

Species	Voucher/culture Nos.	Geographic origin	Substrate	Genbank accession Nos.		
				CAL	ITS	*EF1*
T. afarasin	CBS 130742 = Dis 314f	Cameroon	*Cola altissima* trunk endophyte	FJ442312	FJ442259	FJ463400
T. afarasin	Dis 377a	Cameroon	*Cola* sp. trunk endophyte	KP115277	FJ442665	FJ463322
T. afarasin	CBS 130501 = G.J.S. 06-98	Cameroon	Soil	FJ442353	FJ442630	FJ463327
T. afarasin*	CBS 130755 = IMI 393967	Cameroon	Soil	FJ442388	AY027784	AF348093

	= G.J.S. 99-227					
T. afroharzianum	G. Harman 1295-22 = ATCC 29847 = T22 = GJS 94-26	Colombia and New York[2]	G. Harman, patented biocontrol strain	AF469190	AF469188	AF469194
T. afroharzianum	G.J.S. 00-24	Mexico	Soil	AF442880	AF443922	AF443940
T. afroharzianum*	CBS 124620 = G.J.S. 04-186	Peru	On basidioma of *Moniliophthora roreri* on fruit of *Theobroma*	FJ442370	FJ442265	FJ463301
T. afroharzianum	CBS 124614 = G.J.S. 04-193	Peru	On basidioma of *Moniliophthora roreri* on fruit of *Theobroma*	FJ442372	FJ442233	FJ463298
T. afroharzianum	G.J.S. 05-113	Italy	Wheat seed	FJ442371	FJ442235	FJ463378
T. afroharzianum	G.J.S. 97-263	Japan	Soil	AF442877	AF194010	AF348091

T. afroharzianum	G.J.S. 97-268	Japan	Soil	AF442878	AF194015	AF348105
T. afroharzianum	IMI 393972 = G.J.S. 99-225	Cameroon	Soil	AF442882	AY027781	AF348106
T. aggressivum	CBS 433.95	Northern Ireland	Mushroom compost	FJ442279	FJ442605	AF348097
T. atrobrunneum	CBS 130440 = G.J.S. 04-67	Italy	Soil	FJ442329	FJ442273	FJ463360
T. atrobrunneum	G.J.S. 05-100	Italy	*Castanea sativa* twig	FJ442364	FJ442234	FJ463299
T. atrobrunneum	G.J.S. 05-101	Italy	Soil	FJ442331	FJ442677	FJ463392
T. camerunense*	CBS 137272 = G.J.S. 99-230	Cameroon	Soil	AF442875	AY027780	AF348107
T. camerunense	G.J.S. 99-231	Cameroon	Soil	AF442874	AY027783	AF348108
T. endophyticum	CBS 130730 = Dis 217h	Ecuador	*Theobroma gileri* trunk endophyte	FJ442293	FJ442242	FJ463314
T.	Dis 217o	Ecuador	*Theobroma gileri* trunk	FJ442294	FJ442241	FJ463323

endophyticum			endophyte			
T. endophyticum	Dis 220k	Ecuador	*Theobroma gileri* trunk endophyte	FJ442299	FJ442270	FJ463328
T. guizhouense	DAOM 231435	Rwanda	Soil	FJ577721	EF191296	EF191321
T. guizhouense	G.J.S. 07-18	Ghana	Soil	FJ442355	FJ442641	FJ463390
T. guizhouense	ATCC 90179 = IMI 374787 = G.J.S. 85-119	Indonesia	Dead wood	AF442881	AF443923	AF443941
T. guizhouense	NBRC 30608 = IFO 30608 = G.J.S. 97-28	Japan	Dead branch	FJ442379	DQ018116	AY937440
T. harzianum*	CBS 226.95 = G.J.S 95-43	U.K.	Soil	AF442864	AJ222720	AF348101
T. harzianum	CBS 227.95 = G.J.S 95-40	U.K.	Soil	AF442866	AJ222721	AF348100
T. harzianum	IMI 359823	Northern Ireland	Mushroom compost	AF442865	X73690	AF348092

T. inhamatum*	CBS 273.78 = IMI 287526 = G.J.S. 95-39	Colombia	Soil	AF442891	FJ442680	AF348099
T. lentiforme	CBS 130726 = Dis 110a	Ecuador	*Theobroma cacao* trunk endophyte	FJ442287	FJ442681	FJ851872
T. lentiforme	Dis 167c	Brazil	*Theobroma cacao* trunk endophyte	FJ442365	FJ442269	FJ463309
T. lentiforme	Dis 218e	Ecuador	*Theobroma gileri* trunk endophyte	FJ442296	FJ442220	FJ463310
T. lixii**	CBS 110080 = ATCC MYA-2478 = G.J.S. 97-96 = BPI 745654	Thailand	Decayed *Ganoderma* basidiocarp	AF442872	AF443920	AF443938
T. neotropicale*	G.J.S. 11-185 = CBS 130633 LA11	Peru	*Hevea guianensis* trunk endophyte	KP115279	HQ022407	HQ022771

T. neotropicale	G.J.S. 11-187 = T51	Peru	*Hevea brasiliensis* trunk endophyte	KP115280	FJ884180	FJ967825
T. rifaii	CBS 130745 = Dis 337f	Panama	*Theobroma cacao* trunk endophyte	FJ442315	FJ442621	FJ463321
T. simmonsii	CBS 123799 = IMI 393966 = G.J.S. 90-22	USA, Wisconsin	Decorticated wood	AF442867	AF443915	AF443933
T. simmonsii*	CBS 130431 = G.J.S. 91-138	USA, Maryland	Decaying bark	AF442869	AF443917	AF443935
T. simmonsii	CBS 546.92 = ATCC MYA-2453 = G.J.S. 92-100	USA, Alabama	Decorticated wood	AF442871	AF443919	AF443937
T. simmonsii	CBS 130432 = G.J.S. 94-53 BPI 749348	USA, Illinois	Decorticated wood	AF442868	AF443916	AF443934

* Indicates a type culture.

** Indicates an epitype culture.

[1] CBS = CBS Fungal Biodiversity Centre culture collection, the Netherlands; DAOM. = Agriculture and Agri-Food Canada National Mycological Culture Collection; IMI. = CABI culture collection, UK; ATCC. = American Type Culture Collection, Manassas, Virginia, USA; BPI = US National Fungus Collection; WU = Herbarium WU, Institute of Botany, University of Vienna, Austria; G.J.S. = G. J. Samuels; P.C. = P. Chaverri; Hypo, C.P.K., S and WJ = W. Jaklitsch collection numbers; Dis = H.C. Evans endophyte cultures; LA, CM, PP, T = P. Chaverri endophyte cultures. Where a plant name alone is given, the substrate is twigs or branches and fungi growing on them.

[2] T22 is product of fusion of protoplasts of two cultures: ATCC 60850, which was isolated from soil in Colombia, and ATCC 20707, which was isolated from soil in New York state. See Ahmad and Baker (1987a,b) and Stasz et al. (1988).

Ahmad JS, Baker R. 1987a. Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutants of *Trichoderma harzianum*. Phytopathology 77:358–362.

—, —. 1987b. Rhizosphere competence of *Trichoderma harzianum*. Phytopathology 77:182–189.

Stasz TE, Harman GE, Weeden NF. 1988. Protoplast preparation and fusion in 2 biocontrol strains of *Trichoderma harzianum*. Mycologia 80:141–150.

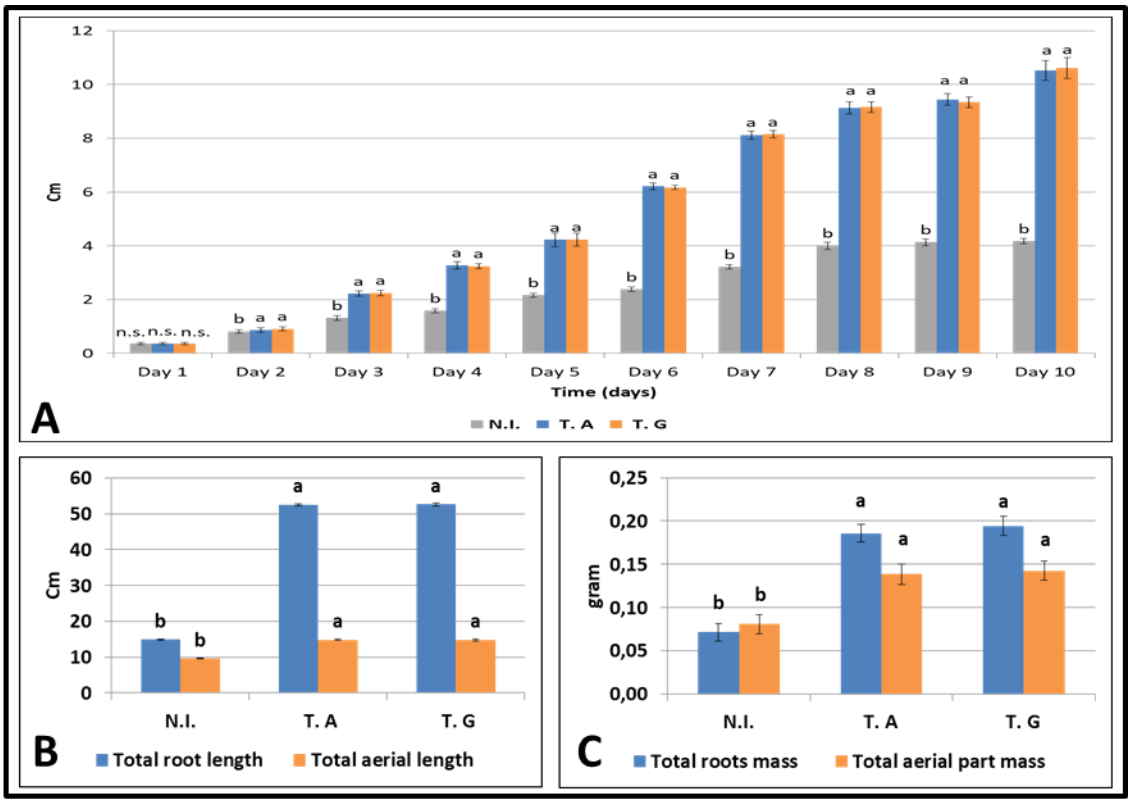
Supplementary Table 2. Fungal strains isolated from Lebanon from different ecological site.

Site	Fugus location	Genus (according to ITS)

Site 1 ICARDA	**Soil**	*Alternaria sp.* (x1)
		Aspergillus sp. (x11)
		Cladorrhinum sp. (x2)
		Talaromyces sp. (x1)
	Root or stem	*Alternaria sp.* (x2)
		Fusarium sp. (x5)
		Mucor sp. (x1)
		Stromatinia sp. (x2)
Site 2 Tal_Amara	**Soil**	*Aspergillus sp.* (x2)
		Penicillium sp. (x1)
	Root or stem	*Boeremia sp.* (x1)
		Mucor sp. (x1)
Site 3 Doueir	**Soil**	*Fusarium sp.* (x1)
		Trichoderma sp. (x1)
		Mucor sp. (x1)
	Root or stem	*Fusarium sp.* (x3)
		Trichoderma sp. (x1)
		Macrophomina sp. (x1)
		Microdochium sp. (x1)

Mucor sp. (x1)

Supplementary Figures



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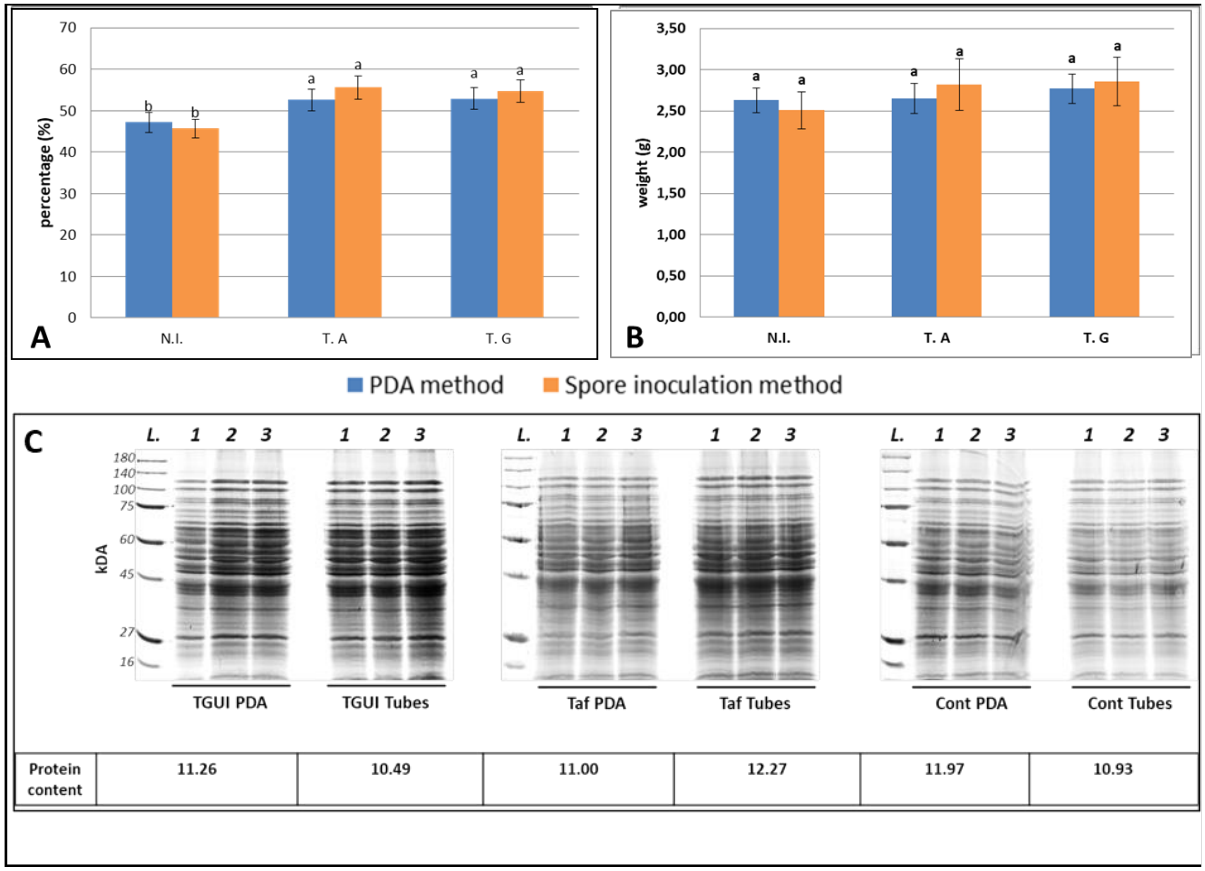
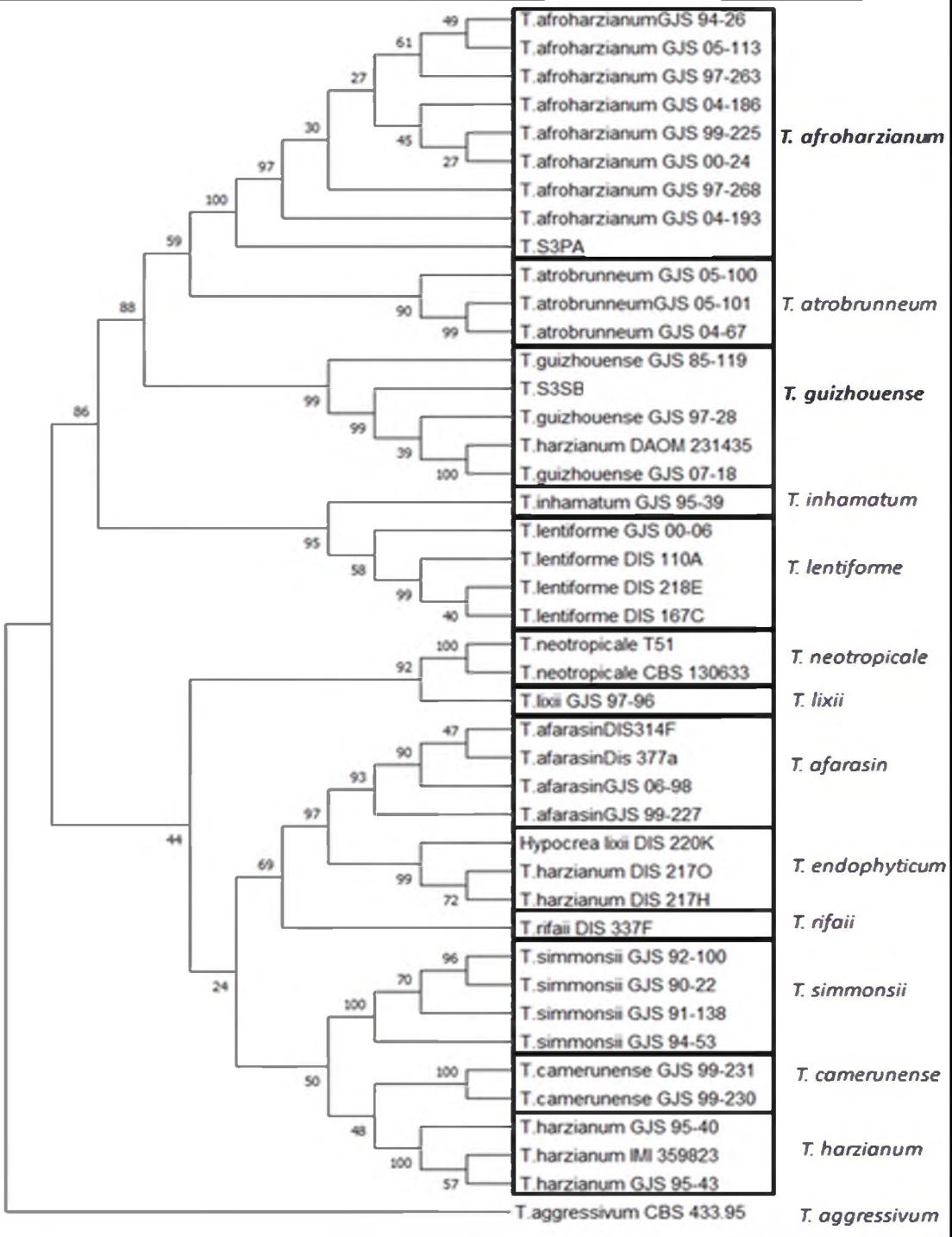


Supplementary Figure 2. (A) Harvest index (%) and (B) 100 grain weight (g). N.I.: non-inoculated; T.G.: wheat plants inoculated with *T. guizhouense*; T.A.: wheat plants inoculated with *T. afroharzianum*. (C) protein analysis (composition and content) of wheat plants non-inoculated (cont) or inoculated with *T. guizhouense* (TGUI) or *T. afroharzianum* (Taf) as influenced by PDA inoculation method (PDA) or the spore inoculation method (Tubes). The protein content of 100 grains in each condition is indicated below the gel pictures. Values in in each graph having different letters are significantly different, at P≤0.01 (ANOVA test).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T.afroharzianum GJS 00-24
T.afroharzianum GJS 97-268
T.afroharzianum GJS 04-193
T.S3PA
T. afroharzianum
T.atrobrunneum GJS 05-100
T.atrobrunneumGJS 05-101
T.atrobrunneum GJS 04-67
T. atrobrunneum
T.guizhouense GJS 85-119
T.S3SB
T.guizhouense GJS 97-28
T.harzianum DAOM 231435
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T. inhamatum
T.lentiforme GJS 00-06
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T.lentiforme DIS 218E
T.lentiforme DIS 167C
T. lentiforme
T.neotropicale T51
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T. neotropicale
T.lixii GJS 97-96
T. lixii
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T.afarasinDis 377a
T.afarasinGJS 06-98
T.afarasinGJS 99-227
T. afarasin
Hypocrea lixii DIS 220K
T.harzianum DIS 217O
T.harzianum DIS 217H
T. endophyticum
T.rifaii DIS 337F
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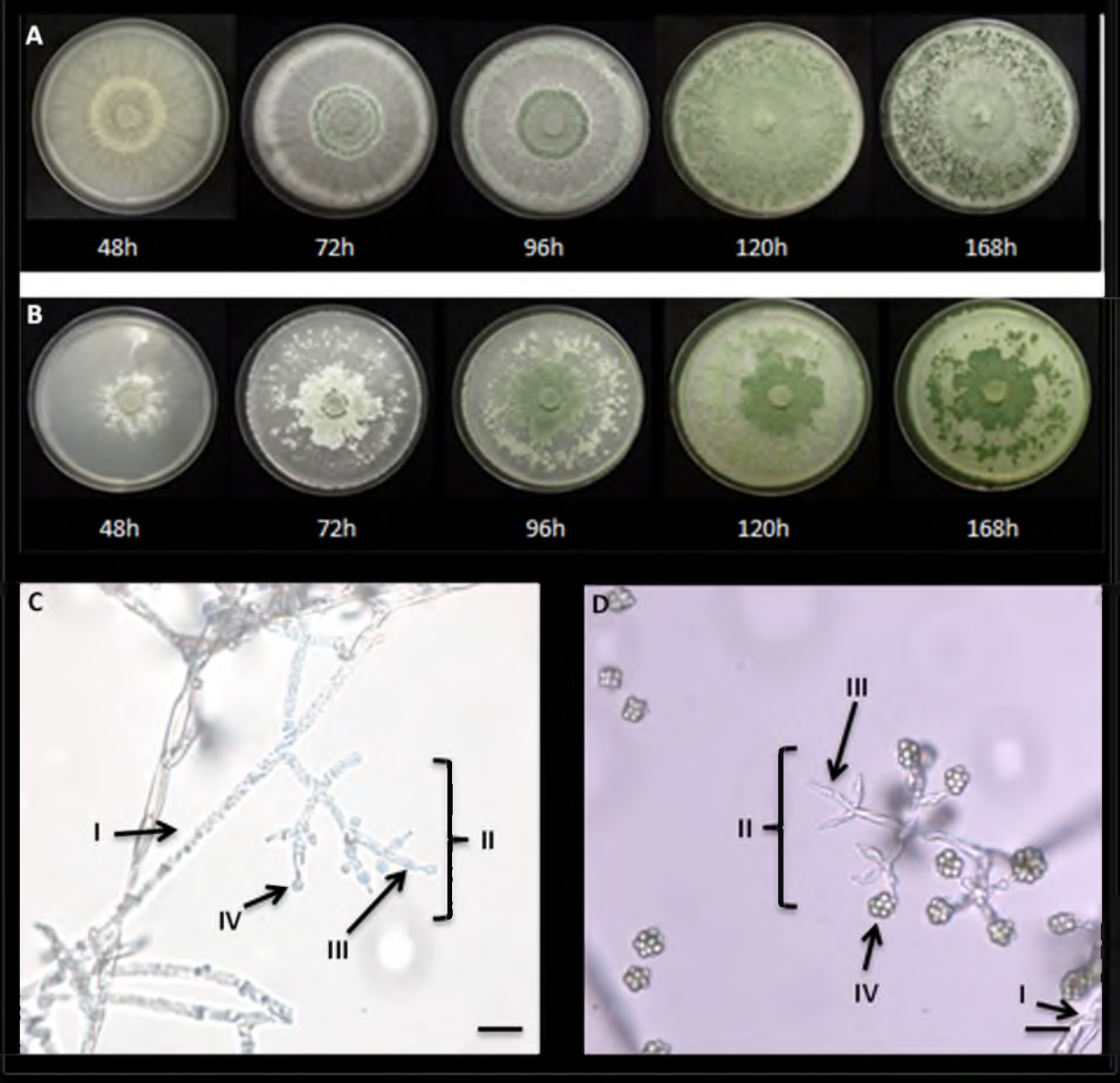
A
48h
72h
96h
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168h
B
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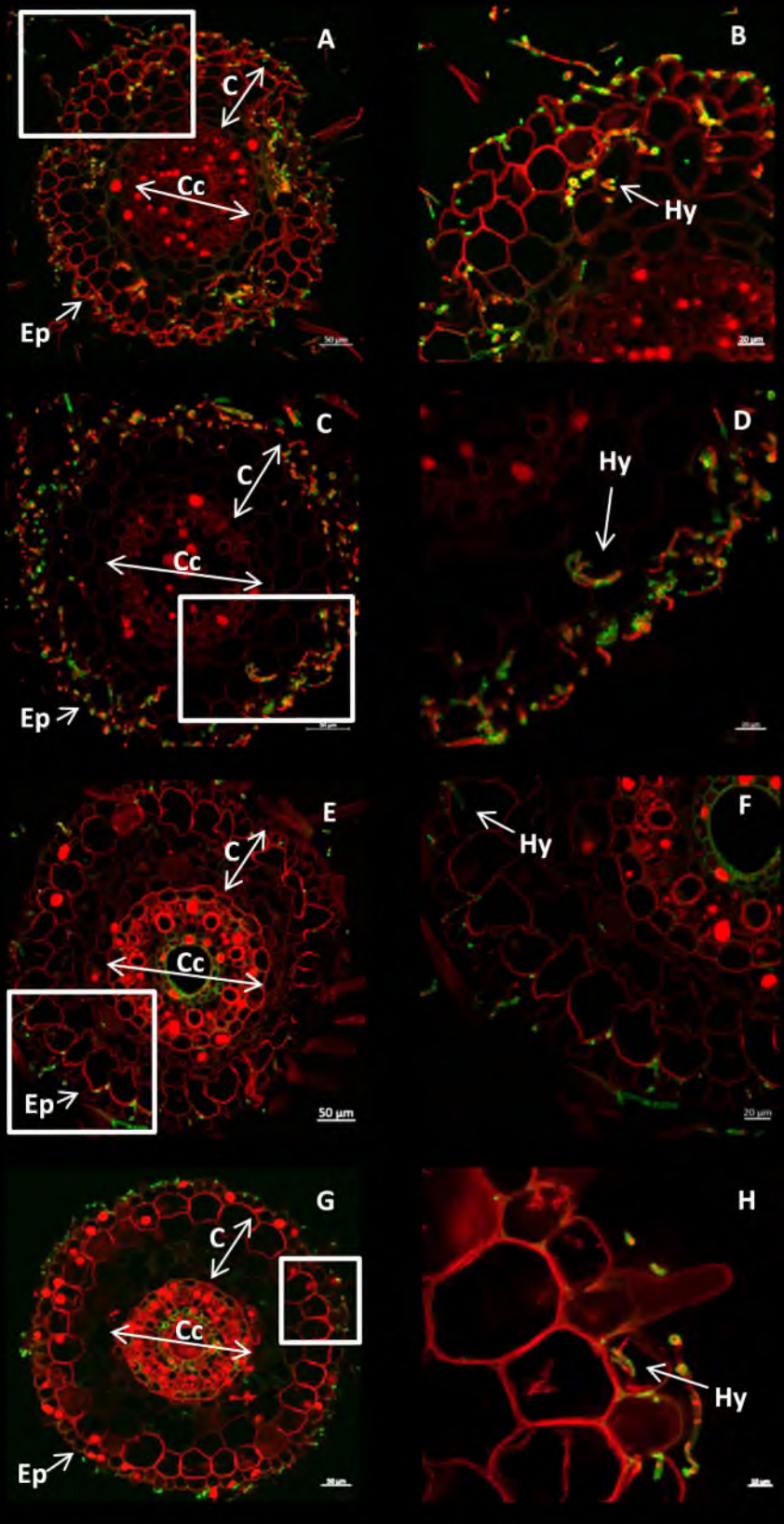
72h
96h
120h
168h
C
I
II
III
IV
D
III
II
IV
I


A
C
Cc
Ep
50 µm
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Hy
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C
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Cc
Ep
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D
Hy
E
C
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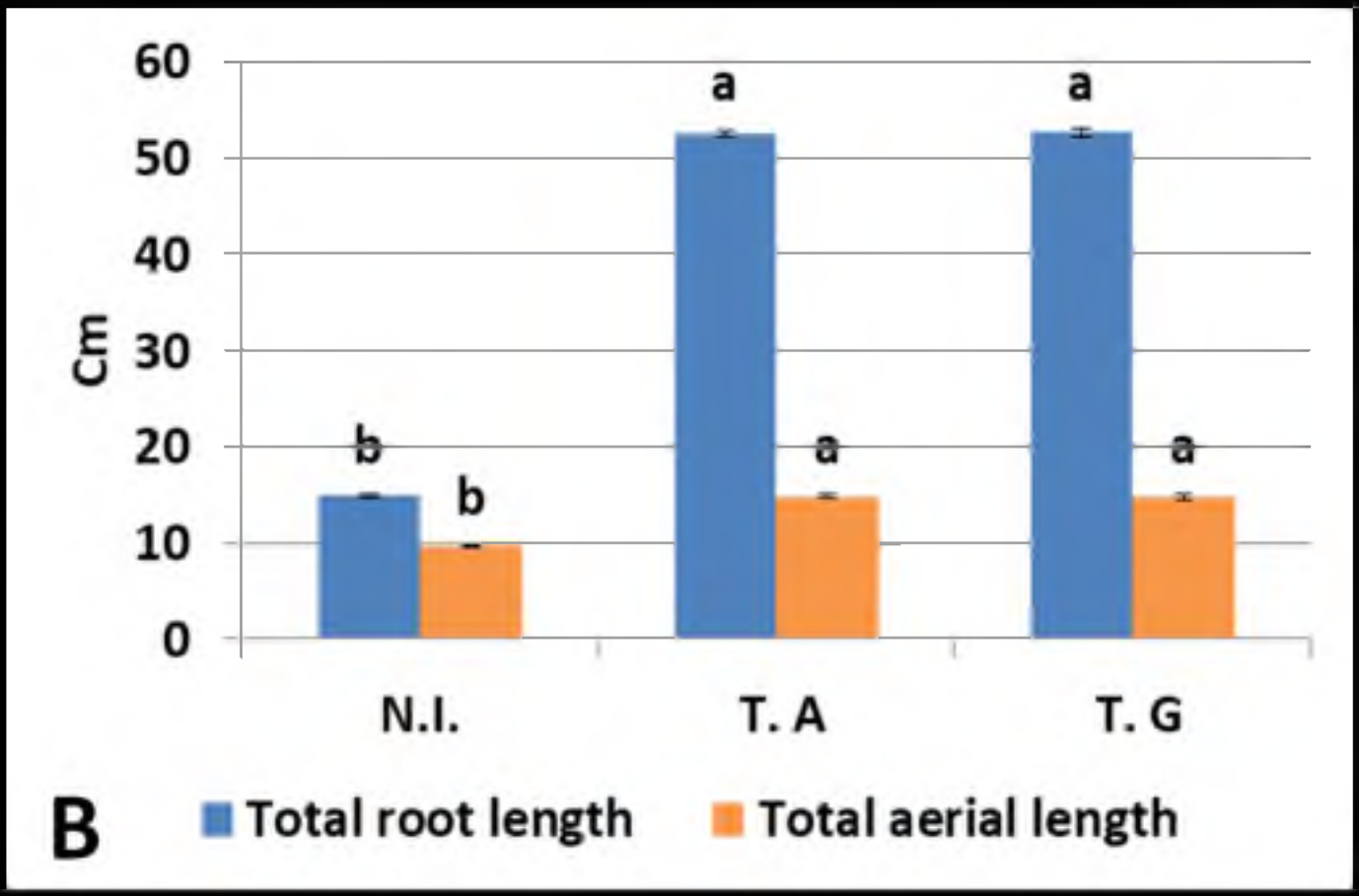
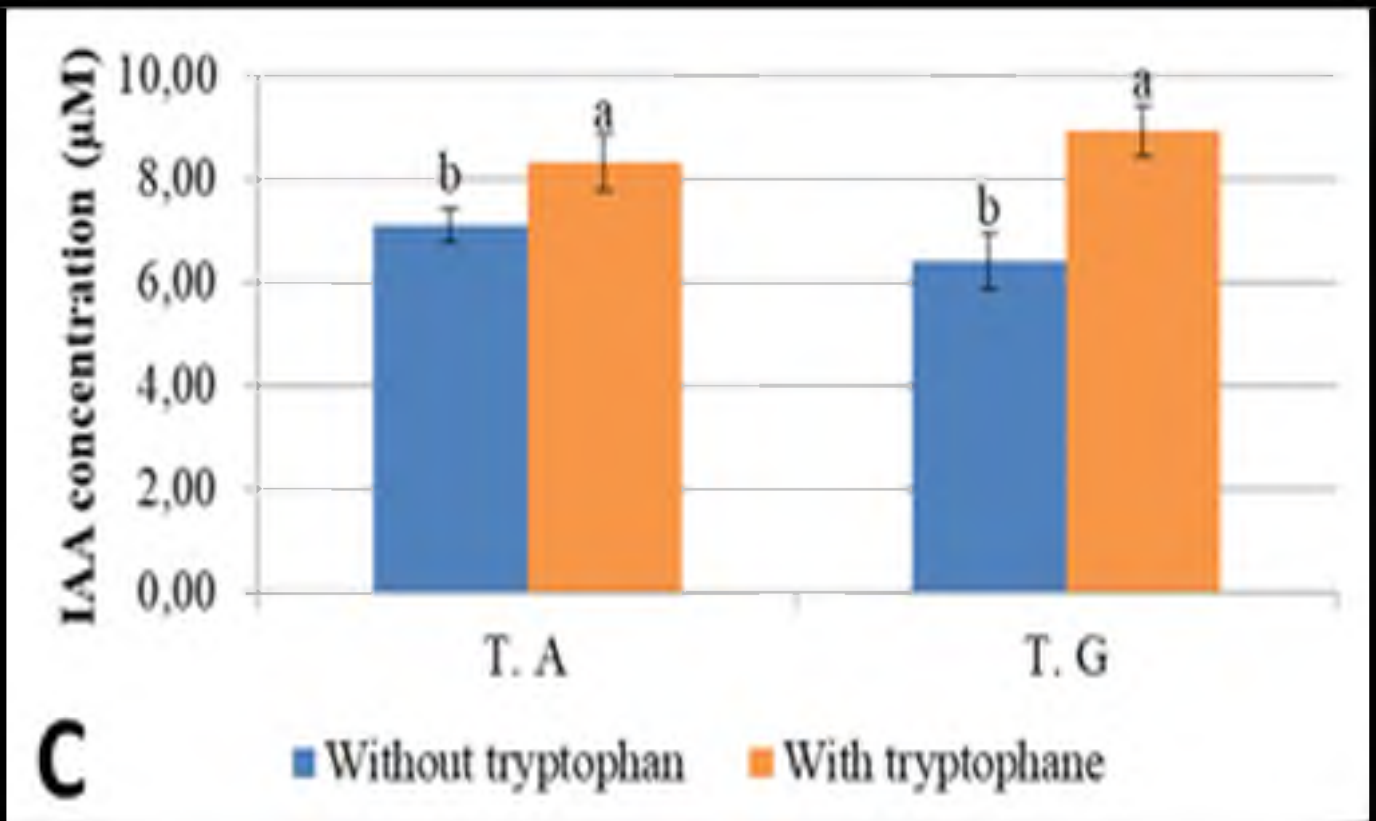
Ep
50 µm
F
Hy
20 µm
G
C
Cc
Ep
50 µm
H
Hy
20 µm

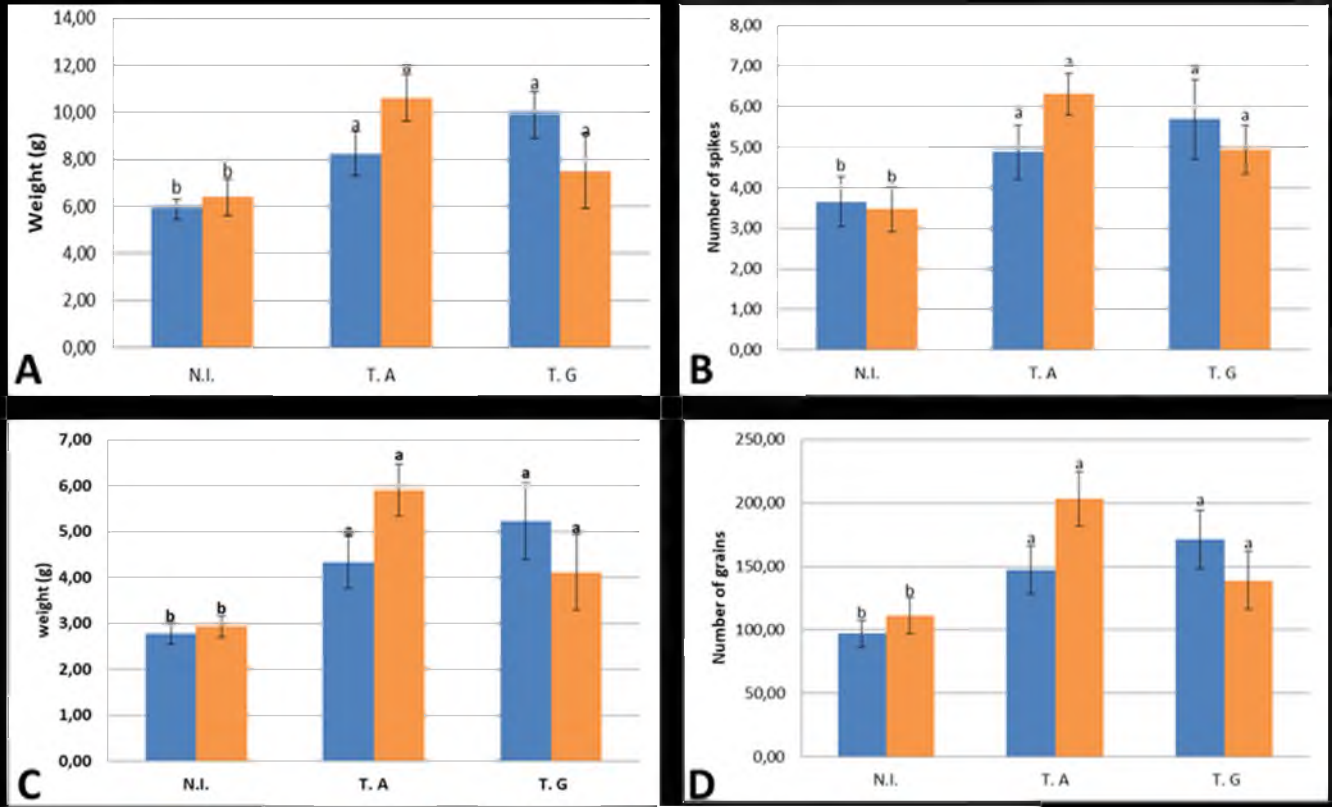

I
II
III
A


60
50
40
30
20
10
0
Cm
a
a
b
b
a
a
N.I.
T. A
T. G
Total root length
Total aerial length

B


IAA concentration (µM)
10,00
8,00
6,00
4,00
2,00
0,00
b
a
b
a
T. A
T. G
Without tryptophan
With tryptophane

C


A
Weight (g)
14,00
12,00
10,00
8,00
6,00
4,00
2,00
0,00
b
b
a
a
a
a
N.I.
T. A
T. G
B
Number of spikes
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
b
b
a
a
a
a
N.I.
T. A
T. G
C
weight (g)
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
b
b
a
a
a
a
N.I.
T. A
T. G
D
Number of grains
250,00
200,00
150,00
100,00
50,00
0,00
b
b
a
a
a
a
N.I.
T. A
T. G
PDA method
Spore inoculation method