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Role of *Trichoderma aggressivum* f. *europaeum* as Plant-Growth Promoter in Horticulture

Brenda Sánchez-Montesinos [1], Fernando Diánez [1,*], Alejandro Moreno-Gavíra [1], Francisco J. Gea [2] and Mila Santos [1]

[1] Departamento de Agronomía, Escuela Superior de Ingeniería, Universidad de Almería, 04120 Almería, Spain; brensam@hotmail.com (B.S.-M.); alejanmoga@gmail.com (A.M.-G.); msantos@ual.es (M.S.)
[2] Centro de Investigación, Experimentación y Servicios del Champiñón (CIES), Quintanar del Rey, 16220 Cuenca, Spain; fjgea.cies@dipucuenca.es
* Correspondence: fdianez@ual.es; Tel.: +34-628188359

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Abstract: The main objective of this study was to determine the capacity of *Trichoderma aggressivum* f. *europaeum* to promote pepper and tomato seedling growth compared to that of *T. saturnisporum*, a species recently characterised as a biostimulant. Consequently, in vitro seed germination and seedling growth tests were performed under commercial plant nursery conditions. Additionally, the effects of different doses and a mixture of both species on seedling growth under plant nursery and subsequently under greenhouse conditions were determined. Furthermore, mass production of spores was determined in different substrates, and their siderophore and indole acetic acid production and phosphate (P) solubilisation capacity were also determined. Direct application of *Trichoderma aggressivum* f. *europaeum* to seeds in vitro neither increases the percentage of pepper and tomato seed germination nor improves their vigour index. However, substrate irrigation using different doses under commercial plant nursery conditions increases the quality of tomato and pepper seedlings. Tomato roots increased by 66.66% at doses of 10^6 spores per plant. Applying *T. aggressivum* f. *europaeum* or *T. saturnisporum* under plant nursery conditions added value to seedlings because their growth-promoting effect is maintained under greenhouse conditions up to three months after transplantation. The combined application of the two species had no beneficial effect in relation to that of the control. The present study demonstrates the biostimulant capacity of *T. aggressivum* f. *europaeum* in pepper and tomato plants under commercial plant nursery and greenhouse conditions.

Keywords: *Trichoderma*; plant growth promotion; tomato; pepper; biostimulant

1. Introduction

The success of applying *Trichoderma* in agriculture results from the multiple benefits that it generates in plants. Thus, the genus *Trichoderma* is characterised by its strong competitive and reproductive potential, presenting high survival rates under unfavourable or abiotic stress conditions, such as salinity [1], water stress [2], or the presence of various toxic chemicals, including fungicides [3], among others. Similarly, *Trichoderma* exhibits high efficiency in the promotion of nutrient uptake [4], the capacity to modify the rhizosphere and root structure in which the fungus is established [5,6], high aggressiveness against plant pathogenic fungi, efficiency in the promotion of plant growth [7–12], and the ability to induce plant defence mechanisms, among many additional benefits [8,9,13]. The properties of *Trichoderma* have generated considerable research interest in these fungi for use in agriculture, and a large number of commercial products have been developed using different *Trichoderma* species [10,14]. Many formulations contain mixtures of different species that provide a wider range of direct and indirect beneficial effects for the plants. Numerous studies have reported the benefits

of *Trichoderma* application for plant growth and even increased production yield. Thus, applying *Trichoderma* species, to both soil and seeds, allows the multiplication of the fungus in conjunction with the developing root system [15]. Its ability to colonise plant roots from the appressorium-like structure directly enhances seed vigour [16] and germination and promotes seedling growth [10,11,17]; thereby, suggesting that these fungi should be applied from the plant nursery stage in the case of horticultural, ornamental, or forest species, which would allow the early colonisation of the roots by *Trichoderma*, before transplanting the seedlings in the field.

It has been reported that plant growth is enhanced in association with *Trichoderma* species similar to that of other plant-growth-promoting microorganisms (PGPMs), but the effects are greater with *Trichoderma* when plants are under biotic, abiotic, or physiological stress conditions [9,18–21]. Recently, *T. aggressivum* f. *europaeum* has been described as a melon seedling growth promoter under saline stress conditions, in addition to its capacity to control *Pythium ultimum*, decreasing the severity of the disease in seedlings [1]. *Trichoderma aggressivum* Samuels & W. Gams is the causal agent of the green mould disease, which causes economic losses in the cultivation of white button mushrooms (*Agaricus bisporus* (J.E. Lange) Imbach) worldwide. There are two subspecies, *T. aggressivum* f. *aggressivum* and *T. aggressivum* f. *europaeum* found in North America and Europe, respectively [22]. *Trichoderma aggressivum*, a fast-growing filamentous fungus, colonises compost and casings used as growth substrates in mushroom cultivation and produces dense white mycelial colonies that change colour to green after sporulation [23]. This aggressive competitor is known to produce metabolites that are toxic to *A. bisporus* [24,25]. In areas colonised by *T. aggressivum*, fruit body formation is retarded, and fruit bodies may be of poor quality because of damage or discolouration [23]. Numerous *Trichoderma* species have been isolated from *Agaricus* compost and *Pleurotus* substrates, such as *T. harzianum, T. longibrachiatum, Trichoderma ghanense, T. asperellu,* and *T. atroviride*, although its aggressiveness has not been determined [26]. Sánchez-Montesinos et al. [1] demonstrated its high mycelial growth and sporulation on roots. Thus, *T. aggressivum* f. *europaeum* is a potential biofertilizer for different crops. In our study, the growth-promoting capacity of this species has been analysed in comparison to that of *T. saturnisporum* Ca1606, which was recently characterised as a biocontrol agent and a seedling growth promoter for different horticultural plants [11,16,27]. Since the effectiveness of microorganisms as growth promoters will depend on the crop, dose and application method, among many other factors, further studies on *T. aggressivum* f. *europaeum* are needed to determine its efficacy.

Consequently, in the present study, *T. aggressivum* f. *europaeum* Tae52481 and *T. saturnisporum* Ca1606, were tested to evaluate: (a) the effects of direct application to seeds of a fungus suspension on root colonisation of tomatoes and peppers and subsequent plant vigour; (b) the promotion of growth and quality of pepper and tomato seedlings under a conventional production system; and (c) the effects of applying different doses and the synergistic effect of both isolates on tomato seedlings and on their subsequent transplantation under greenhouse conditions.

2. Materials and Methods

2.1. Fungal Isolates

Trichoderma saturnisporum Ca1606 (TS), already known for their plant growth promotion properties, were extracted from suppressive soils. TS was cultivated on potato dextrose agar (PDA) for 7 days at 25 °C in lightless conditions. The growth results measured were used to establish a comparison value.

For this study *Trichoderma aggressivum* f. *europaeum* Tae52481 (TA) were isolated from samples of substrate used for *Agaricus bisporus* cultivation at mushroom farms. These fungal spore samples were similarly cultivated on potato dextrose agar (PDA) for 7 days at 25 °C in dark conditions. The corresponding growth results were recorded. The spore suspensions for both samples were prepared using sterile distilled water. A concentration of 1×10^7 spores/mL was achieved with a Neubauer haemocytometer.

2.2. Analysis of Plant Growth-Promoting Attributes

In accordance with the method of Louden et al. [28], by the transference of fungal mycelial discs (5 mm) of active culture onto Chrome-Azurol S (CAS) agar medium, siderophore production was determined. At 24, 48 and 72 h the diameter of the siderophore colony indicative orange halos on blue were measured.

Indole-3-acetic acid (IAA) production was estimated according to the procedure described by Diánez et al. [16]. Five independent replicates of TA and TS were analysed. This process is described as follows. A glucose peptone broth (GPB) of 50 mL, amended with or without L-tryptophan (Sigma-Aldrich) at a concentration of 100 mg L^{-1} was prepared. Flasks containing this broth inoculated with TA and TS were incubated on an orbital shaker at 150 rpm in dark conditions for 7 days at 25 °C. Subsequently the supernatants from each flask, having first being centrifuged for 30 min at 12,000× *g* and filtered through sterile Millipore membranes (pore size 0.22 µm), were collected into sterile test tubes. In order to determine the quantity of IAA, optical density tests were carried out and compared to a standard IAA curve. For both the TA and TS, 3 mL of the culture supernatant and 2 mL (0.5 mol L^{-1} $FeCl_3$ + 98 mL of 35% $HClO_4$) Salkowski reagent were combined and left for 30 min. The intensity of the resulting red pigmentation density was measured at 530 nm using a scanning spectrophotometer for each of the samples.

To determine the quantitative estimation of phosphate solubilisation, a modified version of the Lima–Rivera procedure [29] was followed. Then, 250 mL capacity flasks containing 50 mL National Botanical Research Institute's phosphate (NBRIP) broth, inoculated with two 5 mm pure *Trichoderma* isolates agar disks were agitated at 100 rpm and incubated at 26 °C for 3, 5, 7, 10 and 15 days. As a control the procedure was carried out on uninoculated flasks containing the same NBRIP broth. The experiments were conducted in triplicate.

Using the Fiske and Subbarow method [30] phosphate concentrations in culture supernatants were estimated as equivalent phosphate (µg mL^{-1}), mean values expressed and pH analysed. The total P (phosphate) in the flasks was 10 mg mL^{-1}.

2.3. Mass Production of TA and TS on Solid Substrates

A mixture of two kinds of substrates, one containing buckwheat husk (BH) and oat (O), the other containing BH and rice (R) were tested for the mass multiplication of TA and TS [31]. Different proportions of BH-O (90–10%, 80–20% and 70–30% *v*/*v*) and BH-R (90–10%, 80–20% and 70–30% *v*/*v*) were submerged in 30% *v*/*v* of water for 24 h. Each mixture was sterilised for 1 h at 125 °C twice on consecutive days. Each mixture was placed on a tray and aseptically inoculated by spraying with 5 mL of spore suspension containing 1×10^7 spores mL^{-1} of each isolate. The trays were kept at 25 °C in the dark for 15 days. In total, three samples (2 g) of the fungus-colonised substrate were removed from the trays in each treatment. The samples were successively diluted in sterile distilled water + 0.01% Tween 20® and the number of conidia g^{-1} of the solid substrate was quantified for each replicate using a Neubauer haemocytometer. There were three replications per treatment. The collected spores were used in the different experiments conducted in this study.

2.4. Analysis of Effects of TA and TS on Seed Germination under Laboratory Conditions

Three treatments (control, TA and TS) and four repetitions following a random block experimental design were implemented in this study. For each repetition of the three treatments 50 seeds of tomato (*Solanum lycopersicum* 'Red Cherry') and pepper (*Capsicum annuum* 'Largo de Reus') were germinated on two sheets of sterile distilled water moistened Whatman No. 1 filter paper in (150 mm) Petri dishes. These seeds were first surface sterilized for 5 min with 1.5% sodium hypochlorite (NaOCl), rinsed twice with sterile distilled water and dried under laminar airflow on sterile paper [16]. Germination was achieved by treating the seeds with 50 µL of spore suspension (1×10^5 spores mL^{-1}) of TA, TS or 50 µL of sterile water (control). The trays were placed in a lightless incubator at 25 ± 1 °C, 7 days

for tomato and 10 days for pepper seeds. For each Petri dish treated with one of the three solutions (control, TA and TS), percent germination, root length and shoot length of tomato and pepper seeds were recorded. A Seed Vigour Index (SVI) was calculated as follows: SVI (length) = seed germination% (mean root length + mean shoot length) [32].

2.5. Analysis of Promoter Effects of TA and TS on Pepper and Tomato Seedlings: Experiment 1

The following experiment was conducted in autumn using a completely randomised design at a commercial nursery (Almería, Spain). Pepper (*Capsicum annuum* 'Largo de Reus') and tomato (*Solanum lycopersicum* 'Red Cherry') seeds were sown in 96-cell commercial peat mix filled nursery polystyrene planting trays (70 mL volume) and covered with vermiculite. Trays were relocated to a greenhouse and rinsed with sterile distilled water (control), or a 5 mL (TA or TS) spore suspension per cell at 10^5 spores per plant, after a 2 day (tomato) or 4 day (pepper) period in a germination room (relative humidity (RH) = 95%; 25 °C). Four trays of seedlings for each treatment were cultivated under standard nursery culture conditions (18–28 °C; 75.4 ± 6.7% RH). Then, 20 plants per treatment and control were randomly selected from the four replications at 45 days after sowing across the four replications. Different growth parameters: number of leaves, stem length, stem base diameter, total leaf area and root dry weights, as well as leaf area using the WINDIAS 3.1 of the plants, were measured. The formula: DQI = TDW/((LS/D) + SDW/RDW)) where TDW is the total dry weight (g), LS is stem length (cm), D is stem diameter (mm), SDW and RDW are stem and root dry weight (g), respectively; they were employed to determine the Dickson Quality Index (DQI) [33].

2.6. Analysis of Effects of Applying Different Doses of TA and TS to Tomatoes: Experiment 2

The experimental procedure followed for experiment 2 was similar to that described for experiment 1, although conducted in winter. Again, propagated in substrate appropriately irrigated according to climate and crop necessity under commercial plant nursery conditions and supplemented with a commercial complex nutrient fertiliser, 96 tomato seedlings per replicate of four were treated with three solutions of spore suspension, each with 5 mL of TA, TS conidia and TA + TS (M) (TA D1, TS D1 and M D1: 10^5 spores mL^{-1}; TA D2, TS D2 and M D2: 10^6 spores mL^{-1}; and TA D3, TS D3 and M D3: 10^7 spores mL^{-1}). After 30 days of sowing, twenty plants from each of the three treatment batches and control were randomly selected for harvest. The plants were measured, and data were recorded for the same parameters described in experiment 1. In mid-February a further 25 plants were transplanted into a sandy soil and analysed in mid-May.

In all tests, roots inoculated with *Trichoderma* isolates were collected at the end of the tests. Roots were surface sterilized in 0.1% sodium hypochlorite and washed with sterilised water. Root fragments were placed in PDA medium to determine root colonisation by the fungal isolate.

2.7. Statistical Analysis

The experimental results are presented as the means and standard error (± SE) for the different replicates. Mean separation was carried out using Fisher's Least Significant Difference (LSD) test. The data were tested by one-way analysis of variance (ANOVA) or Student's *t*-test with significance defined as *p*-values less than 0.05 ($p < 0.05$). Statgraphics Centurion 18 Software was utilised for statistical analysis.

3. Results

3.1. Mass Production of Trichoderma Isolates on Solid Substrates

The results are outlined in Table 1. Both isolates grew and sporulated well in all mixtures tested. The proportion of 70 + 30% for buckwheat husk and oats (Figure 1), respectively, and 80 + 20% for buckwheat husk and rice, resulted in significantly higher spore production for both species, followed by

90 + 10% and 70 + 30% of BH + 10% R (Table 1). The lowest spore production rate was observed for 80 BH + 20% O.

Table 1. Mass production of spores on solid substrates (CFU g^{-1}).

Treatments	*T. aggressivum* f. *europaeum*	*T. saturnisporum*
90% BH + 10% O	$6.65 \cdot 10^8 \pm 3.04 \cdot 10^7$ c	$6.48 \cdot 10^8 \pm 2.84 \cdot 10^7$ c
80% BH + 20% O	$5.63 \cdot 10^8 \pm 3.20 \cdot 10^7$ d	$5.17 \cdot 10^8 \pm 6.60 \cdot 10^7$ d
70% BH + 30% O	$1.04 \cdot 10^9 \pm 1.44 \cdot 10^7$ a	$9.98 \cdot 10^8 \pm 5.69 \cdot 10^7$ a
90% BH + 10% R	$8.32 \cdot 10^8 \pm 1.61 \cdot 10^7$ b	$7.88 \cdot 10^8 \pm 6.45 \cdot 10^7$ b
80% BH + 20% R	$1.04 \cdot 10^9 \pm 1.04 \cdot 10^7$ a	$1.02 \cdot 10^8 \pm 6.26 \cdot 10^7$ a
70% BH + 30% R	$8.00 \cdot 10^8 \pm 5.00 \cdot 10^7$ b	$7.12 \cdot 10^8 \pm 4.25 \cdot 10^7$ bc
p-value	0.0000	0.0000

BH: buckwheat husk; O: oat; R: rice; CFU: colony forming unit. Data were analysed by ANOVA and treatment means were compared according to Fisher's Least Significant Difference (LSD) statistical procedure (*F*-test at $p < 0.05$). Different letters indicate significant differences according to the one-way ANOVA test ($p = 0.05$).

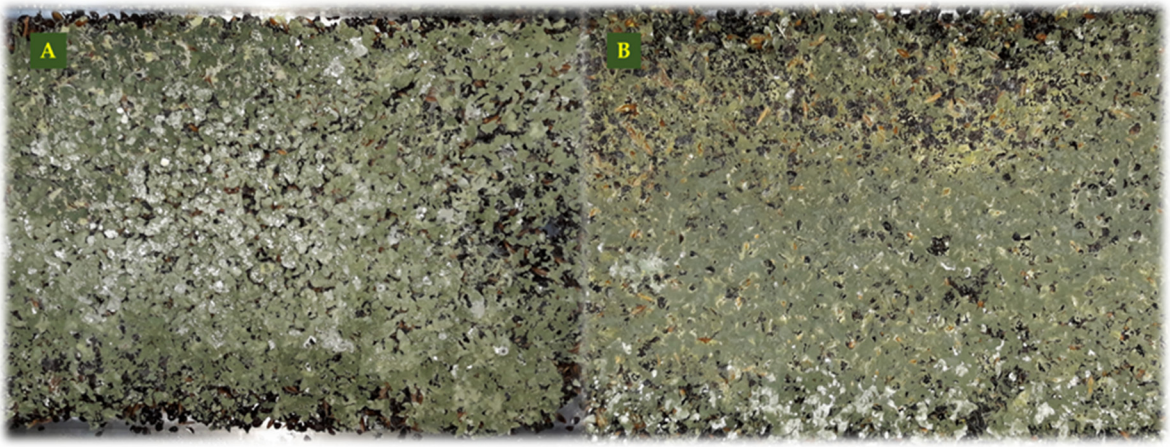

Figure 1. Mass production of (**A**) *Trichoderma aggressivum* f. *europaeum* and (**B**) *Trichoderma saturnisporum* on 70 + 30% for buckwheat husk and oats.

3.2. Siderophore Production, IAA and P Solubilisation

TA and TS siderophore production was observed in the formation of an orange-coloured zone around the fungal colonies at 24 and 48 h, and the production of TA was higher, in both cases. No increase in the diameter of the halo (mm) was detected at 72 h in any isolate (Table 2).

Table 2. Siderophores and IAA production by *Trichoderma* isolates.

Radius of Siderophores Production (mm)				IAA (mg mL^{-1})	
Treatment	**24 h**	**48 h**	**72 h**	**+Trp**	**−Trp**
p-value	0.0000	0.0000	-	0.0068	0.0304
T. aggressivum	9.73 ± 0.89	18.50 ± 1.70	-	0.145 ± 0.011	0.085 ± 0.009
T. saturnisporum	5.45 ± 0.31	9.82 ± 0.56	-	0.199 ± 0.014	0.129 ± 0.021

Values are average of five replications; values after ± represent standard deviation. IAA: indole-3-acetic acid; +Trp: with L-tryptophan; −Trp: without L-tryptophan.

Although both *Trichoderma* strains exhibited an in vitro ability to produce IAA in medium supplemented with and without 100 mg L^{-1} tryptophan during a 7-day period, the production of *T. saturnisporum* was higher under both conditions tested (Table 2). In both TA and TS, IAA production increased in the medium supplemented with tryptophan.

The effects of TA and TS on the soluble phosphate concentration are shown in Figure 2. The initial concentration of P in the medium was used to quantify the concentration of P solubilised by both isolates.

As shown in Figure 2, P solubilisation was significant from the fifth day of incubation, with no significant differences between the two isolates. Furthermore, no change in the pH of the medium was detected, which remained at approximately 6.5–7.

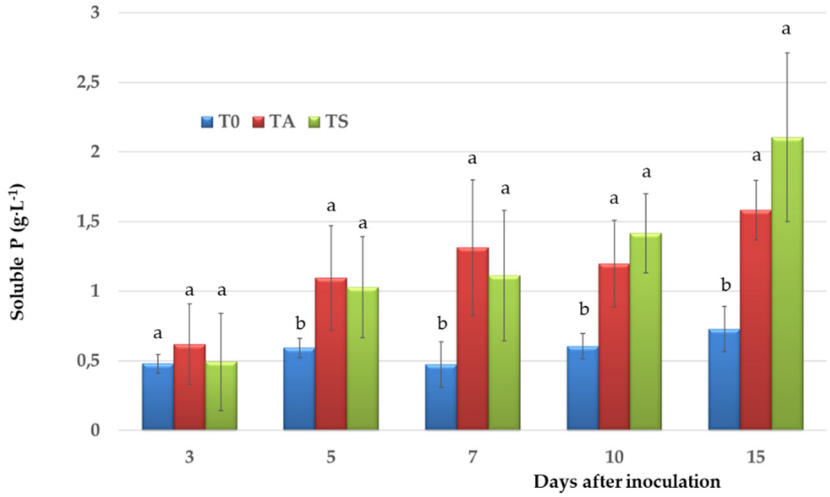

Figure 2. Effects of *Trichoderma aggressivum* f. *europaeum* (TA) and *Trichoderma saturnisporum* (TS) on phosphate solubilisation in National Botanical Research Institute's phosphate (NBRIP) broth containing tribasic calcium phosphate (10 g). T0: NBRIP broth without *Trichoderma* isolates. The results are shown as the average of the three replicates, in g L^{-1}. Mean standard deviation is expressed in the error bar ($n = 3$). For each isolate, columns marked with different letters indicate a significant difference at $p < 0.05$.

3.3. Effects of TA and TS Treatment on Germination and Vigour Index

The results from the in vitro application of TA and TS spores to tomato and pepper seeds are outlined in Table 3. No significant effects on pepper and tomato seed germination percentages were observed in either treatment. However, the application of both *Trichoderma* isolates led to a decrease in the radicle and hypocotyl length (growth) parameters and significantly decreased the SVI in peppers. The tomato seed vigour index was not affected by TA or TS treatment ($p = 0.1918$).

Table 3. Effects of *T. aggressivum* f. *europaeum* and *T. saturnisporum* on tomato and pepper seed germination 7 and 10 days after treatment, respectively.

Treatment	% Germination	Root Length (cm)	Shoot Length (cm)	Seed Vigour Index
		Pepper		
p-value	0.5420	0.0126	0.0010	0.0030
T. aggressivum	83 ± 6.83a	0.64 ± 0.18b	1.66 ± 0.55b	138.14 ± 35.48b
T. saturnisporum	80 ± 3.26a	0.91 ± 0.11b	1.29 ± 0.17b	176.50 ± 18.14b
Control	78 ± 4.61a	2.16 ± 1.01a	1.98 ± 0.56a	320.32 ± 83.36a
		Tomato		
p-value	0.5268	0.0020	0.3154	0.1918
T. aggressivum	89 ± 6.83a	4.53 ± 0.31a	2.97 ± 0.47a	671.80 ± 112.91a
T. saturnisporum	92 ± 7.30a	3.25 ± 0.37b	2.55 ± 0.27a	536.29 ± 76.26a
Control	85 ± 8.32a	3.95 ± 0.28b	2.81 ± 0.29a	580.13 ± 96.37a

Different letters indicate significant differences according to the one-way ANOVA test ($p = 0.05$).

3.4. Effects of Trichoderma Inoculation on Tomato and Pepper Seedlings

The effects of TA and TS application on morphological parameters and DQI are shown in Table 4. Unlike the results from the direct application of both *Trichoderma* to the seeds, the application to the substrate increased the study parameters compared to that of the control, and the results were better in peppers than in tomatoes, with better quality seedlings, according to the DQI values (Figure 3). There were no significant differences after the application of TA and TS in both horticultural plants. The increased percentage assessed in pepper seedlings for each species (TA/TS) was 8%/8.5% for stem length, 12.32/~0.01 for stem diameter, 7.77/5.5 for leaf number, 22.22/25 for shoot dry weight, 36.36/63.63 for root dry weight and 13.83/13.74 for leaf area, respectively. In tomato seedlings, the percentages were 9/6 for stem length, 0.5/1.5 for stem diameter, 6/8.8 for leaf number, 12.5/5.3 for shoot dry weight, 0/–6.6 for root dry weight and 8/9.2 for leaf area. No significant differences in DQI were found in tomato seedlings for any treatment applied with respect to that of the control.

Figure 3. (**A**) Differential growth of pepper seedlings with *T. aggressivum* f. *europaeum* (TA), compared to control. (**B**) Tomato plants grown under field transplantation conditions (60 days).

3.5. Effects of Dose of Application of T. aggressivum f. europaeum and T. saturnisporum

Since no significant plant growth-promoting results were found in tomato seedlings, the effects of applying three doses of both species separately, as well as jointly, were determined. The results are outlined in Table 5, wherein values significantly higher than that of the control are highlighted in green, and negative values in red, for better visualisation.

The increase in the dose of both species improved seedling quality, increasing all study parameters in TA D2 and TS D3 treatments, with respect to that of the control. In treatment TA D2, stem length increased 14.37%, plant diameter 9.4%, leaf number 21.58%, shoot dry weight 16.66% and root dry weight 66.66%. In treatment TS D3, stem length increased 39.05%, plant diameter 15.22%, leaf number 11.55%, shoot dry weight 12.5% and root dry weight 33.33%. Although most treatments favoured the development of seedling shoots, no favourable results were found in roots; therefore, the seedling quality was not improved. The combination or mixture of the two species for the three doses tested did not improve the results compared to that of their separate application.

The results of the study parameters after transplantation of the seedlings into the soil are outlined in Table 5 (Figure 3). Three treatments, TA D1, TA D2 and TS D2, led to a good relationship between tomato shoots and roots, with significantly higher plant quality, compared to that of the control, without a new application of *Trichoderma*. Thus, shoot dry weight increased 43.20%, 22.84% and 29.58% and root dry weight increased 29.94%, 39.32% and 31.51% after the TA D1, TA D2 and TS D2 treatment, respectively. The establishment of the endophytic fungus at the root (Figure 4) enabled its effects to persist after transplantation.

Table 4. Morphological parameters and quality index of pepper and tomato seedlings treated with *T. aggressivum* f. *europaeum* and *T. saturnisporum* at 45 days after sowing.

Treatment	Length of Stem (cm)	Diameter (mm)	Number of Leaves	Aerial Dry Weight (g)	Root Dry Weight (g)	Leaf Area (mm^2)	DQI
			Pepper				
p-value	0.0000	0.0000	0.0092	0.0000	0.0000	0.0001	0.0000
T. aggressivum	29.18 ± 1.36a	4.10 ± 0.24a	7.22 ± 0.76a	0.44 ± 0.06a	0.15 ± 0.30b	86.38 ± 12.58a	0.06 ± 0.01a
T. saturnisporum	29.31 ± 1.75a	3.68 ± 0.22b	7.07 ± 0.82a	0.45 ± 0.06a	0.18 ± 0.09a	86.31 ± 13.22a	0.06 ± 0.01a
Control	27.01 ± 2.07b	3.65 ± 0.23b	6.70 ± 0.72b	0.36 ± 0.04b	0.11 ± 0.03c	75.88 ± 11.15b	0.04 ± 0.01b
			Tomato				
p-value	0.0000	0.4387	0.0031	0.0232	0.0245	0.0295	0.0793
T. aggressivum	27.93 ± 1.99a	3.86 ± 1.84a	4.45 ± 0.50a	0.63 ± 0.09a	0.15 ± 0.02ab	74.63 ± 10.99a	0.06 ± 0.01a
T. saturnisporum	27.17 ± 1.62a	3.90 ± 0.24a	4.57 ± 0.50a	0.59 ± 0.10ab	0.14 ± 0.02b	75,52 ± 13.40a	0.06 ± 0.01a
Control	25.61 ± 2.07b	3.84 ± 0.26a	4.20 ± 0.46b	0.56 ± 0.10b	0.15 ± 0.02a	69.12 ± 6.96b	0.06 ± 0.00a

Different letters indicate significant differences according to the one-way ANOVA test ($p = 0.05$). DQI: Dickson Quality Index.

Table 5. Morphological parameters and DQI of tomato seedlings and plants treated with different doses (10^5, 10^6 and 10^7 spores per plant; D1, D2 and D3, respectively) of *T. aggressivum* f. *europaeum* (TA), *T. saturnisporum* (TS) and mix (M) of two species.

Tomato Seedling						
Treatment	**Length of Stem (mm)**	**Diameter (mm)**	**N° Leaves**	**Aerial Dry Weight (g)**	**Root Dry Weight (g)**	**DQI**
p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
TA D1	135.21 ± 28.79de	3.91 ± 0.46ab	4 ± 0.65b	0.27 ± 0.09a	0.03 ± 0.02cd	0.025 ± 0.01de
TA D2	143.80 ± 32.58d	3.81 ± 0.34b	4 ± 0.51b	0.28 ± 0.07a	0.05 ± 0.018a	0.034 ± 0.01a
TA D3	127.02 ± 25.41e	3.76 ± 0.46b	3.98 ± 0.64b	0.27 ± 0.10a	0.04 ± 0.02ab	0.030 ± 0.01ab
TS D1	141.25 ± 19.83d	3.55 ± 0.49c	3.65 ± 0.60cd	0.23 ± 0.10b	0.03 ± 0.01cd	0.024 ± 0.01de
TS D2	188.44 ± 30.29a	4.07 ± 0.33a	4.25 ± 0.73a	0.28 ± 0.07a	0.03 ± 0.01d	0.021 ± 0.01e
TS D3	174.83 ± 27.78b	4.01 ± 0.45a	3.67 ± 0.63cd	0.27 ± 0.09a	0.04 ± 0.02a	0.030 ± 0.01ab
M D1	157.25 ± 29.14c	3.5 ± 0.39c	3.56 ± 0.62de	0.27 ± 0.061a	0.04 ± 0.01bc	0.026 ± 0.00bcd
M D2	99.08 ± 21.02b	3.13 ± 0.23b	3.35 ± 0.39bc	0.28 ± 0.08a	0.04 ± 0.01bcd	0.029 ± 0.00bcd
M D3	98.54 ± 21.37f	3.11 ± 0.45d	3.33 ± 0.63ef	0.18 ± 0.08c	0.04 ± 0.01ab	0.029 ± 0.00abc
Control	125.73 ± 22.3e	3.48 ± 0.31c	3.29 ± 0.62f	0.24 ± 0.07b	0.03 ± 0.01cd	0.025 ± 0.01cde
Tomato Plants						
Treatment	**Length of Stem (cm)**	**Diameter (mm)**	**Internodes**	**Aerial Dry Weight (g)**	**Root Dry Weight (g)**	**DQI**
p-value	0.0000	0.5379	0.0159	0.0015	0.2769	0.5373
TA D1	101.40 ± 15.06ab	12.11 ± 0.90a	15.00 ± 1.15ab	61.19 ± 11.27a	4.99 ± 0.68ab	3.23 ± 0.24a
TA D2	102.80 ± 9.47a	12.11 ± 1.24a	14.10 ± 1.29ab	52.49 ± 11.26	5.35 ± 0.57a	3.20 ± 0.46a
TA D3	92.00 ± 9.82bc	11.26 ± 1.17ab	14.40 ± 0.84abc	54.74 ± 13.09abc	4.70 ± 1.10abc	3.01 ± 0.58ab
TS D1	77.55 ± 7.76e	11.74 ± 1.18ab	13.90 ± 1.37bc	49.26 ± 14.78bcde	4.40 ± 1.04bc	2.91 ± 0.56ab
TS D2	88.75 ± 10.63cd	11.92 ± 1.57ab	15.20 ± 1.03a	55.37 ± 8.24ab	5.05 ± 1.23ab	3.19 ± 0.42a
TS D3	81.00 ± 12.39de	11.58 ± 1.37ab	13.20 ± 2.57c	51.71 ± 7.97abcd	4.34 ± 1.17bc	3.01 ± 0.94ab
M D1	79.05 ± 13.20e	10.87 ± 1.38b	13.90 ± 1.29bc	47.00 ± 11.38bcde	4.14 ± 1.11bc	2.73 ± 0.64ab
M D2	76.18 ± 11.91e	11.61 ± 0.95ab	13.20 ± 1.23c	45.30 ± 9.79cde	4.23 ± 0.99bc	2.85 ± 0.59ab
M D3	78.45 ± 8.08e	11.22 ± 1.18ab	14.30 ± 1.16abc	40.91 ± 11.56e	4.28 ± 1.37bc	2.72 ± 0.86ab
Control	77.96 ± 7.29e	11.52 ± 2.03ab	14.25 ± 1.16abc	42.73 ± 6.46de	3.84 ± 0.62c	2.60 ± 0.43b

Different letters indicate significant differences according to the one-way ANOVA test ($p = 0.05$). Green: favourable; Red: unfavourable; Orange: no effect compared to control. DQI: Dickson Quality Index.

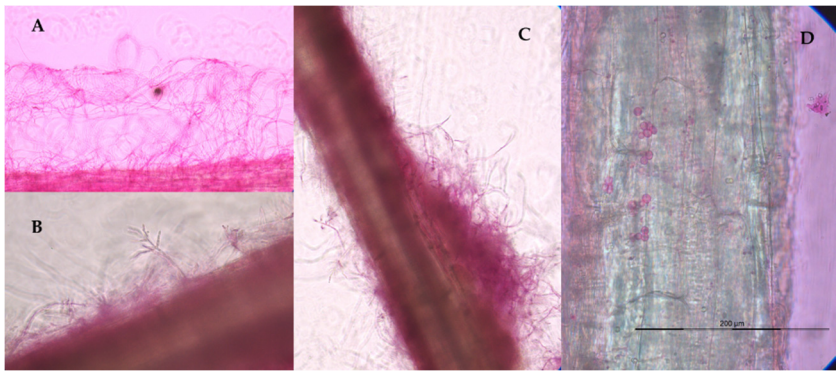

Figure 4. (**A**) Colonization of pepper and tomato roots by *T. aggressivum* f. *europaeum.* (**C**) Mycelium in pepper root (100×). (**B**) Conidiophores and mycelium in tomato root (100×). (**D**) Chlamydospores in pepper root (200×).

4. Discussion

Numerous *Trichoderma* species have been described as plant-growth promoters, including *T. harzianum, T. longipile*, *T. tomentosum*, *T. viride*, *T. koningii*, *T. asperellum, T. aureoviride* and *T. saturnisporum,* among others [34]. This ability to promote growth depends on several factors, including the existence of isolates of the same species that may or may not promote plant growth, or for example, the crop and/or variety to which the species is applied [34]. Similarly, the use of a mixture of species has been extensively studied and commercialised to increase this activity [10]. In this study, the plant growth-promoting capacity of a new species, *T. aggressivum* f. *europaeum*, which is characterised by its rapid growth and sporulation, was analysed and compared to that of *T. saturnisporum*, a species characterised as a plant-growth promoter by Diánez et al. [16,18]. Although Allaga et al. [35] recommend not using species that produce green mould disease, these species do not create any problems in horticultural crops or pose any danger to mushroom crops, as long as they are applied in different geographical areas. Additionally, mushrooms are produced in closed locations and under completely different conditions. Furthermore, plant remains under horticultural production are not used to prepare substrates for mushroom cultivation, as shown in many commercial species; neither are plant remains that have been studied with plant-growth promoters, which may also cause green mould disease, such as *T. harzianum* [36] or *T. longibrachiatum* [37].

The first objective was to obtain viable spores with high yield on low-cost substrates. This product was used for additional tests, which demonstrated that the nutritional composition of the substrates used did not affect the biostimulant capacity of either *Trichoderma* species. Lane [38] determined that the nutrients provided in the medium could affect the biocontrol or biostimulant capacity of the agent. Different substrates have been used for *Trichoderma* spore production, including barley straw [39], wheat, rice, corn kernels [40] or a mixture of substrates, such as wheat straw, bran, cassava, potato starch and sugar beets [41,42], among others. In general, in our study, high yields, expressed as colony forming unit (CFU) g^{-1}, were assessed in all substrate mixtures tested; the yields increased both in 80% buckwheat husk + 20% rice and 70% buckwheat husks + 30% oats. Although in laboratory tests, extraction could be performed without a problem in all mixtures, in the extractor tank, mixtures containing rice adhered to the walls and pipes, complicating the subsequent extraction and filtration processes. For this reason, to develop low-cost production methods for industrial scale-up, rice was rejected as a constituent of the production substrate for TA and TS. A high siderophore and IAA production and P solubilisation by TA and TS compared to other *Trichoderma* species or isolates were demonstrated in our study. These three components play key roles in plant biostimulation by increasing nutrient availability to plants, such as for hormone production [43,44]. However, the direct relationship between IAA production and plant-growth promotion is not yet clear because numerous species can produce IAA, but they do not promote plant growth [45]. Hoyos et al. [45] concluded that IAA production is not a

species-dependent quality of *Trichoderma* and found no direct correlation between biostimulation and IAA and siderophore production or P solubilisation. In turn, Vinale et al. [46] highlighted the effects of siderophore (harzianic acid) production on the germination of tomato seeds and the improved growth of the seedlings even under iron-deficient conditions. Similarly, Qi and Zhao [47] demonstrated that applying *T. asperellum* enhanced cucumber growth by inducing physiological protection under saline stress, and its siderophores played a key role in mitigating the negative effects of salinity.

Many *Trichoderma* species can produce IAA, and high IAA secretion in the presence of tryptophan indicates the importance of tryptophan as a precursor for IAA production [48,49]. Gravel et al. [50] reported that IAA production induced by L-tryptophan increased the fresh weight of tomato shoots and roots. Our results indicate that TA and TS produce much higher amounts of IAA than those assessed by other authors. Accordingly, Saber et al. [48] described IAA production of *T. harzianum* isolates that were 10 times lower than that of *T. aggressivum* f. *europaeum* and *T. saturnisporum* assessed in this study. Bader et al. [51] reported that IAA production ranged from 13.38 to 21.14 μg mL^{-1} in *T. brevicompactum, T. gamsii* and *T. harzianum*. Diánez et al. [16] described a highly similar IAA production for *T. saturnisporum*; therefore, the in vitro production capacity of IAA was preserved despite maintaining the isolate in the laboratory for 10 years. Similarly, phosphate solubilisation by *Trichoderma* species has been described both in vitro and in vivo [52–54]. Recently, Tandon et al. [55] evaluated P solubilisation of different *Trichoderma koningiopsis* isolates under abiotic stress conditions and determined a range from 1.6 to 71 μg mL^{-1}. Bononi et al. [12] found that *Trichoderma* isolated from soils of the Amazon rainforest demonstrated a high potential for phosphate solubilisation, which ranged from 51.7 to 90.3% 10 days after inoculation. Despite their high P solubilisation capacity, some of these isolates inhibited the germination of soybean seeds. In our study, the P solubilisation range of both isolates was lowest on the tenth day of incubation, at 5.9% and 6.16% for TA and TS, respectively.

Applying PGPMs to seeds makes it possible to use a lower concentration of spores while ensuring that the PGPMs are readily accessible at germination and during early developmental plant stages, stimulating healthy and rapid establishment, and consequently, maximising crop production [43]. However, the direct application of different *Trichoderma* isolates or species to seeds (bioprimming) has not always had beneficial effects. In this study, the seed germination rate was not affected by *T. aggressivum* f. *europaeum* or *T. saturnisporum* application. Similar results were found by Azarmi et al. [18] after applying *T. harzianum* isolates to tomato seeds. Hajieghrari et al. [56] demonstrated that direct exposure of corn seeds to *Trichoderma* spores decreased the percentage of seed germination, as well as radicle and shoot length. However, You et al. [57] demonstrated that *T. harzianum* and *T. koningiopsis* isolates significantly enhanced the tomato seed vigour index when they were used to treat tomato seeds. Our results demonstrated that direct *T. aggressivum* f. *europaeum* and *T. saturnisporum* application decreased seed vigour, significantly so in peppers but not in tomatoes. However, the application of either species under commercial plant nursery conditions, via substrate irrigation, similarly enhanced pepper seedling quality significantly, albeit again non-significantly for tomatoes. Optimising the application dose for each species is a factor that should be considered, among other factors, to enable companies and producers to adopt this technology with higher security [58]. Increasing the dose of *T. aggressivum* f. *europaeum* and *T. saturnisporum* applied to tomato seedlings increased most of the study parameters, as well as the DQI value in treatments TA D2, TA D3 and TS D3. The endophytic establishment of *Trichoderma* in plant nurseries may ensure its colonisation once transplanted. As such, in the TA D2 treatment, tomato plants continued to show better quality, without any additional application of *Trichoderma*, and plant quality improved in other treatments with *Trichoderma* applied separately. The poorest results were obtained for mixtures of both species, with no improvement in study parameters for any dose tested, and even a reduction of 21.62%, 10.63% and 25% in stem length, diameter and shoot dry weight of tomato seedlings in the MD3 treatment, respectively. Similar results were found by Liu et al. [59], who reported that the combination of three species, *T. afroharzianum, T. pseudoharzianum* and *T. asperelloides*, decreased the biocontrol and growth-promoting effects in comparison to the application of each species separately.

Although major reductions in the use of chemical fertilisers without production losses is currently difficult in many farming systems, their gradual decrease accompanied by the use of biostimulants or biofertilizers is a tool that can optimise the use of chemical inputs while reducing environmental pollution and food crop contamination.

5. Conclusions

The present study demonstrated, for the first time, the biostimulant capacity of *T. aggressivum* f. *europaeum* in pepper and tomato plants under commercial plant nursery and greenhouse conditions, with similar results to those of *T. saturnisporum*.

6. Patents

This isolate was patented with a Spanish patent number ES2706099: New strain of *T. aggressivum* f. *europaeum*, compositions and applications.

Author Contributions: F.D. and M.S. conceived and designed the experiments; B.S.-M., F.J.G., and A.M.-G. performed the experiments; M.S. analysed the data; F.D. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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