Trichoderma asperellum as a preventive and curative agent to control Fusarium wilt in *Stevia rebaudiana*

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Keywords

Trichoderma asperellum, biological control, antagonism, mycoparasitism, *Fusarium oxysporum*

Abstract

Stevia has been introduced in many countries for the production of sugar-free sweeteners. Concurrently several emerging pathogens are being described on this plant host. One of the latest has been *Fusarium oxysporum*, a well-known soil-borne pathogen causing vascular wilt in many plants. Classical methods to control Fusarium wilt are being questioned, and biocontrol agents gain importance as part of integrated approaches to manage the disease. Different species of *Trichoderma* have been described as optimal candidates to control *F. oxysporum*. However, their effectiveness is generally reported in short-term plants and depend on the time of application. In this sense, we conducted an experiment to assess the preventive or curative potential of the rhizospheric *T. asperellum* UDEAGIEM-H01 strain against *F. oxysporum* on the perennial plant *S. rebaudiana*. After 33 days, *F. oxysporum*-infected stevia seedlings showed 90% of disease incidence whilst only 10% of the *T. asperellum* pre-treated plants and 70% of the post-treated were affected. Dual confrontation assays proved the potential antagonistic effect of *T. asperellum* against *F. oxysporum* and five additional soil-borne pathogens affecting *S. rebaudiana*. Further *in vitro* tests revealed that this new strain of *T. asperellum* produces phytohormones (salicylic and jasmonic acid), and the secretion of cell-wall degrading enzymes (chitinases and cellulases); this ability could be related to its antagonistic and mycoparasitic activity. The present work concluded that *T. asperellum* UDEAGIEM-H01 has a high ability, mainly as a preventive agent, to control *F. oxysporum* in stevia plants showing further antagonistic effects and mycoparasitism on other fungal pathogens.

1. Introduction

Fusarium oxysporum is a soil-borne fungal pathogen that has been detected in most agricultural fields around the world (Dean et al., 2012). This fungus is the causal agent of Fusarium wilt, one of the main vascular plant diseases, that affects more than 120 crops of agricultural and horticultural importance (Akila et al., 2011; Kang et al., 2014; Zhang et al., 2014a; McGovern, 2015; Zhu et al., 2019). Recently, there was a first report on *F. oxysporum* producing vascular wilt in commercial plantations of *Stevia rebaudiana* in Colombia (Díaz-Gutiérrez et al., 2019).

Fusarium wilt management is difficult due to its severity, the long-term survival of the fungus in soils, the wide distribution, and the extensive genetic variability (Dean et al., 2012). The use of disease-resistant material, crop rotation, and chemical pesticides are the most common methods for controlling soil-borne pathogens (Abawi and Widmer, 2000). However, there are limitations related to multiple factors. Most relevant are the decrease in fruit quality and productivity of the Fusarium resistant plants (Molina et al., 2011), the high ability of the fungus to survive for a long time even in the absence of the main host (López-Zapata and Castaño-Zapata, 2019), and pollution issues and degradation of ecosystems, with adverse effects on human health, due to the use of agrochemicals. An alternative to replace these conventional methods is the use of biological control agents (BCAs), which are described as effective and eco-friendly biopesticides and biofertilizers (Fravel, 2005). Although the positive effects of BCAs are mainly reported in annual or short-term plants, this biological disease control strategy is receiving higher attention in perennial crops during the last decades (Ploetz, 2007; Bubici et al., 2019).

Trichoderma species have been used as BCAs to manage plant diseases and control plant pathogenic fungi, including *Fusarium* species (Vos et al., 2015; Saravanakumar et al., 2016; Abbas et al., 2017; Al-Ani, 2018). However, their effects against Fusarium wilt have not been profusely investigated on perennial crops from the tropics and benefits of *Trichoderma* spp. have been only reported on integrated approaches to manage the banana Fusarium wilt disease (Akila et al., 2011; Raza et al., 2017; Bubici et al., 2019).

Several studies have indicated the advantages of the use of *Trichoderma* spp. in biocontrol, such as ubiquitous distribution; rapid colonization of soil, and rapid growth on different substrates (Al-Ani, 2018). The biocontrol effect is related to antagonistic properties towards pathogens by activating one or more mechanisms such as the competition for space, and nutrients, mycoparasitism (physical contact and synthesis of hydrolytic enzymes), antibiosis (production of toxic metabolites), growth promotion, and stimulation of plant defense system (Harman et al., 2004; Brotman et al., 2010; Mohiddin et al., 2010; Zeilinger et al., 2016). Ultimately, higher productions are associated with *Trichoderma* amendments in different crops, which represents a tremendous benefit for sustainable agriculture (Hermosa et al., 2012; Al-Ani, 2018). Related to the Fusarium wilt control in perennial *Musa* plants, contrasting results are reported with *Trichoderma* inducing disease protection and yield increase (Raguchander et al., 1997; Thangavelu and Gopi, 2015), but also failure examples (Wibowo et al., 2013). Therefore, the beneficial effect of *Trichoderma* seems to be context-dependent with results that, for example, can be conditioned by the time of the BCA application (before or after the pathogenic agent) (Ben-Amira et al., 2017). In this sense, the present study was conducted to assess the *in vivo* biocontrol ability of a

rhizospheric *Trichoderma asperellum* strain when it is inoculated before or after the phytopathogen *F. oxysporum* strain in the perennial host *S. rebaudiana.* The *in vitro* antagonistic effect of *T. asperellum* has been also tested against *F. oxysporum* and five other phytopathogenic fungi, all of them isolated from field-decayed *S. rebaudiana* plants. Additional *in vitro* assays have been conducted to study the ability of this *T. asperellum* strain to produce hormones and cell-wall degrading enzymes that would serve to the antagonistic and mycoparasitic activity. As far as we know, this is the first study analyzing the effect of *Trichoderma* against Fusarium wilt on stevia plants.

2. Materials and Methods

2.1. Fungal strains

Trichoderma asperellum strain UDEAGIEM-H01 was used as antagonist against six different phytopathogenic fungi: *Curvularia geniculata* UDEAGIEM-H06, *Stagonosporopsis caricae* UDEAGIEM-H07, *Fusarium equiseti* UDEAGIEM-H08, *Fusarium oxysporum* UDEAGIEM-H11, *Diaporthe phaseolorum* UDEAGIEM-H13, and *Macrophomina phaseolina* UDEAGIEM-H14. The fungal strains were previously isolated from commercial plantations of *Stevia rebaudiana* located in Antioquia, Colombia. The fungal strains UDEAGIEM-H06, UDEAGIEM-H07, UDEAGIEM-H08 and UDEAGIEM-H13 were isolated from leaves, UDEAGIEM-H01 and UDEAGIEM-H11 were isolated from soil and UDEAGIEM-H14 was isolated from root. Fungi were identified by using molecular methods based on the sequencing amplification products of the ITS, EF, calmodulin and β-tubulin regions by PCR. All fungal strains were deposited at the fungal

117 collection of the Interdisciplinary Group of Molecular Studies, located in Medellin
118 (Colombia). Fungi were cultivated on potato dextrose agar (PDA) plates, and incubated at
119 28ºC in darkness for 7 days, to allow abundant conidia production.
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121 *2.2. In vivo antagonistic between T. asperellum and F. oxysporum*
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123 *2.2.1. Plant material*
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125 Two-month-old plants of *Stevia rebaudiana* cv. Morita II were obtained by asexual
126 propagation. Stem cuttings were soaked in a solution with 0.2% indole-3-butyric acid
127 (Mycsa Ag, Inc., Texas, USA) to stimulate rooting and then planted in pots autoclave-
128 sterilized containing coconut fiber substrate (C.I. Sustratos S.A.S, Inc, Antioquia,
129 Colombia). Cuttings were grown under greenhouse conditions at 28 ± 2°C until optimal
130 root development (4 weeks). Plants were fertilized once (day 16) with 200 mg NPK and
131 minor elements (Fercon, Inc, Cauca Valley, Colombia).
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133 *2.2.2. Plant inoculation*
134
135 Conidial suspensions (1 x 10^6 conidia ml^{-1}) of *T. asperellum* and *F. oxysporum* were
136 prepared for soil and stems inoculation of stevia plants, respectively. Roots were carefully
137 dug out, and *T. asperellum* was directly applied to roots by pouring 300 ml of conidial
138 suspension. Stems were wounded, and a 25 µl-drop of the conidial suspension of *F.*
139 *oxysporum* was applied using a sterile inoculating needle. To test the effect of
140 *Trichoderma*, 4 different treatments were carried out: 1) soil inoculated with *T. asperellum*

(control for the biocontrol agent), 2) stem inoculated with *F. oxysporum* (control for the pathogen), 3) soil inoculated with *T. asperellum* and, 6 days later, stem inoculated with *F. oxysporum* (preventive effect), and 4) stem inoculated with *F. oxysporum* and, 6 days later, soil inoculated with *T. asperellum* (curative effect). Preventive and curative effects were defined based on the protocol of Ben-Amira et al. (2017). Control treatment was carried out by inoculating plants with sterile distilled water in soil and wounded stems. Five plants were inoculated per treatment, and the experiment was repeated twice.

The disease incidence (DI) and disease severity (DS) were assessed 33 days after the inoculation. The DI was calculated for each treatment using the formula of Toghueo et al. (2016).

$$DI(\%) = \left(\frac{Number\ of\ infected\ plants}{Total\ number\ of\ plants\ in\ the\ treatment}\right) \times 100$$

The DS was scored for each plant based on the severity of aerial symptoms according to the percentage of foliage with yellowing or necrosis where 0 = No evidence of lesions, 1 =33% of leaf area infected, 2 =34–66% of leaf area infected, 3 = 67–100% of leaf area infected and 4 = dead plant (Sanchez-Hernandez et al., 1998; Ben-Amira et al., 2017). The disease severity index (DSI) was calculated for each treatment using the formula of Promwee et al. (2017).

$$DSI(\%) = \left(\frac{\sum(Scale\ \times Amount\ of\ plants)}{Maximum\ level\ \times Total\ plants}\right) \times 100$$

After the DI and DS assessment, pieces of stems and roots with wilt symptoms compatible with F. *oxysporum* infection were collected specifically at the edge of the affected tissues.

Collected tissues were surface sterilized (70% ethanol, 15s), placed on PDA plates with oxytetracycline (0.9 ml/l), and incubated at 28ºC in darkness for 7 days. Colonies of *F. oxysporum* were identified by morphological characteristics and microscopic examination.

2.3. Hormones quantification on stevia and T. asperellum

2.3.1. Plant material

S. rebaudiana cv. Morita shoots were collected from six months old plants grown in the Plant Physiology Laboratory, at the Autonomous University of Barcelona, (Barcelona, Spain). Explants, consisted on apical buds between 3 – 5 mm, were surface sterilized by dipping in 1.2% (v/v) sodium hypochlorite solution and Tween-20 (3 drops/100 ml solution) for 15 min with constant shaking. Under laminar flow cabinet, the explants were rinsed with sterile distilled water four times and placed on 15 x 10 x 10 cm sterile plastic boxes previously filled with 150 ml of MS supplemented medium. One explant was deposited per box.

2.3.2. Media and plant growth conditions

MS (Murashige and Skoog, 1962) basal medium supplemented with 30 g l^{-1} sucrose, 10 g l^{-1} myoinositol, 4 g l^{-1} thiamine, 0.05 g l^{-1} pyridoxine, 0.05 g l^{-1} nicotinic acid, 0.2 g l^{-1} glycine, and 15 g l^{-1} agar was used for *in vitro* plant growth. The pH of the media was adjusted to 5.7 – 5.8. Plants were placed in a plant growth chamber with a photoperiod of 16 h of light/8 h darkness, light intensity of 153 μmol m^{-2} s^{-1}, and temperature of 25ºC.

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188 *2.3.3. Plant inoculation, and treatments*

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190 An inoculum of *T. asperellum* strain UDEAGIEM-H01 was prepared from a pure culture
191 maintained on PDA plates for 7 days. Ten µl of the conidial suspension (1 x 10^3 conidia ml^{-1}
192) of *T. asperellum* were deposited at the opposite ends of MS boxes containing the plants.
193 Hormone production was analyzed on: 1) *T. asperellum* mycelium after plant contact and,
194 2) Shoots after contact with *T. asperellum.* The experiments were performed for 21 days.
195 Eight stevia plants were inoculated per treatment, and the experiment was repeated twice.

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197 *2.3.4. Determination of SA and JA concentrations*

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199 Analyses of SA and JA were performed following the method developed by Segarra et al.
200 (2006) with some modifications (Llugany et al., 2013). To summarize, around 300 mg of
201 fresh material was ground under liquid N_2 with mortar and pestle and were three times
202 extracted with 750 µl, 625 µl, and 750 µl of methanol–isopropanol–acetic acid (20:79:1,
203 v/v/v), respectively, and centrifuged for 10 min at 10.000 rpm. On the second extraction,
204 125 µl of SA and JA deuterated standards were added (500 and 50 ng ml^{-1}, respectively).
205 The collected supernatants were dried under N_2, resuspended on 250 µl of methanol
206 (100%), and finally filtered through a Spin-X 0.22 µm cellulose acetate filter. SA and JA
207 quantification was done using a standard addition calibration curve with SA and JA
208 solutions (ranging from 50 to 800 and from 5 to 80 ng ml^{-1}, respectively), and extracted as
209 described above. The samples were analyzed using liquid chromatography-mass
210 spectrometry (LC-ESI-MS/MS) QTrap 6500 on a Kinetex C18 column (50 mm length x 2

211 mm internal diameter, 2.6 μm particle size) at a constant flow rate of 0.6 ml min^{-1} using the
212 mobile phase consisting of 0.05% acetic acid in water (Solvent A) and acetonitrile (Solvent
213 B). All the analyses were performed using the Turbo Ionspray source in negative ion mode.
214
215 *2.4. In vitro antagonism of T. asperellum against six phytopathogenic fungi of S.*
216 *rebaudiana*
217
218 *In vitro* antagonism tests were carried out by the dual culture technique described by Bell et
219 al. (1982). Five-millimeter diameter discs of *T. asperellum* and each phytopathogenic
220 fungus (*C. geniculata, D. phaseolorum, F. equiseti, F. oxysporum, M. phaseolina and S.*
221 *caricae*) were obtained from the edge of actively growing 7 days-old cultures in PDA. Each
222 disc was placed at distances opposite to each other (about 60 mm apart) in 90 mm diameter
223 Petri plates containing PDA. Plates inoculated only with *T. asperellum* or each
224 phytopathogenic fungus served as control. The plates were incubated at 28ºC in darkness
225 for 12 days. All the experiments were conducted in quadruplicate. Plates of dual cultures
226 and controls were photographed for 12 days and the images were processed using the
227 Image-Pro Plus 6.0 software (Media Cybernetics Inc., USA) to determine the mycelial
228 growth area (in cm^2) for each isolate. The percentage of inhibition of radial growth (PIRG)
229 was calculated according to the method of Royse and Ries (1978) at the end of the
230 experiment by applying the following formula:
231
232 $$PIRG = \frac{(Rc - Rt)}{Rc} * 100$$

Where, Rc is the radial growth of the pathogen in the control (mm) and Rt is radial growth of the pathogen in the presence of *T. asperellum* UDEAGIEM-H01 (mm).

At the end of the experiment, five-millimeter diameter discs of the pathogen from dual cultures and specifically from the hyphal interaction zone were subcultured in PDA plates to determine the kind of mycoparasitism (fungistatic or fungicidal) induced by *Trichoderma*. Plates were incubated at 28ºC in darkness for 8 days. The fungistatic activity was described when the pathogen was able to grow on the subcultured plate while fungicidal activity was defined when a 100% of growth inhibition was observed.

2.5. Capacity of T. asperellum and F. oxysporum of producing cell wall degrading enzymes

2.5.1. Chitinase assay

The chitinase activity of *T. asperellum* UDEAGIEM-H01 and *F. oxysporum* UDEAGIEM-H11 were performed following the protocol of Agrawal and Kotasthane (2012) with some modifications. The fungi were cultured in a basal medium with colloidal chitin as sole carbon source and supplemented with Bromocresol purple. Colloidal chitin was prepared from commercial chitin (MP Biomedicals) by the method of Rojas-Avelizapa (1999). Chitin degradation was observed by a purple halo contrasting with the yellowish color of the preserved chitin in the medium. The chitinase detection medium was prepared comprising (per liter) 4.5 g of colloidal chitin, 0.3 g of $MgSO_4 \cdot 7H_2O$, 3.0 g of $(NH_4)2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 µl of Tween-80 and 0.15 g of bromocresol purple. Medium pH was adjusted to 4.7 and then autoclaved at

121ºC for 15 min. Five-millimeter diameter mycelial discs of *T. asperellum* and *F. oxysporum,* from actively growing 7 days-old cultures, were placed on plates containing chitinase detection medium. As control was used plates with PDA. Plates were incubated at 28ºC in darkness for 4 days and then photographed. Images were processed using the Image-Pro Plus 6.0 software (Media Cybernetics Inc., USA) to determine the area (in cm^2) of the purple halos around the mycelium. The experiments were conducted in triplicate.

2.5.2. Cellulase assay

The cellulase activity was performed following the method developed by Sharma et al. (2017) with some modifications. Cellulase activity of *T. asperellum* UDEAGIEM-H01 and *F. oxysporum* UDEAGIEM-H11 were screened in a medium with carboxymethyl cellulose (CMC) as a sole carbon source in Congo red-stained agar medium. The cellulose degradation was observed by a yellow halo contrasting with the reddish color of the preserved medium. The cellulase detection medium comprising (per liter) 0.3 g of $MgSO_4.7H_2O$, 3 g of $(NH_4)\ 2SO_4$, 2 g of KH_2PO_4, 15 g of agar and 1% (p/v) of CMC. Five-millimeter diameter mycelial discs of *T. asperellum* and *F. oxysporum,* from actively growing 7 days-old cultures, were placed on the cellulase detection medium plates. As control was used plates with PDA. Plates were incubated at 28ºC in darkness for 4 days and then photographed. Cellulolytic activity was observed by adding Congo red stain (0.1%) for 30 min and then detaining with 1M NaCl. The growth area was determined as described in section 2.3.1. The experiments were conducted in triplicate.

2.6. Statistical analyses

Data were analyzed by the software STATGRAPHICS CENTURION XV (Statpoint Technologies, Inc., Warrenton, VA, USA). Normal distribution, homoscedasticity and the independency of errors was checked prior to the analyses and data were log-transformed when required. All the tested parameters were submitted to one-way ANOVA followed by Fisher's test to check the statistic differences among treatments. Differences at $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Beneficial effect of T. asperellum on Stevia plants inoculated with F. oxysporum

Trichoderma asperellum UDEAGIEM-H01 efficiently reduced the disease incidence and disease severity caused by the artificial inoculation of *F. oxysporum* UDEAGIEM-H11 in stevia seedlings (Fig. 1 and 2). *Trichoderma* species have demonstrated efficacy to reduce the severity of plant diseases by inhibiting phytopathogens through their high antagonistic and mycoparasitic potential (Viterbo and Horwitz, 2010; Hermosa et al., 2012; Shang et al., 2020). In this work, the best antagonistic effect was observed in the preventive treatment when *T. asperellum* was inoculated 6 days before the pathogen with a percentage of disease incidence of 10% and disease severity of 37% (Fig. 1). Additionally, when *T. asperellum* was inoculated 6 days after *F. oxysporum* (curative treatment) the disease incidence and severity were 70% and 63%, respectively. Plants inoculated only with *F. oxysporum* presented 90% of disease incidence and 100% of disease severity, while control plants and

304 those inoculated only with *T. asperellum* showed 0% of disease incidence and disease
305 severity (Fig. 1).
306
307 In this work, we evidenced both a positive effect of *T. asperellum* application on the
308 development of the roots before pathogen attack and also plant recovery in the curative
309 treatment (Fig. 2). *F. oxysporum* inoculations induced reduced growth, leaf chlorosis and
310 necrosis and stem rot that ultimately caused plant wilt on the stevia seedlings (Fig. 2A2).
311 The root system was reduced and affected by rotting (Figs. 2B2 and 2C2). In the curative
312 treatment, leaf chlorosis and necrosis were decreased and the root system was less affected
313 (Figs. 2A5, 2B5 and 2C5), while in the preventive treatment, the general symptomatology
314 induced by the pathogen was lower than in the curative one (Figs. 2A4, 2B4, and 2C4).
315 Although literature describes plant growth promotion after *Trichoderma* application, we did
316 not observe a boost in plant shoots in BCA treated plants (data not shown). Control plants
317 and plants inoculated only with *T. asperellum* did not show aerial symptoms and presented
318 optimal root development (Figs. 2A1 and A3, 2B1 and B3 and 2C1 and C3). *F. oxysporum*
319 was effectively re-isolated from roots and stem of plants artificially inoculated with the
320 pathogen (Fig. 2D2) except the preventive and *T. asperellum* treatments where only
321 *Trichoderma* was recovered (Figs. 2D3 and 2D4). In the curative treatment, mycelium
322 growth of both pathogen and biocontroller were observed (Fig. 2D5). These results
323 demonstrate the efficiency of *T. asperellum* UDEAGIEM-H01 as a beneficial fungal
324 biocontrol agent against *F. oxysporum* in mesocosm conditions with mainly preventive, but
325 also partially curative activity.
326

Important suppression of soil-borne plant diseases has been associated with *Trichoderma* spp. The pathogen-protective effect of *Trichoderma* has been reported in mono- and dicotyledonous crops, but mainly annual species (Harman, 2006; Ploetz, 2007). In the last 10 years, more than 400 works have been published related to the use of *Trichoderma* and other biocontrol agents on the management of Fusarium wilt, but less than 30 focused on perennial species. Literature demonstrated the increased resistance to this pathogen attack due to *Trichoderma* applications in citrus (Camprubí et al., 1995), pinus (López-López et al., 2016) and olive trees (Ben-Amira et al., 2017), but the most cited ones focused on banana (Sharavanan et al., 2003; Thangavelu et al., 2004; Nel et al., 2006; Zhang et al., 2014b; Raza et al., 2017). As far as we know, only one publication described the deleterious effect of *F. oxysporum* on *S. rebaudiana* (Díaz-Gutiérrez et al., 2019) and the present work is the first biocontrol strategy to counteract the disease in this economically important crop. These good results will offer a sustainable way to combat Fusarium wilt on this perennial and tropical host eluding classical control methods based on agrochemicals.

Root colonization of *Trichoderma* can triggers local and systemic resistance against many plant pathogens (Contreras-Cornejo et al., 2014). *Trichoderma*-induced resistance may include both the SA (Harman et al., 2004) and the JA/ET signaling cascades (Druzhinina et al., 2011) in plants that can be activated separately or simultaneously (Salas-Marina et al., 2011). Therefore, reduction of disease incidence due to *Trichoderma* inoculation can be related to enhanced levels of salicylic acid (SA) and jasmonic acid (JA) (Shoresh et al., 2010). In this study, the production of SA and JA was analyzed on the mycelium of *T. asperellum* and shoots of stevia when they interacted on MS medium. According to our results, the levels of both hormones were higher in the mycelium than in the stevia shoots

(Fig. 3). Furthermore, *Trichoderma* produced higher amounts of SA, 8 times more than JA. Although in lower amounts, our *T. asperellum* strain was able to induce the synthesis of SA in the aerial parts of stevia after the BCA root colonization. Despite the reduced concentrations of SA in stevia shoots, the possible induction of systemic acquired resistance (SAR) through this hormone should not be dismissed. However, to confirm this assertion the levels of hormones on mature plant-*Trichoderma* interactions should be further studied.

3.2. *In vitro antagonism of T. asperellum* against stevia pathogens

The pathogens tested in this study were isolated from decayed stevia plants or soil around affected plants. *F. oxysporum* was firstly reported from Colombian stevia plantations affected by vascular wilt on a recent publication of our group (Díaz-Gutiérrez et al., 2019). The resting five pathogens have been described as causal agents of serious diseases in different crops (Estrada and Sandoval, 2004; Goswami and Punja, 2008; Dissanayake et al., 2015; Stewart et al., 2015; Al-Ahmadi et al., 2018; Herrera-Téllez et al., 2019) but, as far as we know, only *M. phaseolina* has been previously associated to disease symptoms in this host (Koehler and Shew, 2017). *S. rebaudiana* has become an emerging crop cultivated for the production of glycosides world-wide recognized by their extreme sweetener potential (Brandle et al., 1998). The expansion on its cultivated area will contribute to the emergence of new pathogens and, in the last decade, several soil-borne pathogens have been recently described such as, *Pythium* spp (Koehler et al., 2017), *Sclerotium rolfsii* (Koehler and Shew, 2014a), *Sclerotinia sclerotiorum* (Koehler and Shew, 2014b), and *Verticillium dahliae* (Farrar et al., 2000).

Dual confrontation assays demonstrated the antagonistic behavior of *T. asperellum* UDEAGIEM-H01 through *in vitro* growth inhibition on the six tested phytopathogenic fungi (*C. geniculata, D. phaseolorum, F. equiseti, F. oxysporum, M. phaseolina,* and *S. caricae*) (Fig. 4). The growth inhibition of phytopathogenic fungi by *T. asperellum* UDEAGIEM-H01 ranged from 24% to 96%. (Table 1). The less affected pathogen was *D. phaseolorum* with 24% of inhibition, while the growth for the other five species was reduced by more than 50% (Table 1 and Fig. 4). An extreme inhibition was observed for *F. equiseti* which growth was almost completely aborted. The dual culture kinetics indicated that the six pathogen species grew slower compared to cultures without confrontation but also that the growth was species-dependent (Fig. 4). *C. geniculata*, *F. equiseti* and *S. caricae* colonized exponentially the plate, while *D. phaseolorum* and *F. oxysporum* grew at very slow rates, and *M. phaseolina* showed an exponential growth along seven days. The rate of *T. asperellum* growth indicated exponential growth faster, regardless of the kind of culture (with or without pathogen) and regardless of the pathogen species (Fig. 4). However, after the first hyphal interaction between this BCA strain and the phytopathogenic fungi (Suppl. Mat. Fig. 1), *T. asperellum* growth was reduced and a stationary growth phase was observed (Fig. 4). After 12 days, *T. asperellum* grew and sporulated over the phytopathogenic fungi, except in the dual culture with *C. geniculata* where did not show sporulation (Fig. 4A). The growth inhibition of phytopathogenic fungi can be attributed to competition for space and nutrients of the culture medium but also to direct contact through mycoparasitism (Brotman et al., 2010; Vos et al., 2015; Ben-Amira et al., 2017; Juliatti et al. 2019). Inhibition of plant pathogen growth is commonly related to *Trichoderma* spp (Hermosa et al., 2012; Shang et al., 2020). On the same line, our results

399 indicate that *T. asperellum* UDEAGIEM-H01 strain is a promising BCA candidate against
400 *C. geniculata, D. phaseolorum, F. equiseti, M. phaseolina,* and *S. caricae.* However,
401 further *in vivo* experiments confronting *T. asperellum* UDEAGIEM-H01 and these five
402 phytopathogenic fungi have to be carried out to confirm its potential ability.
403
404 The fungicidal or fungistatic action of *T. asperellum* was verified by sub-culturing the
405 mycelia of the phytopathogenic fungi. The test exhibited only *T. asperellum* growth from
406 dual cultures with *D. phaseolorum, F. equiseti, F. oxysporum, M. phaseolina,* and *S.*
407 *caricae* indicating fungicidal activity of *T. asperellum* against these five pathogenic strains
408 as their growth were completely inhibited in the sub-cultured plates. However, fungistatic
409 activity was described in subculture of *C. geniculata* where growth of pathogen and
410 antagonist were observed (Suppl. Mat. Fig. 2).
411
412 A part from competition for space and nutrients, this *T. asperellum* strain exhibited
413 mycoparasitic activity given the evidenced inhibition zone in dual cultures of *T. asperellum*
414 against *F. oxysporum* and *M. phaseolina* (Figs. 5A and 5C). Microscopic examination (at
415 40X magnification) showed that the *T. asperellum* hyphae grew adhered and coiled around
416 the hyphae of the two phythopathogenic fungi and branching toward adjacent hyphae
417 causing morphological deformities as flattening and crumpling (Figs. 5B and 5D). Other
418 studies have shown hyphal coiling and morphological deformities in dual cultures of *T.*
419 *harzianum* with *F. solani, Alternaria solani,* and *Pythium ultimum* (Ben-amira et al., 2017;
420 Mazrou et al., 2020).
421

Trichoderma spp. can localize potential preys through the constitutive secretion of low levels of cell wall-degrading enzymes (CWDEs) (Benitez et al., 2004; Harman et al., 2004; Vos et al., 2015). *Trichoderma* mycoparasitism involves the secretion of cell wall-degrading enzymes (CWDEs) such as chitinases, proteases and glucanases (Harman, 2006; Sanchez et al., 2019). In this study, *T. asperellum* UDEAGIEM-H01 was able to grow and metabolize chitin as a sole source of carbon and energy showing a uniform growth with little sporulation in an area up to 42 cm^2 (Fig. 6A and B). *T. asperellum* transformed the yellow color of the medium to purple indicating chitinase activity (Fig. 6A). Similarly, *T. asperellum* UDEAGIEM-H01 was able to growth and metabolize cellulose as sole source of carbon and energy showing a uniform growth with little sporulation (Fig. 6A). A yellow discoloration in the Congo red stained agar medium was observed when *T. asperellum* grew in an area up to 34 cm^2, indicating cellulase activity (Fig. 6A and C). Color changes in both media were coincident with the growth lines suggesting that the enzymes are produced uniformly by actively-growing-hyphae but not spreading through the media. Cellulase and chitinase production in *T. asperellum* UDEAGIEM-H01 were higher compared to *F. oxysporum* secretion of CWDEs (Fig. 6). Chitinase and cellulose secretion of *T. asperellum* UDEAGIEM-H01 would degrade or hydrolyze the cell wall of host fungal and fungal-like pathogens, releasing oligomers that induce further *Trichoderma* CWDEs, and directional growth towards the prey, to enable physical attack (Benitez et al., 2004; Woo et al., 2006; Mukherjee et al., 2012; Monfil and Casas-Flores, 2014). Other authors have already highlighted the importance of CWDEs on the *Trichoderma* biocontrol ability against phytopathogenic fungi (Harman et al., 1993; Viterbo et al., 2001; Bambharolia et al., 2012).

445 The beneficial effect of *Trichoderma* spp. varies according to the strain, the type of
446 pathogen, the state of the plant and the environmental conditions (Brimner and Boland,
447 2003; Cordier and Alabouvette, 2009; Mohiddin et al., 2010). Therefore, isolating and
448 selecting native *Trichoderma* strains would favor the optimal performance of the
449 biocontroller expecting a better disease control (Howell, 2003). Thus, *T. asperellum*
450 UDEAGIEM-H01 strain isolated from soil of *S. rebaudiana* plants may enhance its
451 biocontroller effect on the incidence of disease and severity disease caused by *F.*
452 *oxysporum*, mainly when it is applied of preventive form.
453
454 **Conclusion**
455
456 The present work demonstrated the disease resistance of stevia plants infected by *Fusarium*
457 *oxysporum* after amendment of *T. asperellum* isolate UDEAGIEM-H01. Interestingly, this
458 strain of *T. asperellum* had been isolated from the rhizospheric soil of a commercial
459 plantation of stevia. The previous colonization of plant root by this *Trichoderma* isolate
460 was the best method to control the fungal disease although certain curative abilities were
461 also observed. In dual culture experiments, *T. asperellum* UDEAGIEM-H01 was able to
462 inhibit the growth of *F. oxysporum* and five other pathogenic organisms (*C. geniculata, D.*
463 *phaseolorum, F. equiseti, M. phaseolina,* and *S. caricae*). All the phytopathogenic fungi
464 had been isolated from decayed stevia plants or rhizospheric soil around them. This *T.*
465 *asperellum* strain exhibited additional abilities such as mycoparasitic activity as well as
466 salicylic acid and jasmonic acid production and strong hydrolytic capacity of chitin and
467 cellulose. Taken together our findings suggest that this *T. asperellum* strain is a powerful
468 biocontrol agent against soil-borne pathogens challenging commercial stevia crops.

Acknowledgment

The authors thank the COLCIENCIAS Administrative Department of Science, Technology and Innovation, for its financial support in calls 727/2015 national doctoral (FP44842-124-2017) and calls 656/2014 “Es Tiempo de Volver” (FP44842-505-2014). FUNDAGIEM Foundation for the support of research in the Interdisciplinary Group of Molecular Studies, for its technical support, and MAEC-AECID scholarships for University and Scientific Cooperation for Development, academic year 2015-2016. Work performed at UAB benefited from project BFU2016-75176-R.

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Figure captions

Fig 1. Disease incidence (DI) and disease severity (DS) caused by *F. oxysporum* on *S. rebaudiana*. Control: Control plants. *T. asperellum*: *T. asperellum* inoculated plants. *F. oxysporum*: *F. oxysporum* inoculated plants. Preventive treatment: *T. asperellum*-treated plants followed by *F. oxysporum* inoculation. Curative treatment: *F. oxysporum* infected plants followed amended with *T. asperellum*. Different letters indicate significant differences between treatments for each variable ($P < 0.05$).

Fig 2. Symptoms of vascular wilt disease caused by *F. oxysporum* on *S. rebaudiana* 33 days after inoculation. A. Full plant. B. Stem and roots. C. Crown detail using stereoscopy (6.4X magnification) and D. Conidia of fungal isolates using optical microscopy (40X magnification). 1. Control plant. 2. *F. oxysporum*-inoculated plant. 3. *T. asperellum*-inoculated plant. 4. Preventive treatment (*T. asperellum*-treated plants followed by *F. oxysporum* inoculation). 5. Curative treatment (*F. oxysporum* infected plants followed amended with *T. asperellum)*. A representative image of each treatment is illustrated from a set of ten plants per treatment (divided in two sequential experiments with 5 plants/assay).

Fig 3. Levels of salicylic acid (SA) and jasmonic acid (JA) on *T. asperellum* mycelium and stevia shoot after interaction. Different letters indicate significant differences between plant and BCA ($P < 0.05$).

Fig 4. Dual culture growth kinetics of *T. asperellum* against each phytopathogenic fungi. A. *C. geniculata*. B. *D. phaseolorum*. C. *F. equiseti.* D. *F. oxysporum.* E. *M. phaseolina.* F. *S. caricae.* Each graphic contains an image of the *in vitro* antagonism between *T. asperellum* (left part of the plate) and phytopathogenic fungi (right part of the plate) after 12 days of growth.

Fig 5. Dual cultures of *T. asperellum* (left part of the plates) and phytopathogenic fungi (right part of the plates) exhibiting inhibition growth zone and microscopic examination of mycoparasitim (40X magnification). A and B. *F. oxysporum.* C and D. *M. phaseolina.*

Fig 6. Enzymatic production of *T. asperellum* and *F. oxysporum.* A. Morphological view on PDA, chitinase medium (chitin-bromocresol purple) and cellulase medium (carboxymethyl cellulose-congo red), respectively. Area of mycelium growth (in cm^2) of *T. asperellum* and *F. oxysporum* on the chitinase medium (B) and the cellulase medium (C) compared to growth on the PDA medium (control). Different letters indicate significant differences between treatments 4 days after plate inoculation ($P < 0.05$).

Supplementary Fig 1. First hyphal interaction between *T. asperellum* (left) and phytopathogenic fungi (right) 5 days after plate inoculation. A. *C. geniculata*. B. *D. phaseolorum.* C. *F. equiseti*. D. *F. oxysporum*. E. *M. phaseolina*. F. *S. caricae.*

Supplementary Fig 2. Antagonistic activity of *T. asperellum* on phytopathogenic fungi. A. *C. geniculata*. B. *D. phaseolorum.* C. *F. equiseti*. D. *F. oxysporum*. E. *M. phaseolina*. F. *S. caricae.*

844 **FIGURE 1**

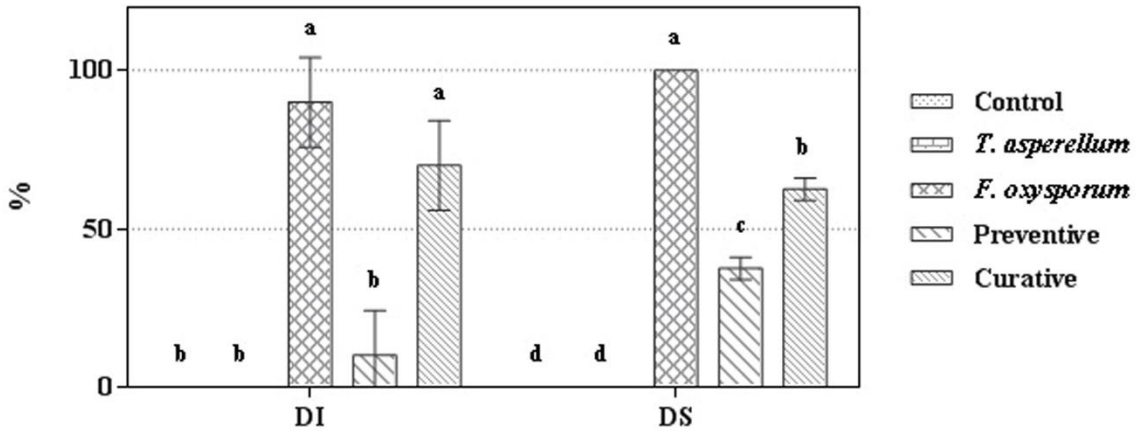

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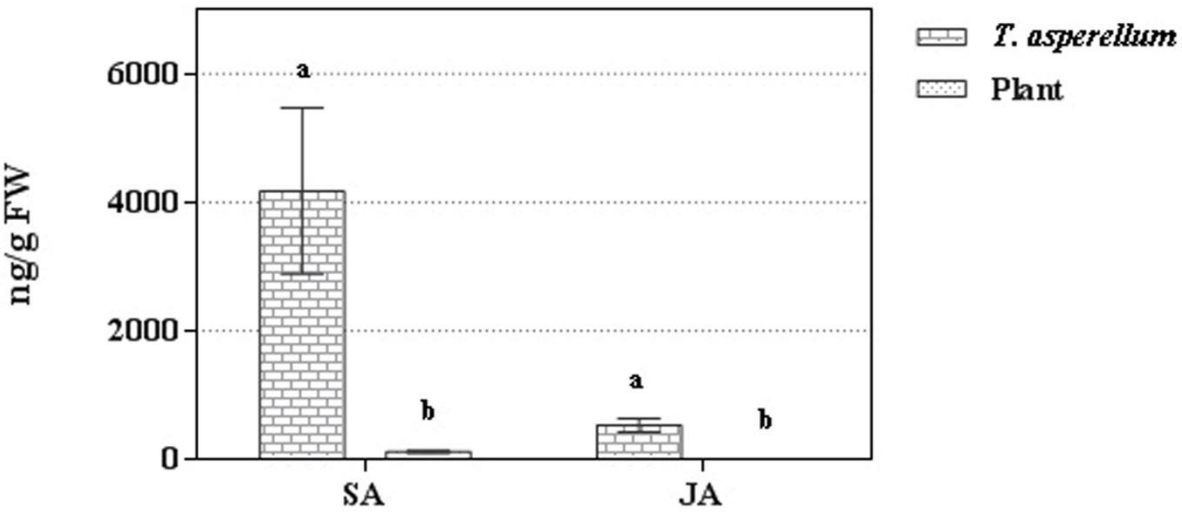
FIGURE 2


Control
F. oxysporum
T. asperellum
Preventive
Curative
A1.
A2.
A3.
A4.
A5.
B1.
B2.
B3.
B4.
B5.
C1.
C2.
C3.
C4.
C5.
D1.
D2.
D3.
D4.
D5.

851 **FIGURE 3**



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854 **FIGURE 4**

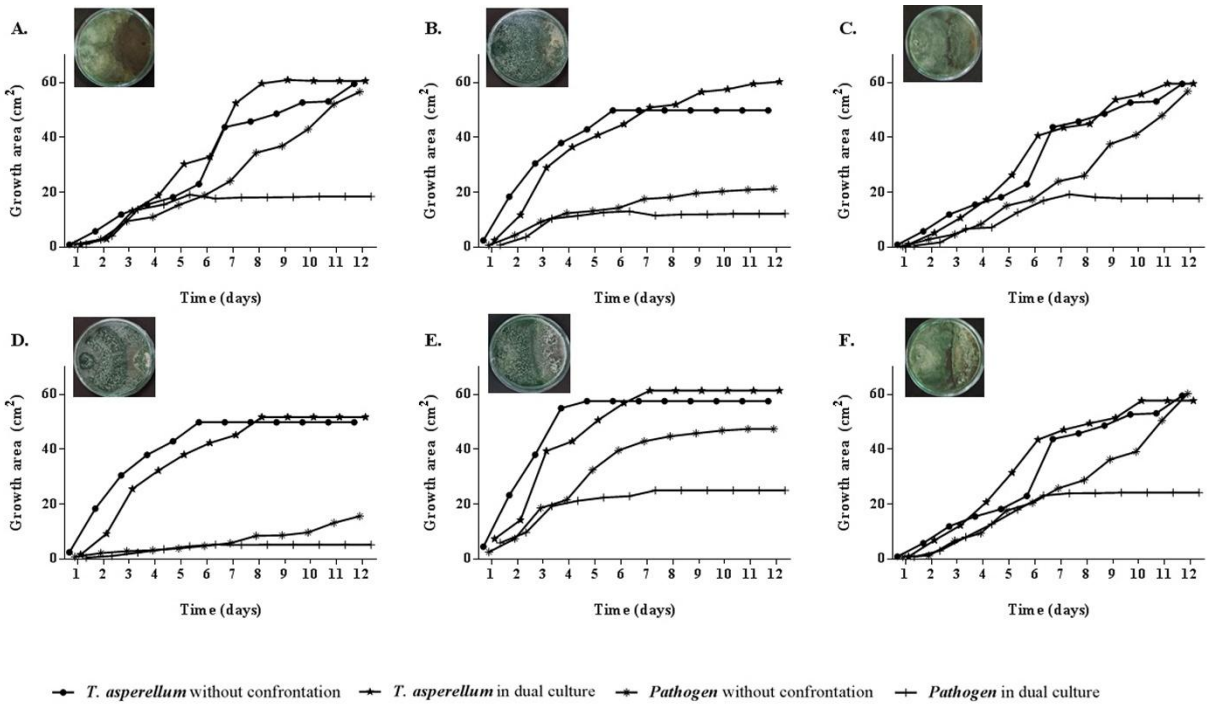

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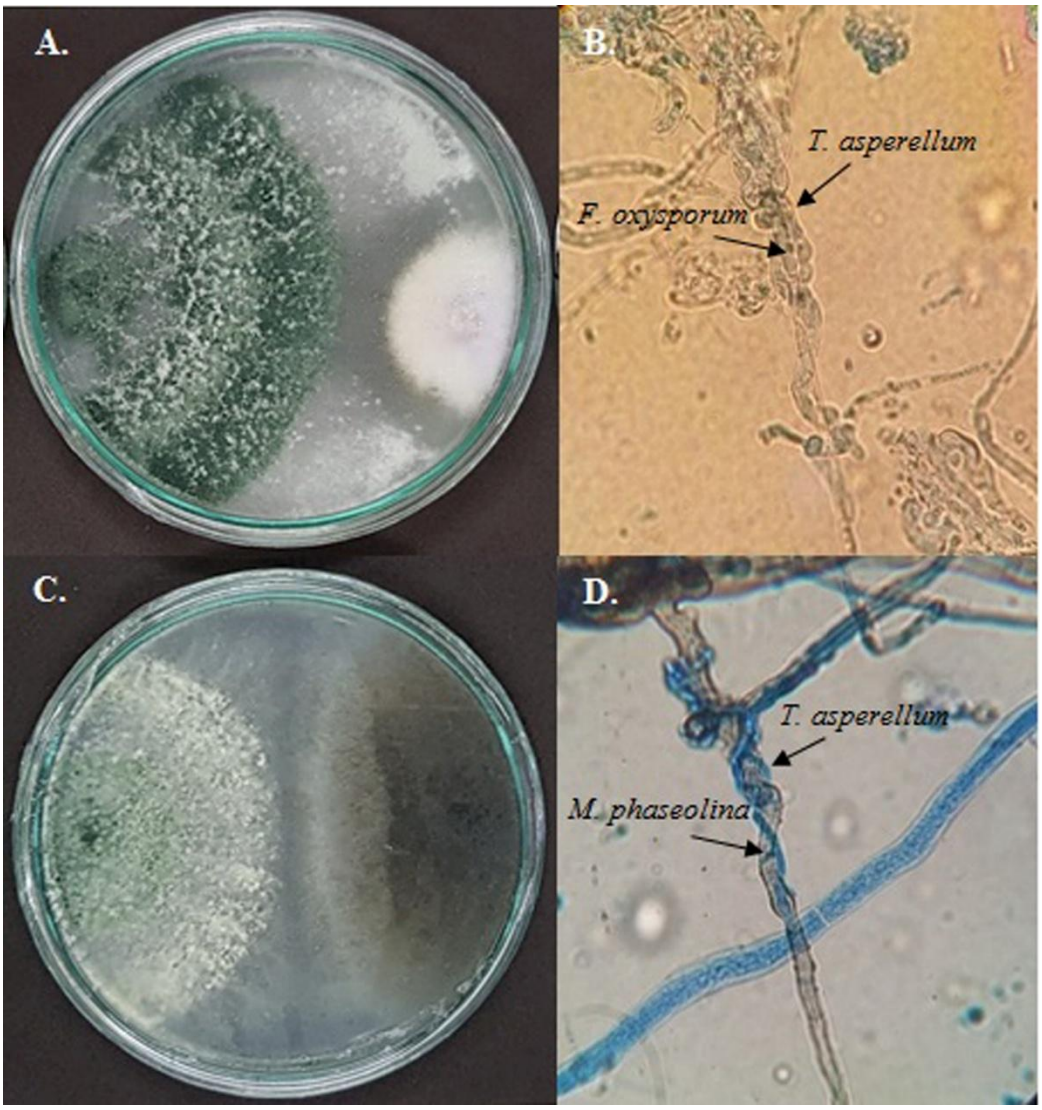
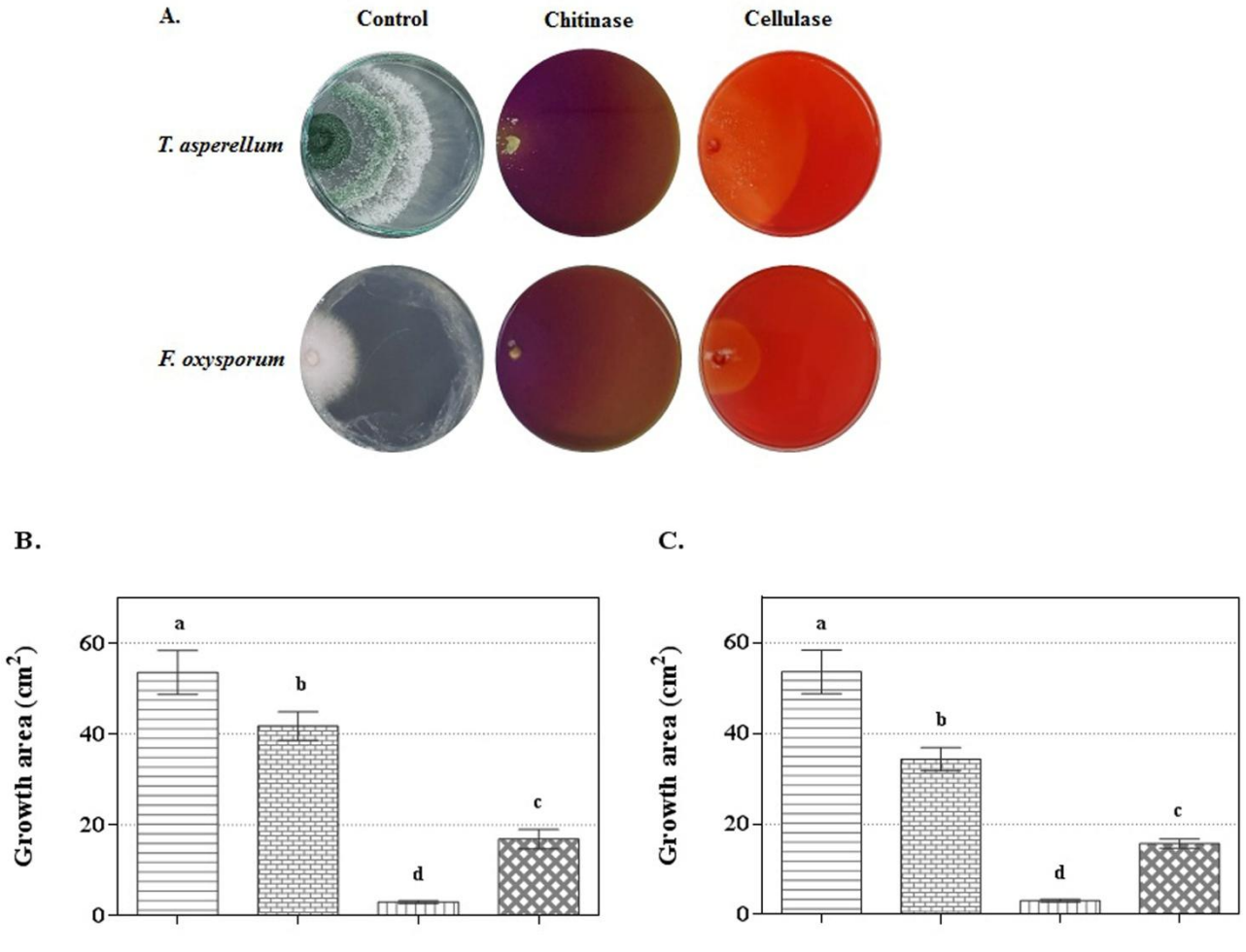
FIGURE 5



862 **FIGURE 6**



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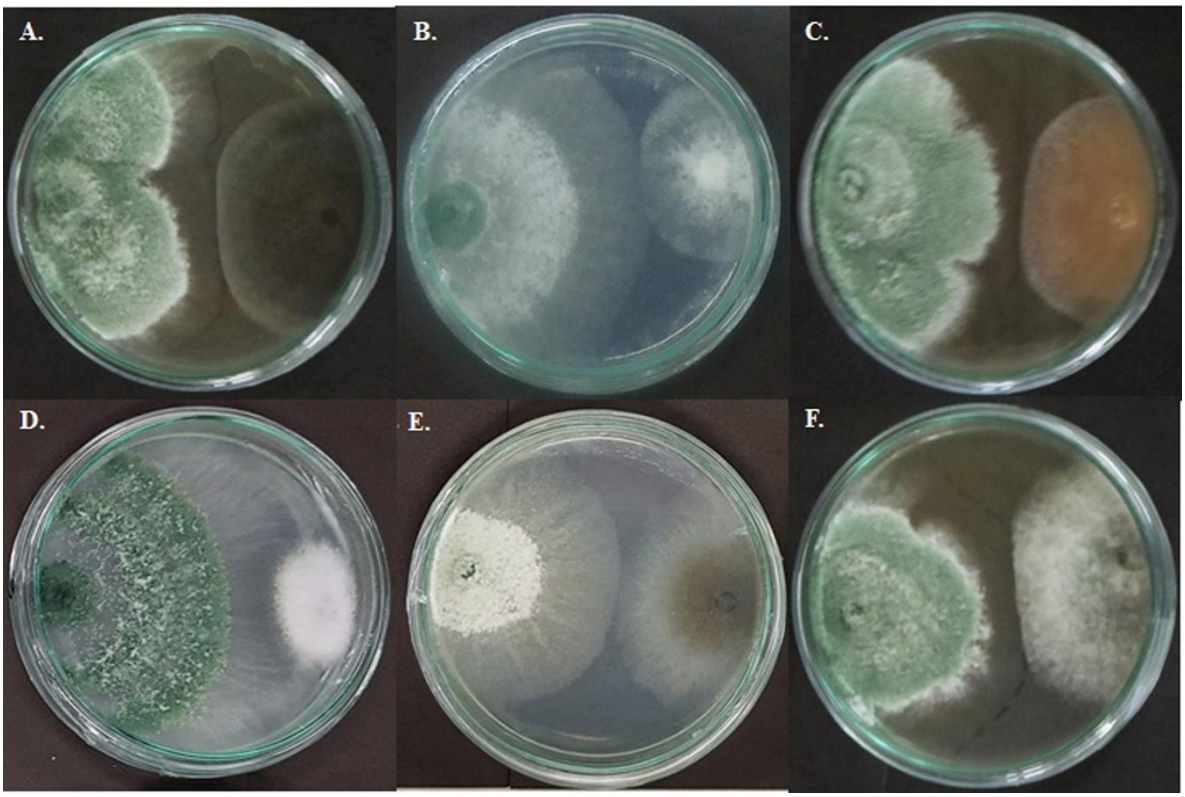
SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2