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# Native *Trichoderma* Isolates from Soil and Rootstock to *Fusarium* spp. Control and Growth Promotion of *Humulus lupulus* L. Plantlets

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**Abstract:** *Fusarium* genus is a wide host phytopathogen causing significant losses in multiple crops, including hops. There is limited information on the sustainable management of *Fusarium* spp. in hop fields. *Trichoderma* is an endophytic fungus used in agriculture as a biological control agent (BCA) and as a plant growth promoter. It has been used to antagonize *Fusarium* spp. in other crops. The objective of the current study was to identify indigenous hop field *Trichoderma* isolates with biocontrol and hop growth promotion capabilities. Three isolates of *Fusarium* and eleven autochthonous *Trichoderma* isolates collected from sustainable hop fields were evaluated in this work. Direct confrontation tests (the physical interaction between the pathogen and BCA and their competition for space and nutrient resources) and membrane tests (the capacity of the BCA to produce metabolites or enzymes through a cellophane film and inhibit the development of the pathogen) assessed the antagonism of these *Trichoderma* isolates against *Fusarium culmorum*, *F. sambucinum*, and *F. oxysporum*. A bioassay with hop plantlets inoculated with a spore suspension of *Trichoderma* was performed to assess its hop growth enhancement. *T. hamatum* (T311 and T324), *T. virens* T312, and *T. gamsii* T327 showed high growth inhibition of *Fusarium* spp. phytopathogens and high plant growth promotion. Native *Trichoderma* isolates from sustainable hop-producing soils have great potential as BCAs and hop growth promoters.

**Keywords:** hops; biological control; antifungal activity; plant-growth promotion; soil microorganisms; sustainable agriculture; direct confrontation; dual culture; membrane assay



**Citation:** Porteous-Álvarez, A.J.; Fernández-Marcos, A.; Ramírez-Lozano, D.; Mayo-Prieto, S.; Cardoza, R.E.; Gutiérrez, S.; Casquero, P.A. Native *Trichoderma* Isolates from Soil and Rootstock to *Fusarium* spp. Control and Growth Promotion of *Humulus lupulus* L. Plantlets. *Agriculture* **2023**, *13*, 720. https://doi.org/10.3390/agriculture13030720

Academic Editors: Beata Janowska and Maciej Bosiacki

Received: 14 February 2023
Revised: 16 March 2023
Accepted: 16 March 2023
Published: 21 March 2023



## 1. Introduction

Hops (*Humulus lupulus* L.) are long-lived, perennial, herbaceous, and dioecious climbing plants cultivated worldwide for the female flower cones used in the brewing and pharmaceutical industries. Hops are affected by several pests and diseases that can cause significant economic losses [1]. The main pests and diseases affecting hops are mildew, powdery mildew, *Verticillium*, *Fusarium*, aphids, and two-spotted spider mites. They all cause significant reductions in the production and quality of cones, affecting farmers economically [2].

There is a concern to control the main pests and diseases affecting hop fields and help reduce economic losses. Detailed research has been done on the management of aphids on hops [1–5]. Several research groups are currently studying the biology and management of powdery mildew [6–10]. There is a rising concern among hop farmers about the presence of *Fusarium* spp. in hop fields and the ways to control it. *Fusarium* spp. in hops has been described as a problem in Europe [11,12], the USA [13], and Brazil [14].

*Fusarium* spp. is a wide host phytopathogen causing significant losses in multiple crops, including hops. It survives in the soil or on living plant material and accesses the plant through wounds or natural openings in the plant tissue at ground level. *Fusarium sambucinum* Brondeau can cause *Fusarium* canker or wilt and *Fusarium* cone tip blight [15]. *F. oxysporum* Schltdl. and *F. culmorum* (Wm. G. Sm.) Sacc. can cause hop wilting [16]. In *Fusarium* cone tip blight, a necrosis at the tip of the cone is observed. In *Fusarium* canker and wilt, the affected area of the stem swells while, near the crown, the stem narrows again. Infected branches can be covered with white-pink or red-brown mycelium during the growth or sporulation of the fungus on the stem surface [12,17,18].

There is limited information about the management of these infections with sustainable solutions in hop fields. The need to find new sustainable and environment-friendly solutions against phytopathogens in different crops has led to the search for biocontrol agents (BCA) belonging to genera such as *Bacillus*, *Beauveria*, *Fusarium*, *Pseudomonas*, *Streptomyces*, or *Trichoderma* [19–22]. Many research reports show possible sustainable solutions to control *Fusarium* spp. using *Trichoderma* spp. in vitro or in crops including melon or wild apple [23–28], but no similar studies have been conducted to control *Fusarium* spp. on hops.

*Trichoderma* spp. is an important endophytic fungal genus, a fast-growing secondary opportunistic invader, avirulent [29,30], and beneficial to agriculture because it protects crops against phytopathogens like *Rhizoctonia solani*, *Fusarium* spp., *Sclerotinia*, and *Alternaria* [26,31–33] and promotes plant growth and development, as reported by many authors over time [24,34–36]. The benefits to agriculture are associated with the production of antibiotics and plant growth regulators (PGRs) [37] and the induction of systemic resistance in the plants [38]. *Trichoderma* can promote plant growth due to different mechanisms like mobilizing nutrients in the soil, producing siderophores, solubilizing phosphorus or synthesizing PGRs [24,34,35,39]. The increment of the plant biomass has been associated to with synthesis of auxin-derivative compounds like indole-3-acetic acid (IAA) or other PGRs like gibberellic acid ($GA_3$) [34,40–43]. Moreover, indigenous microorganisms co-evolve with a particular crop over time and adapt to environmental changes. Isolates collected from a given crop may provide better biocontrol capabilities than isolates collected from a different plant species [44]. It is essential to develop rational management against pests and diseases in our crops. To do so, select autochthonous *Trichoderma* isolates with beneficial characteristics for the plant, such as plant growth promotion and biocontrol of pathogens.

The aim of this study was to identify autochthonous isolates of *Trichoderma* adapted to hop-producing fields that show biocontrol and plant growth promotion abilities and to evaluate their antagonism against native *Fusarium* isolates and their hop plant growth promotion activity.

## 2. Materials and Methods

### *2.1. Trichoderma and Fusarium Isolates and Culture Collection*

The present assay was performed with three *Fusarium* isolates collected in the hop-producing area of San Román de la Vega, León (Spain), from fields presenting *Fusarium*-like symptoms. The *Trichoderma* isolates used in the study were collected from four sustainable hop-producing fields (Table 1) [45]. All isolates were stored in the collection of pathogens and antagonists at the 'Laboratorio de Diagnóstico de Plagas y Enfermedades Vegetales' (LDPEV), Universidad de León, Spain.

The Nucleospin Plant II kit (Macherey-Nagel, Düren, Germany) was used to extract the genomic DNA from 100 mg of each fungal isolate, following the manufacturer's protocol for fungi. The resulting extracts were eluted in 50 µL of sterile water, and a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to estimate the DNA concentration. Solutions of 50 µL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 1.5 U of DreamTaq DNA polymerase (Thermo Scientific), 200 nM for each dNTP, 400 nM for each primer, and 50 ng of DNA were used to amplify the sequences. For the identification of the *Trichoderma* isolates analyzed in the present work, a fragment corresponding to the ITS region was amplified using the oligonucleotides ITS5 (5′-

GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). For the identification of *Fusarium* spp. in the present work, an internal fragment of the gene tef1 was amplified using the oligonucleotides EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′). The PCR products were purified using the NucleoSpinExtract II kit (Machery-Nagel, Düren, Germany), and sequenced afterwards using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an automatic capilar sequencer ABI 3130xl (Applied Biosystems). All the steps were performed in accordance with the manufacturer's instructions. In order to identify the fungal isolates, the sequences obtained were analyzed and compared with those in the NCBI Genbank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov, accessed on 17 March 2023) using the BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST, accessed on 17 March 2023). The generated sequences were deposited at DDBJ/ENA/Genebank. Accession numbers are specified in Table 1.

**Table 1.** *Trichoderma* and *Fusarium* isolates collected from hop-producing plots in León (Spain).

| ID Treatment | Identified as | % Identity | Origin | Location | Accession Number |
|---|---|---|---|---|---|
| T311 | *T. hamatum* [1] | >99% | Soil | Gavilanes de Órbigo | OQ590011 |
| T324 | *T. hamatum* [1] | >99% | Rootstock | San Román de la Vega | OQ589841 |
| T312 | *T. virens* [1] | >99% | Soil | Gavilanes de Órbigo | OQ590010 |
| T317 | *T. virens* [1] | >99% | Rootstock | Nistal | OQ589506 |
| T327 | *T. gamsii* [1] | >99% | Soil | Seisón de la Vega | OQ589844 |
| T316 | *T. rossicum* [1] | >99% | Rootstock | Gavilanes de Órbigo | OQ589503 |
| T328 | *T. rossicum* [1] | >99% | Soil | Gavilanes de Órbigo | OQ589861 |
| T329 | *T. harzianum* [1] | >99% | Soil | San Román de la Vega | OQ589868 |
| T314 | *T. spirale* [1] | >99% | Soil | Nistal | OQ589492 |
| T319 | *T. spirale* [1] | >99% | Rootstock | Nistal | OQ589706 |
| T323 | *T. brevicompactum* [1] | >99% | Soil | San Román de la Vega | OQ589712 |
| F076 | *F. culmorum* [2] | >99% | Soil | San Román de la Vega | OQ625436 |
| F103 | *F. oxysporum* [2] | >99% | Soil | San Román de la Vega | OQ632904 |
| F079 | *F. sambucinum* [2] | >99% | Rootstock | San Román de la Vega | OQ632903 |

[1] The isolates were identified by ITS regions. [2] The isolates were identified by EF regions.

### 2.2. In Vitro Antifungal Assays-Direct Confrontation

The direct confrontation assay evaluated the in vitro physical interaction between the pathogen and BCA and their competition for space and nutrient resources [32]. The inoculum of pathogen and BCA was obtained from the growing edge of 7 day cultures growing on potato-dextrose-agar medium (PDA, Sigma-Aldrich, St. Louis, MO, USA) at 25 °C and collected using a 6 mm diameter cork borer. The plugs were sown on the same Petri plate with PDA medium, with a 55 mm distance between plugs. Each treatment was represented by 4 replicates. Growth diameters of the pathogen were measured after five days, taking two measures of the colony: r1, the radium of the colony growing towards the BCA, and r2, the radium growing away from the BCA; the latter served as a control for the growth of the pathogen against the growth of the colony closer to *Trichoderma*. The percentage of growth inhibition caused by *Trichoderma* was calculated using Equation (1):

$$\text{Growth inhibition (\%)} = (r2 - r1)/r2 \cdot 100. \quad (1)$$

### 2.3. In Vitro Antifungal Assays-Membrane Assay

The membrane assay evaluated the capacity of the BCA to produce metabolites or enzymes through a cellophane film and inhibit the development of the pathogen without direct contact [32]. Inoculum plugs were collected as described above. On a Petri plate with PDA medium, a cellophane film was placed in contact with the medium, and a 6 mm *Trichoderma* plug was placed on the center of the membrane and left to grow for 48 h. After that time, the film was removed, and a 6 mm plug of the *Fusarium* isolate was placed on the

center of the plate. The growth of the fungi was evaluated five and eight days later, taking two orthogonal measurements of the growth diameters. Four replicates were evaluated per treatment. Plates with no *Trichoderma* served as a control. The data are presented as a percentage of pathogen growth inhibition compared to the growth on control plates.

### *2.4. In Vivo Assay-Trichoderma Growth Promotion Activity*

The in vivo assay evaluated the growth promotion of *H. lupulus* L. 'Columbus' plantlets by *Trichoderma* isolates under controlled climatic conditions. The bioassay was performed with one-month-old plantlets obtained from aerial parts of 'Columbus' cultivar. After one month, the plantlets formed their own root systems. Plantlets were grown in 100 mL volume pots with non-autoclaved peat substrate (Exclusive Substrate, Gebr. Brill Substrate GmbH & Co. KG, Georgsdorf, Germany) and were watered weekly.

The bioassay was performed in a climate chamber at 25 °C and 70% relative humidity (RH) during the day and 15 °C and 75% RH during the night, with a photoperiod of 16:8 h day:night.

Ten plants per treatment were selected, and their initial development was measured. All plants presented one string at the beginning of the assay, with a similar average number of nodes (5.4 + 1.9) in all treatments at the beginning of the trial. Figure 1 shows an example of two plantlets with the initial development of one string and five to six pairs of leaves, or nodes.

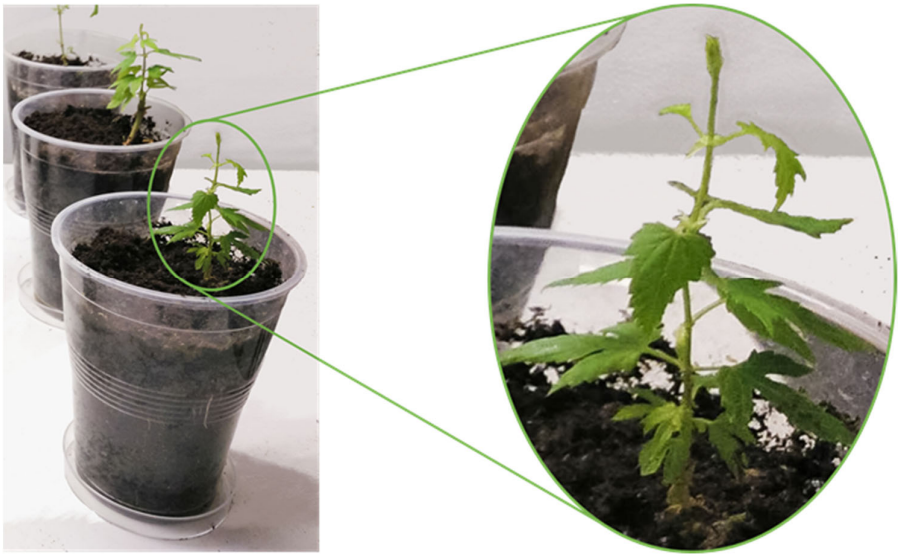


**Figure 1.** Hop plantlets with one string and five–six nodes per plant.

Plantlets were inoculated with 5 mL of each *Trichoderma* spore solution ($1 \times 10^7$ sp/mL). Plants inoculated with 5 mL of water were used as control plants. They were kept in the growing chamber under the climate conditions described above for two months. Plant growth was evaluated one and two months after inoculation by tallying the number of strings and the number of nodes per string, counting from the base of the plant to the last pair of leaves distinct from the tip bud.

Two months after the inoculation, soil samples were collected from the surface and the soil surrounding the rootstock of the plants. Soil samples were spread on Rose Bengal chloramphenicol agar medium (Conda Laboratory, Torrejón de Ardoz, Madrid, Spain) and incubated at 25 °C in the dark to confirm Koch´s postulates, as *Trichoderma* growth was observed in the culture media with soils inoculated with *Trichoderma* and there was no growth of *Trichoderma* on control pots.

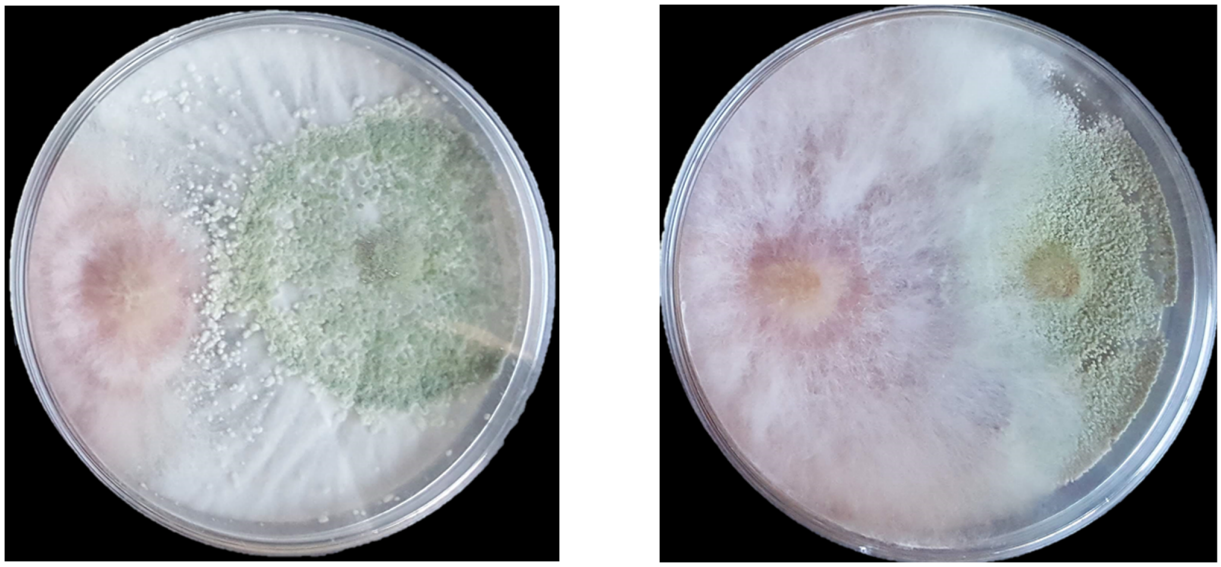
### *2.5. Statistical Analysis*

All data were compared by analysis of variance (ANOVA) and compared by Fisher's least significant difference (LSD) ($p < 0.05$) post hoc tests using SPSS (IBM SPSS Statistics for Windows, Version 26.0, Armonk, NY, USA).

## 3. Results

### *3.1. In Vitro Antifungal Assay-Direct Confrontation*

Direct confrontation assays evaluated the competition for the media resources and space of the *Trichoderma* isolates against *Fusarium* spp. In the current study, the isolates performed differently with each pathogen isolate (Figure 2).



(**a**) (**b**)

**Figure 2.** Direct confrontation of *F. culmorum* with (**a**) *T. hamatum* T311 (65.8% of growth inhibition) and (**b**) *T. rossicum* T328 (49.7% of growth inhibition). In the image, *Fusarium* sp. is on the left, and *Trichoderma* spp. is on the right.

Figure 3 represents the mean percentages of growth inhibition of the *Fusarium* spp. assayed. Regarding the direct confrontation results, the isolates of *T. hamatum* (T311 and T324) showed the highest values of growth inhibition against *F. culmorum* (65.8% and 66.3%) and *F. oxysporum* (47.5% and 50.6%), respectively. Against *F. sambucinum*, *T. hamatum* T311 exhibited the highest inhibition percentage, 46.3%, without significant differences with *T. hamatum* T324, 36.5%. The isolates of the species *T. virens* (T312 and T317) showed high inhibition rates against the *Fusarium* isolates, but these values were lower than the performance of the *T. hamatum* isolates. *T. virens* isolates performed similarly against all *Fusarium* isolates, having no significant differences among each other. *T. virens* T317 had no significant differences with *T. hamatum* in the growth inhibition of *F. culmorum* (61.0%) and *F. sambucinum* (35.8%). *T. gamsii* T327 showed 43.2% and 42.8% growth inhibition against *F. sambucinum* and *F. oxysporum*, respectively, with no significant differences to *T. hamatum* (T311 and T324). The inhibition percentage shown by *T. gamsii* T327 against *F. culmorum* (54.0%), although high, was significantly lower compared to the performance of *T. hamatum* (T311 and T324). *T. rossicum* (T316 and T328) showed the lowest values on growth inhibition of *Fusarium* spp. compared to the rest of the *Trichoderma* isolates. *T. rossicum* T328 presented the lowest inhibition percentages, 13.4% and 11.2% against *F. sambucinum* and *F. oxysporum*, respectively. *T. rossicum* T328 showed 49.7% growth inhibition in *F. culmorum*. *T. harzianum* T329 caused a 52.0% growth inhibition of *F. culmorum* without significant differences with *T. virens* T312 and *T. gamsii* T327. Against *F. oxysporum*, *T. harzianum* T329 had a 30.0% growth inhibition, without significant differences with *T. virens* (T312 and T317). Against

*F. sambucinum*, *T. harzianum* T329 showed a high inhibition percentage (36.6%) without significant differences to *T. hamatum* (T311 and T324).

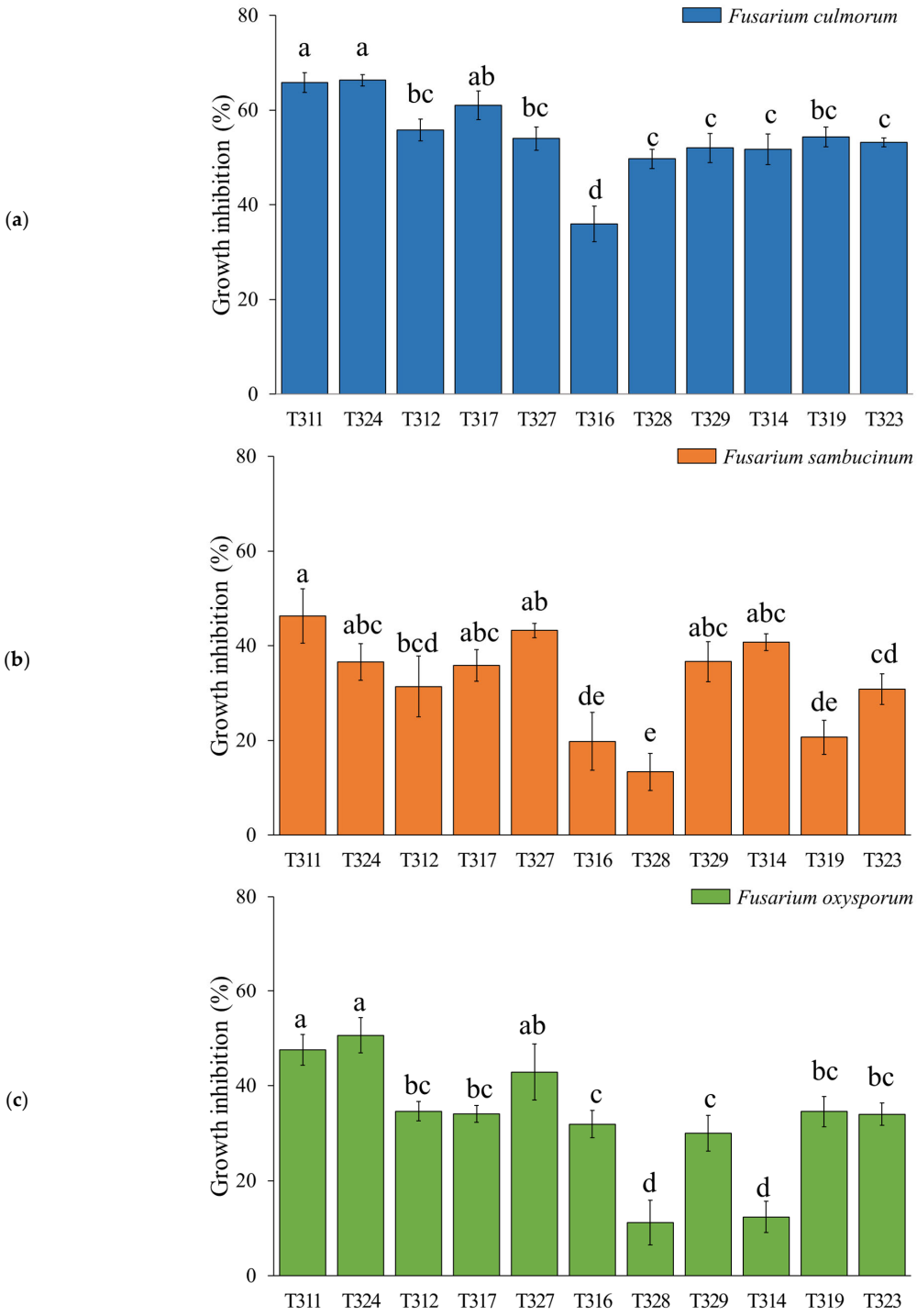



**Figure 3.** Growth inhibition percentages of the *Trichoderma* isolates against (**a**) *F. culmorum*, (**b**) *F. sambucinum*, and (**c**) *F. oxysporum* in vitro by direct confrontation. Mean values ± SE. Columns within the same chart with different letters indicate significant differences (Fisher's LSD $p < 0.05$).

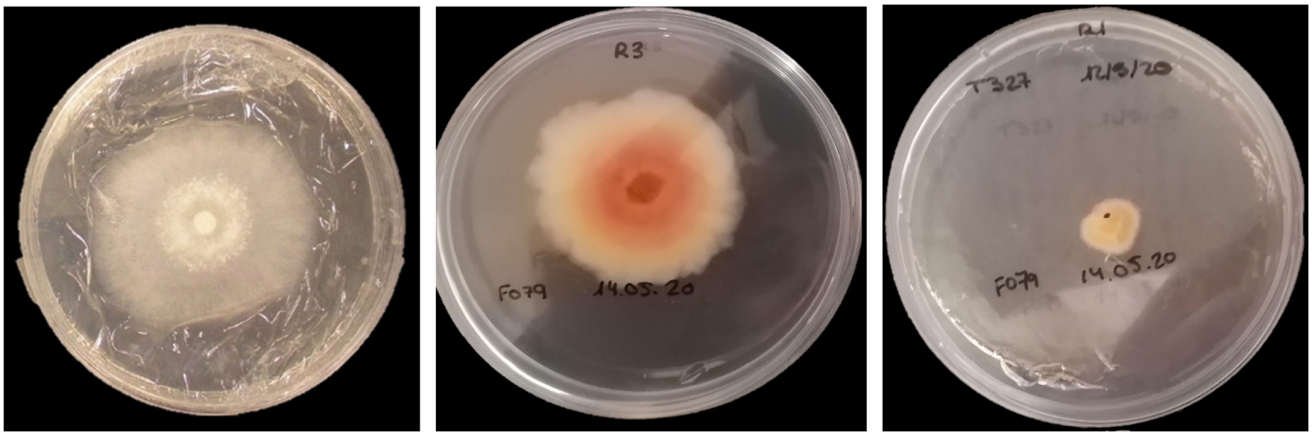
The isolates of *T. spirale* (T314 and T319) showed significantly similar inhibition percentages for *F. culmorum* (51.7% and 54.3%) but interacted differently against the other *Fusarium* species. *T. spirale* T314 had high inhibition percentages against *F. sambucinum* (40.7%) without significant differences with *T. hamatum* (T311 and T324), and *T. spirale* T319 showed a higher inhibition percentage against *F. oxysporum* (34.6%) without significant

differences with *T. virens* (T312 and T317) and *T. gamsii* T327. *T. brevicompactum* T323 presented a 53.1% growth inhibition of *F. culmorum* and 30.8% and 34.0% growth inhibition against *F. sambucinum* and *F. oxysporum*, respectively, without significant differences to *T. rossicum* T316.

Overall, the isolates of *T. hamatum*, (T311 and T324) showed a high inhibition percentage of the three *Fusarium* isolates. *T. virens* (T312 and T317) and *T. gamsii* T327 presented high inhibition values but lower than the *T. hamatum* isolates, with significant differences against *F. oxysporum* in the case of *T. virens* and *F. culmorum* in *T. gamsii* T327. *T. rossicum* (T316 and T328) showed significant differences with the top isolates but still presented high inhibition percentages for *F. culmorum*. *T. harzianum* T329 had adequate growth inhibition rates against *F. culmorum*, *F. sambucinum*, and *F. oxysporum*.

### 3.2. In Vitro Antifungal Assay-Membrane Assay

The membrane assays evaluated the capacity of each *Trichoderma* isolate to produce metabolites and enzymes with antibiosis activity under laboratory conditions. Figure 4a shows how *Trichoderma* spp. grew on the cellophane membrane without physical contact with the PDA medium. Figure 4b shows how *F. sambucinum* grew naturally on PDA medium in a negative control plate, and Figure 4c shows the inhibited growth of *F. sambucinum* on PDA after removing the cellophane with the *T. gamsii* T327 colony.




(**a**) (**b**) (**c**)

**Figure 4.** (**a**) *Trichoderma* spp. growing on the membrane without physical contact to the medium, (**b**) *F. sambucinum* growing on a negative control on potato-dextrose-agar medium (PDA, Sigma-Aldrich, St. Louis, MO, USA), and (**c**) *F. sambucinum* growing on PDA after removing the membrane with *T. gamsii* T327 (74.9% of growth inhibition).

Figure 5 represents the growth inhibition in the membrane assays of all the *Trichoderma* isolates against *Fusarium* spp. *T. gamsii* T327 showed good performance in production of metabolites with antifungal activity against all the pathogens assayed and an outstanding growth inhibition percentage for *F. sambucinum* (74.9%). *T. hamatum* (T311 and T324) presented greater antibiosis against *F. culmorum* (53.3% and 41.8%) and *F. sambucinum* (63.9% and 64.1%) but low antibiosis against *F. oxysporum* (21.8% and 17.2%). *T. virens* (T312 and T317) showed high growth inhibition values against *F. sambucinum* (64.4% and 67.5%) and *F. oxysporum* (44.9% and 42.8%) but lower inhibition rates against *F. culmorum* (28.7% and 23.0%). *T. spirale* (T314 and T319) had their highest inhibition rates against *F. sambucinum* (38.1% and 35.0%) without significant differences between *T. spirale* T319 and *T. harzianum* T329. Against *F. oxysporum*, *T. spirale* (T314 and T319) presented a low inhibition percentage (19.0% and 18.0%), with no significant differences to *T. hamatum* (T311 and T324). *T. harzianum* T329 showed low inhibition percentages against all the *Fusarium* isolates assayed, with the highest value being 29.2% in *F. sambucinum*. *T. brevicompactum* T323 had a high inhibition rate in *F. sambucinum* (58.5%) without significant differences to

*T. hamatum* and *T. spirale* and a 45.0% inhibition rate against *F. culmorum* and *F. oxysporum*. The isolates of *T.* rossicum (T316 and T328) had the lowest values against all the *Fusarium* isolates, all under 20.0% growth inhibition, with significant differences with the rest of the *Trichoderma* isolates.

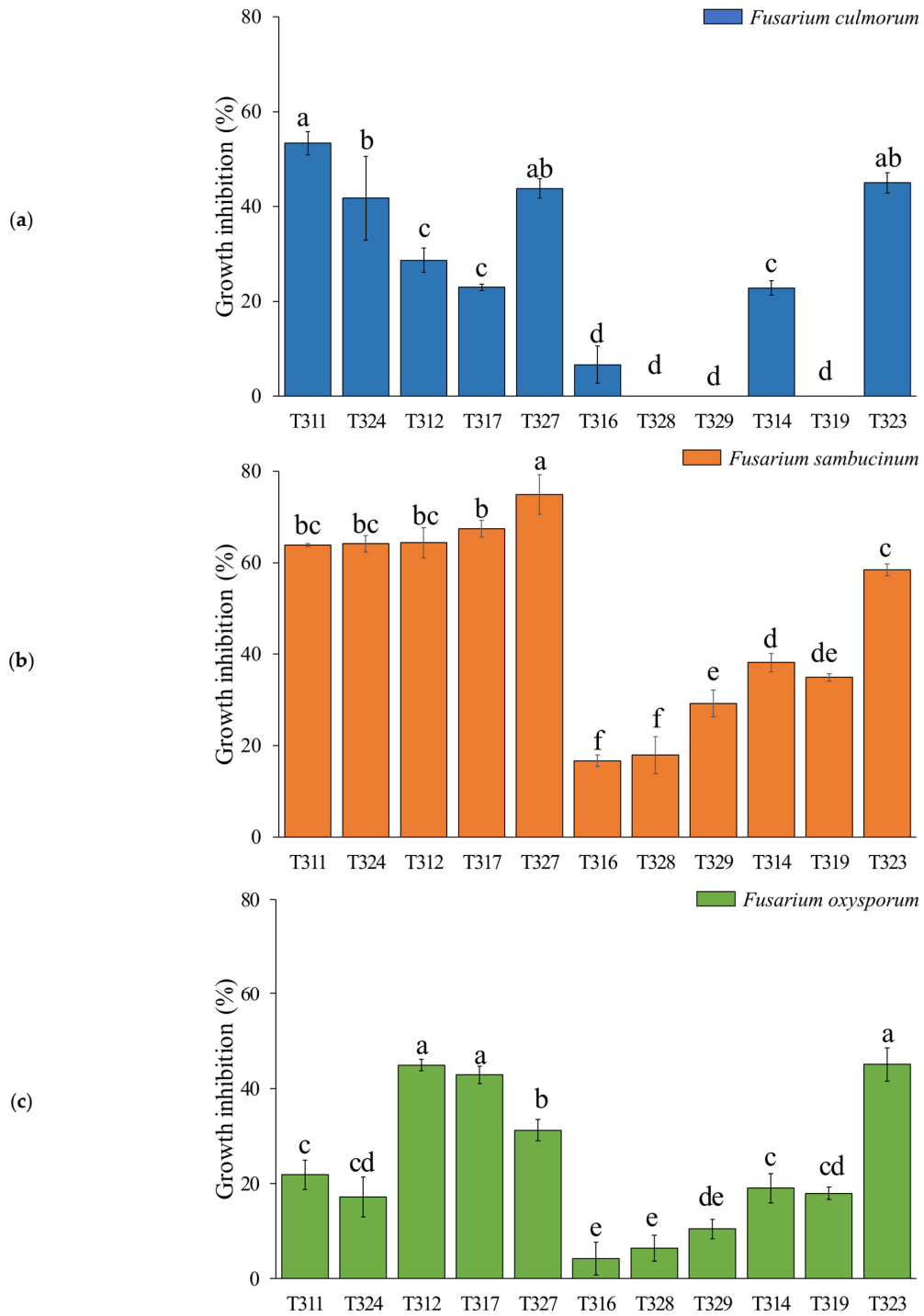



**Figure 5.** Growth inhibition percentages of the *Trichoderma* isolates against (**a**) *F. culmorum*, (**b**) *F. sambucinum*, and (**c**) *F. oxysporum* in antifungal membrane assays. Mean values ± SE. Columns within the same chart with different letters indicate significant differences (Fisher's LSD, $p < 0.05$).

Altogether, *F. culmorum* and *F. oxysporum* showed a lower influence by *Trichoderma* metabolites, whereas *F. sambucinum* was more susceptible, as the percentages of inhibition had higher values. In general, the metabolite secretion of *T. hamatum* (T311 and T324) and

*T. gamsii* (T327) showed greater antibiosis against *F. culmorum* and *F. sambucinum*. The metabolites secreted by *T. virens* (T312 and T317) presented high growth inhibition values against *F. sambucinum* and *F. oxysporum*. *T. harzianum* T329 had low antagonism against the *Fusarium* isolates assayed, and the isolates of *T. rossicum* (T316 and T328) showed the lowest antibiosis against all the *Fusarium* isolates. *T. rossicum* (T316 and T328) and *T. harzianum* T329 showed the lowest antibiosis against all the *Fusarium* isolates.

### *3.3. In Vivo Assays-Trichoderma Growth Promotion*

The bioassays in the climate chamber evaluated the growth promotion features of the *Trichoderma* isolates on 'Columbus' cultivar plantlets under controlled conditions. Plants used in the trial presented a significantly similar development on the day of inoculation; all plants had the same number of strings and a similar number of nodes distributed evenly throughout the treatments. Therefore, treatments were compared among them without further consideration in the following measurements. Figure 6 shows hop plantlets in the climate chamber during the assay.

**Figure 6.** Hop plantlets in the climate chamber during the growth promotion assay.

Figure 7 represents the mean values of the development measures taken one and two months after the treatments. After one month, plants in the *Trichoderma* treatments did not significantly increase their development over the controls (CC), except for *T. rossicum* T328 that significantly increased the number of nodes per plant (Figure 7b) and *T. virens* T312 that significantly increased the number of nodes per string (Figure 7c). After one month, *T. gamsii* T327-treated plants, presented the highest number of strings, showing four times the initial number of strings. Treatments with *T. rossicum* T328, *T. gamsii* T327, and *T. spirale* T319 showed the biggest enhancement in the number of nodes on the hop plantlets after one month. *T. virens* T312 caused a significant enhancement in the number of nodes per string compared to CC.

After two months from the inoculation, the CC plants showed lower development compared to most *Trichoderma* treatments. *T. hamatum* (T311 and T324), *T. virens* T312, *T. gamsii* T327, *T. rossicum* T328, and *T. harzianum* T329—treated plantlets presented a significant developmental enhancement, as shown by higher numbers of strings (over three strings per plant) and the number of nodes (more than ten nodes per plant) compared to CC (1.2 strings and 3.8 nodes). Treatments with *T. hamatum* T324, *T. gamsii* T327, and

*T. rossicum* T328 showed more than four nodes per string, significantly higher than CC (2.3 nodes per string).

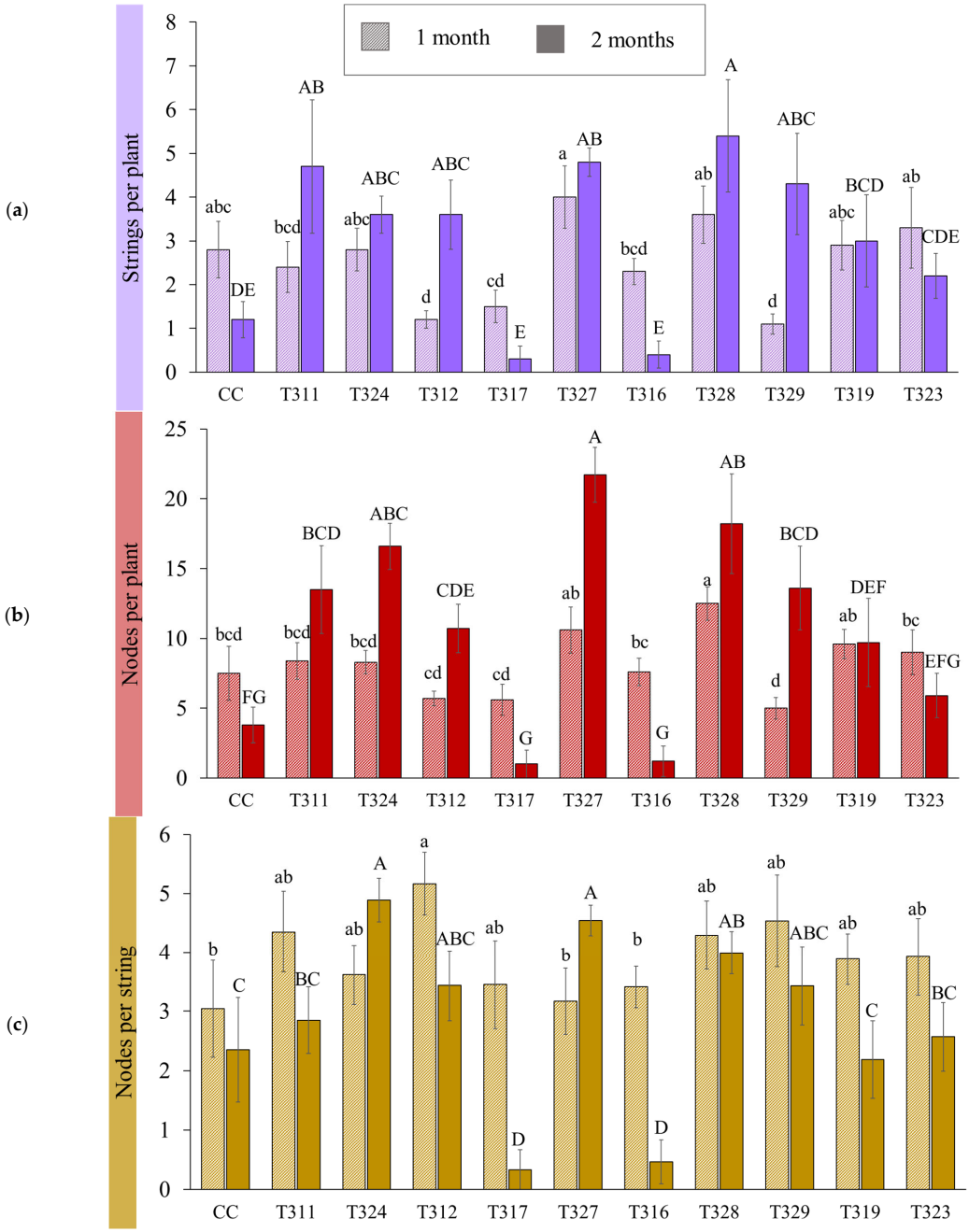



**Figure 7.** Growth development measures: (**a**) strings per plant, (**b**) nodes per plant, and (**c**) nodes per string (down). Mean values ($n$ = 10) ± SE. The mean values after one month are represented in stripes, and after two months in solid colors. Columns with different lower-case letters indicate significant differences after one month (Fisher's LSD $p < 0.05$). Columns with different capital letters indicate significant differences after two months (Fisher's LSD, $p < 0.05$).

*T. brevicompactum* T323 and *T. spirale* T319 showed low growth promotion abilities in hop plantlets without significant differences from CC plants. The isolates of *T. rossicum* T316 and *T. virens* T317 presented the lowest values of plant growth development, with no significant differences to CC plants regarding strings and nodes per plant.

## 4. Discussion

*Trichoderma* is widely used as a BCA and is reported to show antagonistic effects against phytopathogenic microorganisms but also beneficial effects on the host plants, such as the production of plant growth metabolites, nutrient uptake, or induction of defense mechanisms on the host plant [30,37]. The selection of a *Trichoderma* isolate has been commonly based on their in vitro antagonistic activity as a purpose for its application as a BCA, but it is interesting to select them also by their ability to interact and promote plant growth. In the current study, different autochthonous *Trichoderma* isolates collected from soil samples of sustainable hop fields interacted in vitro with the phytopathogens *F. sambucinum*, *F. culmorum*, and *F. oxysporum* and in vivo with 'Columbus' cultivar plants.

Two methods were explored to evaluate the antagonistic potential of the *Trichoderma* isolates. Firstly, through direct confrontation, *Trichoderma* spp. competed for space and nutrients, inhibiting the development of the pathogen [25,32,46]. Secondly, membrane assays, metabolites, and enzymes produced by the *Trichoderma* isolates are released through a cellophane membrane into the growing medium with no physical contact with the fungi, and their activity against the pathogens can be evaluated [32,47,48].

Growth of all *Fusarium* spp. analyzed was inhibited in direct confrontation assays with *Trichoderma* spp. The isolates of *T. hamatum* (T311 and T324) showed the best response against all *Fusarium* isolates. *T. virens* (T312 and T317) and *T. gamsii* (T327) presented high inhibition values against all *Fusarium* isolates but significantly lower than the *T. hamatum* isolates. *T. harzianum* T329 showed good direct confrontation skills against *F. sambucinum*. These data agree with those of other authors showing the ability of *T. harzianum* and *T. hamatum* to inhibit *F. oxysporum* growth in vitro [24,25]. *T. rossicum* T328 showed the lowest values, however, and caused nearly 50% growth inhibition in *F. culmorum*. These results agreed with those of Ji et al. [27], where *T. rossicum* inhibited *Fusarium* sp. growth in vitro by 41% in wild apples.

In the membrane assays, *T. hamatum* T311 and *T. gamsii* T327 were the isolates with higher antagonism by metabolite production against *F. culmorum* and *F. sambucinum*. These results agreed with previous findings describing the in vitro inhibition of *R. solani* growth by *T. gamsii* metabolites [48,49] and *F. oxysporum* [26]. *T. virens* (T312 and T317) showed antifungal activity against *F. sambucinum* and *F. oxysporum*. This species has proven its inhibitory capacity of *Fusarium* spp. and other pathogens such as *R. solani*, *Botrytis cinerea*, *S. sclerotiorum*, etc. [50]. *T. harzianum* T329 showed low inhibition against the isolates of *Fusarium* assayed, which contrasts with previous works with other isolates of the same species [26,51–53]. Vinale et al. [54] observed that the production of secondary metabolites by *T. harzianum* could be affected by the interaction with the phytopathogen. The low interaction of this isolate of *T. harzianum* against *Fusarium* spp. could be related to the fact that the metabolites produced in interaction with these pathogens have no antibiosis effect against them, as the antagonism is not species-dependent [49], or to the inability of producing secondary metabolites under laboratory conditions [55,56]. *T. rossicum* T316 and T328 showed very low inhibition percentages against all *Fusarium* spp. analyzed, indicating that they may not produce antibiotic compounds under laboratory conditions.

Regarding plant-growth promotion activity, the isolates *T. hamatum* (T311 and T324), *T. virens* T312, *T. gamsii* T327, *T. rossicum* T328, and *T. harzianum* T329 had a good overall performance inducing hop growth in the early stages of development. In agreement with our findings, *T. hamatum*, *T. harzianum*, *T. virens*, and *T. gamsii* showed good plant promotion performances reported by different authors in muskmelon, tomato, and *Arabidopsis*, among others [23,24,27,34,35,57]. *T. harzianum* induced a significant increase in root density, plant height, and number of buds when applied to hemp plants [36]. *T. rossicum* was used together with *T. harzianum* as a biofertilizer to promote plant growth in wild apple plants [27]. *T. hamatum* isolates showed favorable results in growth promotion and antagonism assays, in accordance with Martínez-Medina et al. [24] in melons.

The isolates *T. rossicum* (T316 and T328) presented similar performances in the in vitro assays but had very different outcomes in plant growth, similarly to the isolates *T. virens*

(T312 and T317). This could be explained by the origin of the isolates. *T. virens* T312 and *T. rossicum* T328 were collected from soil samples, whereas *T. virens* T317 and *T. rossicum* T316 were collected from rootstock samples. Mayo-Prieto et al. [48] claimed that *Trichoderma* isolates from soil samples may have better biocontrol abilities than isolates from other sources. Calvet et al. [58] observed that the inoculation of *T. aureoviride* alone had no effect on plant growth, contrary to the inoculation of *Trichoderma* with the arbuscular mycorrhizal fungus *Glomus mosseae*. Similar observations were made by Siddiqui and Mahmood [59] with *T. harzianum*. This symbiotic effect on *Trichoderma*-plant interaction may be due to the alteration of the rhizosphere by the arbuscular mycorrhizal fungi, making their presence essential to obtain agronomic benefits from the plant growth promoter fungi [60]. Regarding *T. rossicum* T328 and its results in the antagonism assays and plant promotion, we may consider its lack of abilities to produce antibiosis under laboratory conditions [55], and the Trichoderma-plant-pathogen interaction may be considered for further assays to determine its biocontrol capacities.

The results obtained in this work identify the ability of *Trichoderma* spp. to control *Fusarium* spp. and promote plant growth. Hoyos-Carbajal et al. [39] claimed that *Trichoderma* spp. was able to produce auxin-type PGRs like IAA. Contreras-Cornejo et al. [34] observed that plant biomass enhancement was due to the auxin-dependent mechanisms triggered by *T. viride* in *Arabidopsis*. Nieto-Jacobo et al. [43] observed similar results in *Arabidopsis* with different *Trichoderma* species. Bader et al. [42] observed that the *Trichoderma* isolates that presented a higher production of IAA also showed high antagonism against *F. oxysporum* in tomatoes. The present work evaluated the ability of the BCA to control and promote plant growth. There are good perspectives on some of the isolates assayed, although we consider that further studies must be conducted to characterize the mechanisms of the selected isolates and to understand the effects of the BCA not just in plantlets but also in adult plants and cone yield.

The present work evaluates the interaction between a single pathogen and a single BCA; however, every isolate exhibited different modes of action that may have complementary effects if used in a mixture or combination. The work of Duffy et al. [61] demonstrated that the combination of *T. koningii* and *P. fluorescens* showed enhanced biocontrol in wheat than *T. koningii* alone. Ji et al. [33] observed that the combination of *T. rossicum* and *T. harzianum* promoted wild apple growth. However, before any further consideration on *Trichoderma* combinations, it is important to know how they interact among themselves and with the existing non-pathogenic microbiome, due to the complexity of rhizosphere interactions [62]. Further research should be conducted to better understand the implications of *Trichoderma* against hop pathogens.

Thus, the results of these experiments showed the possible use of *Trichoderma* spp. as a BCA in hop plants. It opens a new line of research to evaluate different species of *Trichoderma* in hops, identify volatile compounds and PGRs produced that benefit the hop plant cultivation in plantlets and adult plants, and observe the impact this BCA may have on the hop cone yield.

## 5. Conclusions

To summarize, *T. hamatum* (T311 and T324) and *T. gamsii* T327 showed control responses against *Fusarium* spp. and growth promotion of hop plantlets. *T. hamatum* T311 exhibited the highest control of *F. culmorum* through metabolite secretion, *T. gamsii* T327 against *F. sambucinum*, and *T. virens* T312 against *F. oxysporum*. *T. rossicum* T328 and *T. harzianum* T329 showed little antagonism in vitro but exhibited plant growth promotion features. Some *Trichoderma* isolates collected from sustainable hop-producing soils can act as sustainable solutions to evaluate new disease control strategies and induce hop promotion.

*Trichoderma* native isolates collected from sustainable hop-producing soils compete for space and nutrients and mycoparasitize *Fusarium* isolates, showing an inhibited development in the pathogen. *Trichoderma* native isolates from sustainable hop-producing soils have great potential as BCAs and hop growth promoters. The native isolates of *T. hamatum* (T311

and T324), *T. virens* T312, and *T. gamsii* T327, all from sustainable hop-producing soils, can serve to develop a sustainable solution to control diseases and enhance hop development.

**Author Contributions:** Conceptualization, P.A.C. and S.M.-P.; methodology, A.F.-M. and A.J.P.-Á.; software, A.J.P.-Á.; validation, A.F.-M. and S.M.-P.; formal analysis, A.J.P.-Á. and A.F.-M.; investigation, A.F.-M., D.R.-L. and A.J.P.-Á.; resources, S.G. and P.A.C.; data curation, A.J.P.-Á.; writing—original draft preparation, A.J.P.-Á.; writing—review and editing, A.J.P.-Á., S.M.-P., R.E.C. and P.A.C.; visualization, A.J.P.-Á., D.R.-L. and P.A.C.; supervision, S.M.-P., S.G. and P.A.C.; project administration, S.G. and P.A.C.; funding acquisition, P.A.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the Ministerio de Universidades (Spain), grant number FPU19/03650 to A.J.P.-Á., and the Ministerio de Agricultura, Pesca y Alimentación (Spain), Quality Hops Operational Group, Innovations in the cultivation of hops in Spain to improve the sustainability of farms (2019/00179/001).

**Institutional Review Board Statement:** Not applicable.

**Acknowledgments:** The authors thank the field workers in charge of the hop farms and laboratory technicians for their help and collaboration in the necessary tasks.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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