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QUALITATIVE AND QUANTITATIVE ENZYMATIC PROFILE OF NATIVE *Trichoderma* STRAINS AND BIOCONTROL POTENTIAL AGAINST *Fusarium oxysporum* f.sp. *cubense* RACE 1

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ABSTRACT

Trichoderma is a ubiquitous fungal genus in the rhizosphere, often utilized as biofertilizer and biocontrol agent that may release enzymes involved in the control of certain fungal phytopathogens. This research evaluated the biotechnological potential of fifteen native *Trichoderma* strains that were previously isolated from the rhizosphere of maize cultivated at the Estado de Mexico (Mexico). The radial growth rate of each fungal strain was assessed as well as the qualitative analysis of enzyme activity on specific culture media. Then, eight prominent strains were selected for evaluating their capability of degrading four complex substrates (filter paper, newspaper, starch, and chitin), and their antagonism towards five isolates of *Fusarium oxysporum* f.sp. *cubense* Race 1 (FocR1). Eight out of the fifteen *Trichoderma* strains showed high radial growth rate (0.137-0.149 cm/h), and good qualitative enzyme activities (cellulase, pectinase, and chitinase) when compared to a positive control strain *T. reesei* CDBB-H-356. The best eight strains (EMV6SIC2, EMV6SIC4, EMV6SIC5, EMV6SIC6, EMV6SIC7, EMV6SIC8, EMMVrSIC4, and EMMVSIC2) showed good inhibition to the five FocR1 isolates (ranging from 3 to 54%), and good capability for degrading cellulosic substrates (filter paper and newspaper) via production of active enzymes. Data were analyzed by one way analysis of variance (ANOVA), followed by a multiple comparison test (Tukey, α=0.05). In conclusion, some native *Trichoderma* strains have good biotechnological potential for being directed to the industry since they produce cellulases; at the same time, these fungal strains may release chitinases by which fungi may control and degrade the cell wall of FocR1 isolates.

Keywords: biocontrol, *Trichoderma*, *Fusarium*, cellulase, pectinase, chitinase

INTRODUCTION

Trichoderma is a cosmopolite fungus widely studied due to its agroecological potential, high adaptation to extreme environments, and production of metabolites (enzymes, plant growth promoting compounds, volatile compounds, among others) for biotechnological and environmental purposes (**Sharma *et al*., 2017; Sharma and Sharma, 2020**). *Trichoderma* species produce certain enzymes that are able to degrade solid organic residues, by which they may significantly contribute on both mineralization and recycling of industrial residues (**Idris *et al*., 2017; Marques *et al*., 2017**). The later also generates byproducts with added values such as enzymes, sugars, biofuels, among others (**Behera *et al*., 2017; Hernández-Melchor *et al*., 2019**).

Trichoderma reesei is one of the most utilized species for industrial purposes since it produces cellulose-degrading enzymes, but the cost of culture media may represent a limitation for these biotechnological processes (**Fang and Xia, 2015**). Thus, for this enzyme production, several substrates like lignin-rich residues have been utilized like wheat straw, sugarcane bagasse, used paper sludge's, newspaper residues, and wood residues (**Guoweia *et al*., 2011; Bohacz and Kornillowicz-Kowalska, 2020**) by using fermentative systems with solid substrates or submerged cultures (**Kumar *et al*., 2014; Ajijolakewu *et al*., 2017**). Furthermore, endoglucanases, exoglucanases, and β-glucosidases are the most relevant enzymes for cellulose degradation, which require specific conditions of pH and temperature; therefore, several biotechnological strategies have been designed like submerged batch culture in reactors for optimizing the process, and reducing operational costs (**Fenila and Shastri, 2016; Weiss *et al*., 2020**).

In addition, *Trichoderma* species are directed for biocontrolling fungal phytophatogens due to several mechanisms of action such as antibiosis, mycoparatisism, competition of space and nutrients, and production of secondary metabolites (**Argumedo-Delira *et al*., 2009; Bunbury-Blanchette and Walker, 2019; De la Cruz-Quiroz *et al*., 2018**). During mycoparasitism, *Trichoderma* releases enzymes like proteases, chitinases and glucanases that cause the hydrolysis of target fungal cell walls (**Gruber and Seidl-Seiboth, 2012; Harman *et al*., 2012; García-Espejo *et al*., 2016; Silva *et al*., 2019**). Thus, several species of *Trichoderma* have been directed for the biocontrol of soil-borne fungi that cause diseases in important agricultural crops like tomato, rice, maize, banana, and avocado, among others (**Diánez *et al*., 2016; Kiriga *et al*., 2018**). Therefore, *Trichoderma* is one of the most effective and successful biocontrol agent for agriculture, and some species are part of more than 60% of the biofungicide formulations already registered worldwide (**Charoenrak and Chamswarng, 2016; Gorai *et al*., 2020**).

The utilization of *Trichoderma* strains in agriculture and biotechnology has increased, but the isolation and the identification of new and promising species are still needed, as well as the characterization and evaluation of biological effectiveness of fungal-derived metabolites. Due to the effectiveness of this fungal genus under several environments, it is relevant to study the enzyme profile of native *Trichoderma* strains as potential agents of biocontrol. In addition, the evaluation of the capability of *Trichoderma* species for producing specific enzymes is desirable, especially when fungi are applied in submerged cultures in which agro-industrial residues are utilized as a carbon source. Thus, this study evaluated the biotechnological potential of native *Trichoderma* strains previously isolated from maize rhizosphere, and their antagonism towards *Fusarium oxysporum* f.sp. *cubense* Race 1 (FocR1).

MATERIAL AND METHODS

Fungal strains

The growth of fifteen strains of *Trichoderma* were reactivated; these strains were previously isolated from maize roots and rhizosphere soil at two plant phenology stages [vegetative growth (V6 stage) and maturity] collected from Estado de Mexico, Mexico (**Herrera-Jiménez *et al*., 2018**), obtained from a previous Research Grant RHIZOMAIZ. Fungal preservation was performed in test tubes with potato dextrose agar (PDA Baker®) and in cryopreservation vials with 1 mL of 0.01% Tween 20 solution (**Herrera-Jiménez *et al*., 2018).** In addition, the strain of *Trichoderma ressei* CDBB-H-356 donated by the National Collection of Microbial Strains and Cellular Cultures (Instituto Politecnico Nacional) registered at the WFCC (World Federation for Culture Collection) was utilized as positive control for enzyme bioassays.

Five strains of *Fusarium oxysporum* f.sp. *cubense* Race 1 (FocR1) were utilized. These banana phytopathogens were obtained from the Mycology Laboratory of the Centro Nacional de Referencia Fitosanitaria (CNRF) belonged to the Servicio Nacional de Sanidad, Inocuidad y Calidad Agroalimentaria (SENASICA, Mexico). These fungal phytopathogens are identified as CNRF-MIC17188, CNRF-MIC17189, CNRF-MIC17190, CNRF-MIC17191, and CNRF-MIC17192.

Both fungal genera *Trichoderma* and FocR1 were propagated on Petri dishes containing PDA, and incubated at 28 ºC for 5 days. Afterwards, *Trichoderma* cultures were exposed to light conditions at room temperature for two days to induce sporulation.

DNA extraction, PCR amplification and molecular identification of *Trichoderma* strains

The genomic DNA from the 15 *Trichoderma* strains was extracted from mycelium samples by using three solutions: 1) 10 mM TRIS, 60 mM NaCl, 5% Sucrose, and 10 mM EDTA; 2) 300 mM Tris, 1.25% SDS, 5% Sucrose, and 10 mM EDTA; and 3) 3M Potassium acetate. The quality and quantity of DNA was determined by gel electrophoresis in a 1% agarose gel, and nanodrop (BioSpec-Nano, Shimazdu®), respectively. The universal primers ITS1 (forward) and ITS4 (reverse) were used for amplifying the ITS rDNA region. The PCR reaction was performed as suggested in the protocol of the kit Taq PCR Master Mix adjusting the mix reaction to 25 µL. The amplifying conditions included a denaturing stage at 94 °C and 30 cycles at 94 °C for 45 s, and alignment at 51 °C and 72 °C for 45 s. The PCR products were verified by gel electrophoresis in 1% agarose gel and subsequently, sequenced by means of the Sanger method performed at the Laboratorio de Servicios Genómicos (LANGEBIO, CINVESTAV).

All sequences were edited with the program Codon Code Aligner (CodonCode Corporation, www.codoncode.com) and aligned in MEGA (**Kumar *et al*., 2016**). Furthermore, sequences were analyzed with the BLAST program (Basic Local Alignment Search Tool). Later, after the sequence edition, fragments of 416 bp were obtained and compared at the GenBank database, and registered. The GenBank access code for fungal strains were included in Table 1.

Determination of the radial growth rate of *Trichoderma* strains

The radial growth rate of all *Trichoderma* strains was estimated on PDA medium in Petri dishes (8.5 cm diameter). A 3 mm-disk of PDA with active growth of the each fungus was set by triplicate at the center of the Petri dish, and incubated at 28°C. The fungal radial growth rate was measured every 24 h until fungi reach the border of the Petri dishes (**García-Espejo *et al*., 2016**).

Qualitative analysis of cellulase, chitinase and pectinase enzymes on culture media

All qualitative analysis of enzymes were carried out in Petri dishes (8.5 cm of diameter). The cellulase activity was determined on culture medium containing 5 mL of the Winogradsky saline solution [5 g/L K_2HPO_4, 2.5 g/L $MgSO_4$, 2.5 g/L NaCl, 0.5 g/L $Fe(SO_4)_3$], 1 g NH_4NO_3, and 1 mL of micronutrient solution (0.05 g/L $KMoO_4$, 0.05 g/L $MnSO_4$, 0.05 g/L Na_3BO_3, 0.05 g/L $CoNO_3$, 0.05 g/L $CdSO_4$, and 0.05 g/L Cu_2SO_4), pH 6.5 (**Winogradsky, 1950**), in which 10 g de carboxymethylcellulose was added (**Ordoñez-Valencia, 2014**). The culture medium was poured in Petri dishes, and a 3 mm agar-disk with the corresponding fungal growth was set at the center of each plate by triplicate. Fungal cultures were incubated at 28 °C for 4 days. The halo of cellulose hydrolysis was detected by adding 15 mL of Congo red solution (1%) for 15 min, and then decanted for further application of 15 mL of 1 M NaCl solution for 15 min; then, the hydrolysis halo was measured.

The chitinase activity was determined by preparing 10 g of colloidal chitin (Sigma®, C-9213) mixed with 100 mL of 85 % phosphoric acid. This mix was kept at 4 °C for 24 h, and occasionally stirred. Therefore, the chitin was precipitated with an excess of distilled water, and filtered throughout two layers of cheese cloth, and washed until reaching a neutral pH. The filtrate was sterilized at 121 °C for 18 min, and kept at 4°C until further utilization (**Romero-Cortes *et al*., 2016**). To determine the positive chitinase reaction, a modified basal medium (BM) consisted on 0.3 g/L $MgSO_4 \cdot 7H_2O$, 3.0 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 1.0 g/L monohydrated citric acid, 30 g/L agar, 4.5 g/L of colloidal chitin, and 0.15 g/L bromocresol purple. The pH was adjusted to 2.8 with the sodium acetate buffer sterilized at 121 °C for 18 min (**García-Espejo *et al*., 2016**). Petri dishes were inoculated with a 3 mm agar disk with active growth of the corresponding fungus by triplicate, and incubated at 28 °C until a change of color from bright yellow to intense violet was observed (**García-Espejo *et al*., 2016**).

The determination of pectinase activity was performed by using the culture medium proposed by **Hankin *et al.* (1971)**, and consisted on preparing two solutions. Solution A consisted on 2 g/L yeast extract, 10 g/L apple or orange pectin and 30 g/L agar. Solution B included 2 g/L $(NH_4)_2SO_4$, 4 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 0.1 mg/L $FeSO_4 \cdot 7H_2O$, 1 mg/L $CaCl_2$, 10 µg/L H_3BO_3, 70 µg/L $MnSO_4$, 50 µg/L $CuSO_4$, 10 µg/L MoO_3, with a pH of either 7 or 5. Two equal volumes of both solutions were mixed and sterilized at 121 °C for 18 min, and the culture medium was poured in Petri dishes. The *Trichoderma* inoculation was performed as previously described, and plates were incubated at 28 °C for 3 days. Therefore, the Petri dishes were added with lugol for 5 min, and this solution was decanted for observing the corresponding clear halo denoting pectinase activity. In this assay, two types of pectinase activity were detected: the pectatelyase activity (pectate transeliminase E.C.4.2.2.2) at pH 7, and the polygalacturonase activity (endo-polygalacturonase E.C. 3.2.1.15) at pH 5. Pectatelyase is usually characterized under alkaline pH, whereas polygalacturonase is detected in acid pH values. By adjusting the pH values in culture medium it was possible to differentiate between lyase and polygalacturonase activity (Hankin *et al*. 1971).

Quantitative analysis of enzyme activities by using four substrates

The degradation kinetics of four substrates [filter paper, newspaper, starch, and chitin (Sigma®)] were determined by growing the *Trichoderma* strains in a submerged culture for 14 days at 30 °C, and 200 rpm. Each test was carried out by triplicate with the corresponding substrate and with the fungal strain. For this, 50 mL-Falcon tubes were utilized; each tube contained 35 mL of basal minimum medium (BMM) [6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 2.64 g/L $(NH_4)_2SO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.015 g/L $CaCl_2$, 3 g/L $MnSO_4$, 3 g/L $ZnSO_4$], with a pH 4.8, adjusted with a 0.05 M citrate buffer (**García-Espejo *et al*., 2016**). Afterwards, the respective substrate to be degraded was added: 1% w/v of starch, 1% w/v of chitin, one strip (1x11cm) of filter paper, and one strip (1x11cm) of newspaper. Each tube was inoculated with a spore suspension ($1x10^6$ spores/mL) of the corresponding fungal strain. In addition, test tubes with the corresponding substrates without fungal inoculum were set as blanks (controls). Every three days during 14 days, 1.5 mL aliquots were taken for determining the corresponding enzyme activities that are described as follows.

The total cellulase activity was evaluated by means of the bioassay with filter paper (FPase), and with the endoglucanase activity (CMCase) performed by the DNS technique (**Miller, 1959; Ghose, 1987**), and by using D-Glucose as blank. For the FPase, a blank test tube and one problem tube were used. Both tubes were added with 1 mL of citrate buffer pH 4.8, one strip of filter paper Whatman No.1 (1x6 cm), and incubated for 5 min at 50 °C. Then, an aliquot of 0.5 mL of the supernatant of the corresponding fungal culture was added only for the problem tube. Tubes were incubated for 60 min at 50 °C, and subsequently were set at room temperature, and 2 mL of DNS solution were added. At the end, 0.5 mL of the supernatant of fungal culture was added to the blank tube. Thus, tubes were incubated at 90 °C for 5 min, and cooled in iced water.

For estimating the CMCase activity, two tubes were also utilized (blank tube and problem tube). Both tubes were added with 0.5 mL of a 2% de carboxymethyl cellulose (CMC) solution in a 0.05 M sodium citrate buffer, pH 4.8, and incubated for 5 min at 50 °C. Afterwards, a 0.5 mL of the supernatant of the fungal culture was only added to the problem tubes. The tubes were incubated for 30 min at 50 °C, and subsequently set at room temperature, and added with 2 mL of the DNS solution. At the end, 0.5 mL of the fungal supernatant was added to the blank tube. Tubes were incubated at 90 °C for 5 min, and cooled with iced water.

The chitinase activity was performed by means of the method described by **Vargas-Hoyos and Gilchrist-Ramelli (2015)**. The production of sugars was determined with the method of **Miller (1959)** using N-acetyl-D-glucosamine (GlcNAc) as blank. For the enzyme quantification, two tubes were also utilized (blank tube and problem tube). Both tubes were added with 0.1 mL of 0.25% colloidal chitin solution in a 0.05 M sodium citrate buffer, pH 4.8, and incubated for 5 min at 40 °C. Afterwards, a 0.05 mL of the supernatant of the fungal culture was only added to the problem tube. The tubes were incubated for 30 min at 40 °C, and subsequently set at room temperature, and added with 3 mL of the DNS solution. At the end, 0.05 mL of the fungal supernatant was added to the blank tube. Tubes were incubated at 90 °C for 5 min, and cooled with iced water.

For the three enzymatic activities from each tube, corresponding aliquots of 250 µL were taken and placed in 96-well clear microplates (Costar®) to measure absorbance readings at 540 nm with a microplate reader (Synergy 2, Biotek®). Each unit of enzyme activity was defined as the amount of the enzyme required to release 1 µmol/min of the reducing sugar, and expressed as equivalents of

glucose under our experimental conditions. The enzyme activities were expressed in terms of international units per liter (IU/L). Each enzyme assay was performed by triplicate.

Biocontrol assays of *Trichoderma* strains against five strains of FocR1

The biocontrol evaluation was based on performing bioassays of confrontation of eight selected *Trichoderma* strains against five isolates of FocR1. These bioassays considered the utilization of dual cultures in which a 3 mm-agar disk of active growth of the respective FocR1 strain was set in one extreme of the Petri dish containing PDA, incubated at 28 °C for 24 h. Afterwards, a 3 mm-agar disk with active growth of the respective *Trichoderma* strain (five replicates) was set in the opposite extreme of the Petri dish; plates were incubated at 28 °C until fungal confrontation was completed. The growth of both fungi was evaluated every 24 h. In addition, three replicates of either antagonist (*Trichoderma* strain) or pathogen (FocR1 strain) were set as controls. The inhibition percentage of the growth of FocR1 due to each *Trichoderma* strain was estimated by using the method proposed by **Vargas-Hoyos and Gilchrist-Ramelli (2015)**.

Statistical analysis

Analysis of variance (ANOVA) and the multiple mean comparison test (Tukey, $P \leq 0.05$) were used to assess significant differences among parameters like the capability of degrading four complex substrates (filter paper, newspaper, starch and chitin), and the antagonism of eight *Trichoderma* strains against five isolates of *Fusarium oxysporum* f.sp *cubense* Race 1. As complement of this analysis, it was proposed to use a descriptive tool for examining and displaying patterns in multivariate data called "Radial plot". This type of graph is practical and profoundly useful for a wide variety of data. The common feature of radial plots is that they are a circular graphing method and have a series of rays projecting from a central point to different variables. The analyses were processed using the Origin Pro 8.0 statistical software and SigmaPlot® software 14.0 (**Saary, 2008; *Orea et al*., 2017; Porter and Niksiar, 2018; López Pérez *et al*., 2020**).

RESULTS AND DISCUSSION

Taxonomic identification of *Trichoderma* strains

The fifteen *Trichoderma* strains were labeled as EMV6SIC1, EMV6SIC2, EMV6SIC4, EMV6SIC5, EMV6SIC6, EMV6SIC7, EMV6SIC8, EMMVrSIC2, EMMVrSIC4, EMMVS2C3, EMMVSIC2, EMMVrSIC3, EMMVS3C1, EMMVrS2C3, and EMMVS2C1. Table 1 shows the taxonomic identification of each fungal strain achieved from phylogenetic analysis. Thirteen of these strains corresponded to *T. harzianum*, and the two remaining strains (EMMVSIC2 and EMMVS2C1) were identified as *T. tomentosum* and *T. gamsii*, respectively.

Besides having a good potential for biocontrolling fungal phytopathogens for crops (for instance, cocoa, tomato, potato) (**Torres-De la Cruz *et al*., 2015; Al-Hazmi and TariqJaveed, 2016; Contina *et al*., 2017**), *T. harzianum* has a biotechnological importance due to the production of important enzymes for industrial purposes like cellulases (**Libardi *et al*., 2017; de Souza *et al*., 2018**), lacases (**Ranimol *et al*., 2018**), xylanases (**Lopez-Ramirez *et al*., 2018**), among others. Similarly, *T. tomentosum* is able to degrade Azo colorants (**He *et al*., 2018**) and polycyclic aromatic hydrocarbons (**Marchand *et al*., 2017**), whereas, *T. gamsii* produces volatile organic compounds related to the biocontrol (**Chen *et al*., 2016; Galletti *et al*., 2020**), and growth inhibition of *F. oxysporum* f. sp. *cepae* (**Bunbury-Blanchette and Walker, 2019**).

Radial growth rate and qualitative enzyme activity

Table 1 shows the radial growth rate of the *Trichoderma* strains, which ranged from 0.091 to 0.150 cm/h. The fast growing strains were EMV6SIC2, EMV6SIC5, EMV6SIC6, EMV6SIC7, EMV6SIC8, EMMVrSIC4, EMMVSIC2, and EMMVrSIC3. The radial growth rates of these fungal strains were 1.1 fold higher than that obtained for *T. reesei* CDBB-H-356 (0.137 cm/h).

The *T. reesei* strain was used as referential fungus due to its high enzyme production (**Li *et al*., 2019; Weiss *et al*., 2020**), and for comparing the qualitative enzyme activity determined for the *Trichoderma* strains. All *Trichoderma* strains had positive qualitative detection of cellulase, chitinase and pectinase (pectatelyase and polygalacturonase) activities. Table 1 shows the diameter of halos which ranged from 2.7-6.2 cm, 3.3-7.2 cm, and 3.9-7.4 for cellulase, pectatelyase and polygalacturonase activities, respectively. All values were higher than those detected for the referential *Trichoderma* strain (3.2 cm of cellulose, 3.3. cm of pectatelyase, and 3.9 cm of polygalacturonase). In addition, a 100 % of chitin degradation (detected by color changes) was observed in shorter time (4-6 days) than *T. reesei* (11 days).

From results of the radial growth rate and the qualitative enzyme detection, eight strains were selected (EMV6SIC2, EMV6SIC4, EMV6SIC5, EMV6SIC6, EMV6SIC7, EMV6SIC8, EMMVrSIC4, and EMMVSIC2) whose radial growth rate ranged from 0.142 to 0.149 cm/h. In addition, these fungal strains showed greater halos of cellulase and pectinase hydrolysis than those observed for the referential strain (*T. reesei* CDBB-H-356), as well as less time for chitin degradation. The later indicates that our *Trichoderma* strains release important enzymes that may degrade complex organic substrates.

Table 1 Radial growth rate and qualitative enzyme activities for 15 *Trichoderma* strains isolated from maize roots cultivated in the Estado de Mexico

Keys of *Trichoderma* isolates	Fungal identification	GenBank access codes	Radial growth rate (cm/h)	Cellulase Halo diameter (cm)	Chitinase 100% color change	Pectatolyase activity pH 7 Halo diameter (cm)	Polygalacturonase activity pH 5 Halo diameter (cm)
EMV6SIC2	*T. harzianum**	MK652840	0.144	4.9 ± 0.2	Day 5	4.9 ± 0.1	3.9 ± 0.8
EMV6SIC4	*T. harzianum**	MK652843	0.137	5.7 ± 0.1	Day 5	4.8 ± 0.1	5.6 ± 0.1
EMV6SIC5	*T. harzianum**	MK652837	0.143	5.4 ± 0.5	Day 5	7.2 ± 0.2	6 ± 0.9
EMV6SIC6	*T. harzianum**	MK652836	0.149	5.7 ± 0.6	Day 5	4.2 ± 0.6	5.9 ± 0.1
EMV6SIC7	*T. harzianum**	MT920962	0.142	5.2 ± 0.1	Day 5	4.5 ± 0.1	6.2 ± 0.6
EMV6SIC8	*T. harzianum**	MK652833	0.147	5.1 ± 0.1	Day 4	5 ± 0.6	6.2 ± 0.3
EMMVrSIC4	*T. harzianum**	MK652847	0.148	4.4 ± 1.7	Day 4	6.4 ± 1.3	5.5 ± 0.3
EMMVSIC2	*T. tomentosum**	MK652832	0.147	4.4 ± 0.6	Day 4	3.3 ± 0.3	5.4 ± 0.3
EMV6SIC1	*T. harzianum*	MK652836	0.135	4.5 ± 0.9	Day 5	5 ± 0.1	5.2 ± 0.03
EMMVrSIC2	*T. harzianum*	MK652848	0.104	2.7 ± 0.1	Day 5	4.5 ± 1.1	5.1 ± 0.5
EMMVS2C3	*T. harzianum*	MK652834	0.132	6.2 ± 0.1	Day 5	4.5 ± 0.04	5.8 ± 0.2
EMMVrSIC3	*T. harzianum*	MK652834	0.15	4.8 ± 0.1	Day 4	5.6 ± 0.03	7.4 ± 0.5
EMMVS3C1	*T. harzianum*	MK652841	0.126	4.6 ± 0.5	Day 4	6.2 ± 0.8	6.9 ± 0.5
EMMVrS2C3	*T. harzianum*	MK652851	0.091	5.3 ± 0.4	Day 5	5.2 ± 0.1	5.4 ± 0.3
EMMVS2C1	*T. gamsii*	MK652850	0.125	5.8 ± 0.1	Day 6	5.7 ± 1.9	6 ± 0.1
***T. reesei* CDBB-H-356**			0.137	3.2 ± 0.04	Day 11	3.3 ± 0.1	3.9 ± 0.2

Means ± standard errors. Asterisks denote the selected eight strains for next experimental stage.

Quantification of the enzyme activity on complex substrates in submerged cultures

Table 2 shows the results of the quantified enzymatic activities (FPase, CMCase and chitinase) of the selected eight *Trichoderma* strains as well as of the reference strain *T. reesei* CDBB-H-356 after 14 days, using newspaper, filter paper, starch and chitin as carbon sources. Thus, the mean comparison test showed significant differences ($P \leq 0.05$) among fungal treatments for each carbon source (Table 2).

Table 2 Quantitative enzyme activity for eight strains of *Trichoderma* after 14 days, previously selected due to their qualitative enzyme activity and radial growth rate (See Table 1).

***Trichoderma* strains/ Substrate**	**Substrate-induced enzyme activity**		
	Cellulase activity on filter paper (FPase) (IU/L)	Carboxymethyl cellulase (CMCase) (IU/L)	Chitinase activity (IU/L)
Newspaper			
EMV6SIC2	497.6 ab ± 158.7	1039.9 a ± 239.8	620.3 d ± 86.4
EMV6SIC4	996.4 d ± 134.4	7061.3 bc ± 306.5	513.7 c ± 136.0
emv6sic5	887.3 cd ± 65.1	14788.6 d ± 453.2	575.4 cd ± 145.7
EMV6SIC6	922.3 cd ± 30.9	9271.7 c ± 319.9	322.9 bc ± 56.7
EMV6SIC7	863.9 c ± 60.7	3403.6 b ± 143.3	547.4 c ± 101.4
EMV6SIC8	906.7 cd ± 26.9	3881.9 bc ± 323.1	289.3 b ± 35.7
EMMVrSIC4	372.9 a ± 61.8	1678.9 ab ± 321.5	280.9 b ± 11.9
EMMVSIC2	696.3 b ± 99.2	8529.5 cd ± 205.5	306.1 ab± 26.7
Trichoderma reesei CDBB-H-356	774.3 bc ± 52.7	7612.4 cd ± 432.2	216.4 a ± 35.0
Filter paper			
EMV6SIC2	879.5 a ± 157.8	6249.8 a ± 360.7	306.1 b ± 46.5
EMV6SIC4	934.0 ab ± 223	11784.9 bc ± 401.0	552.9 d ± 68.0
EMV6SIC5	1608.1 d ± 247.9	29692.1 e ± 415.1	468.8 cd ± 130.7
EMV6SIC6	926.2 ab ± 89.2	7188.5 b ± 336.7	519.3 d ± 70.1
EMV6SIC7	1152.2 b ± 161.3	13438.1 c ± 466.3	297.7 a ± 35.7
EMV6SIC8	1101.8 bc ±126.4	13922.6 c ± 447.6	373.5 c ± 71.4
EMMVrSIC4	832.7 a ± 167.1	19015.6 cb ± 179.8	328.6 bc ± 19.4
EMMVSIC2	1428.9 c ±214.2	28656.5 de ± 89.9	345.4 bc ± 35.0
Trichoderma reesei CDBB-H-356	1206.8 bc ± 67.4	22188.9 d ± 369.5	306.1 b ± 38.8
Chitin (Sigma®)			
EMV6SIC2	493.7 ab ± 140.5	789.8 a ± 82.1	547.3 c ± 48.6
EMV6SIC4	1144.4 b ± 362.8	1573.1 d ± 82.1	710.1 d ± 146.7
EMV6SIC5	1117.2 b ± 347.1	1327.6 c ± 313.9	833.5 de ± 59.1
EMV6SIC6	1510.7 cd ± 115.7	1413.3 cd ± 132.2	850.3 e ± 179.9
EMV6SIC7	1584.8 d ± 115.7	1480.8 cd ± 421.5	457.6 bc ± 71.4
EMV6SIC8	1193.7 b ± 167	895.1 b ± 270.1	432.4 b ± 59.5
EMMVrSIC4	376.8 a ± 8.26	844.4 ab ± 247.9	356.6 ab ± 95.2
EMMVSIC2	353.4 a ± 35.7	887.3 b ± 71.1	322.9 a ± 45.6
Trichoderma reesei CDBB-H-356	1424.9 c ± 33.1	1370.4 c ± 330.7	474.4 bc ± 23.8
Starch			
EMV6SIC2	1371.3 b ± 388.5	1087.6 ab ± 261.8	356.6 ab ± 44.5
EMV6SIC4	1313.2 b ± 82.6	1420.5 c ± 380.9	609.1 bc ±134.6
EMV6SIC5	973.1 a ± 192.7	1710.0 cd ± 317.7	771.8 d ± 145.2
EMV6SIC6	1140.5 ab ± 307.5	1198.9 ab ± 344.6	648.3 c ± 84.7
EMV6SIC7	1502.9 c ± 236.2	2262.5 d ± 365.3	317.4 a ± 63.7
EMV6SIC8	1416.1 bc ± 479.4	1378.5 b ± 198.4	322.9 a ± 37.6
EMMVrSIC4	1880.9 d ± 278.3	1348.0 b ± 192.7	552.9 b ± 134.9
EMMVSIC2	1586.0 cd ± 384.6	715.8 a ± 57.8	474.4 ab ± 23.8
Trichoderma reesei CDBB-H-356	1311.9 b ± 252.2	3554.9 e ± 220.6	536.1 bc± 71.4

*Mean values (± standard errors) in a column followed by different letters for fungal treatment at each complex substrate, are significantly different (Tukey; p≤0.05).

Figure 1 shows a radar plot that depicts the results of enzyme activities (cellulase and chitinase) achieved from the eight *Trichoderma* strains and the referential strain, for degrading four complex substrates (filter paper, newspaper, starch, and chitin). Graphs denote particular patterns of enzymatic activity on the four substrates at different scales, by which the following order was estimated: Carboxymethyl cellulase (CMCase) > Cellulase activity on filter paper (FPase) > Chitinase activity (Figure 1).

Table 3 Inhibition percentages of eight *Trichoderma* strains on the growth of five strains of *Fusarium oxysporum* f.sp. *cubense* Race 1.

***Trichoderma* strains / *Fusarium oxysporum* strains**	**Fungal growth inhibition (%)**				
	CNRF-MIC17191	CNRF-MIC17189	CNRF-MIC17192	CNRF-MIC17188	CNRF-MIC17190
EMMVrSIC4	14.2	6.9	3.4	18.8	39.4
EMMVSIC2	16.4	3.7	33.9*	53.0*	8.6
EMV6SIC2	11.4	16.1	15.6	23.7	5.1
EMV6SIC4	18.1	15.4	2.4	30.2	24.0
EMV6SIC7	7.4	19.1	20.0	33.0	4.0
EMV6SIC8	16.4	15.1	10.8	3.3	4.6
EMV6SIC5	20.0*	22.6*	19.3	36.1	52.2*
EMV6SIC6	11.9	12.7	4.0	24.4	27.7
Trichoderma reesei CDBB-H-356	27.5	14.5	24.3	25.4	54.4

Asterisks denote the highest growth inhibition achieved for each strain of FocR1. n=3.

Enzyme activities showed variations depending on sampling time, fungal strain, and target substrate (Figure 2). At 14th day (Table 2), the highest values for CMCase were achieved for the strain EMV6SIC5 in which the enzyme activities were as follows: 29692.1 IU/L on filter paper, and 14788.6 IU/L with newspaper;

whereas the highest values of CMCase produced by the strain EMV6SIC7 was achieved when starch was added (2262.5 IU/L), and for the strain EMV6SIC4 occurred when chitin was used as substrate (1573.1 IU/L). In the same time, the highest values of FPase were presented for different strains. No significant differences were observed for FPase activities among strains EMV6SIC4, EMV6SIC5, EMV6SIC7 and EMMVrSIC4 whose values were 996.4, 1608.1, 1584.8 and 1880.9 IU/L in the presence of newspaper, filter paper, chitin and starch, respectively.

In regards chitinase activity, at 14th day, the highest value was 620.3 IU/L for the strain EMV6SIC2 in the presence of newspaper. The strains EMV6SIC5 and EMV6SIC6 showed values of chitinases of 771.8 IU/L and 850.3 IU/L, respectively, when either starch or chitin was added in the submerged culture. Moreover, the strain EMV6SIC4 had high chitinase values (552.9 IU/L) when filter paper was applied to the submerged culture (Table 2).

The values of CMCase obtained in the present study were 20 to 200 times higher than those reported by **García-Espejo *et al.* (2016)** who utilized carboxymethylcellulose as a carbon source for *T. inhamatum* BOL12QD grown in agitation and stationary conditions. Conversely, the chitinase activity detected for our *Trichoderma* strains was 200 times lower than those values reported by **Ting and Chai (2015)** with *T. harzianum* in presence of colloidal chitin, in which the achieved enzyme activity was of 354970 IU/L.

The FPase activity depended on the carbon source (filter paper, newspaper, chitin or starch) and on the *Trichoderma* strain. Thus, the FPase activity was similar (1608 IU/L) or 1.5 times higher (1880 IU/L) than those values reported by **de Souza *et al.* (2018)** who demonstrated similar FPase for *T. harzianum* (1630 IU/L) when either cellulose or lactose was used as carbon source. In this regard, our results differed from values reported in the literature, but the nature and complexity of the substrate significantly influence on the induction of the enzyme production of certain fungal species (**Gelain *et al*., 2015; Li *et al*., 2019; Bohacz and Kornillowicz-Kowalska, 2020**). In the same manner, some cultural conditions of bioreactors like temperature, stirring, pH, and others, may also affect both production and effectiveness of fungal-derived enzymes (**Delabona *et al*., 2012; Gutiérrez-Rojas *et al*., 2015; Adnan *et al*., 2019**).

The cellulase and chitinase activities detected for *Trichoderma* strains acting on the four substrates, denote the capability of degrading polysaccharides rich in glucose and N-acetyl-D-glucose-2-amine derived from cellulose and chitin. Then, the *Trichoderma* strains are potential candidates for being used as biocontrol agents against fungal phytopathogens whose cell wall possesses these two carbon sources. For instance, the cell wall of *F. oxysporum* consists on glucose and N-acetyl-glucosamine, and mannose, galactose, uronic acids, and glycoproteins as well (**Schoffelmeer *et al*., 1999; Saravanakumar *et al*., 2016**), and the mycoparasitic contribution of the *Trichoderma* species is based on releasing extracellular enzymes (chitinase and cellulase) that are able to destroy the structure of the cell wall of the fungal pathogen (**Infante *et al*., 2009; Zeilinger *et al*., 2016; Bunbury-Blanchette and Walker, 2019; Silva *et al*., 2019**).

Biocontrol assays of *Trichoderma* strains against isolates of FocR1

Table 3 shows the inhibition percentages of eight *Trichoderma* strains and *T. reesei* CDBB-H-356 against five isolates of the phytopathogen FocR1. All FocR1 isolates had reduced growth due to the confrontation with the *Trichoderma* strains. This growth inhibition ranged from 3 to 54 %, and the greatest inhibition percentages were achieved by *T. harzianum* EMV6SIC5, and *T. reesei*. In particular, *T. ressei* has been described as a good antagonist to fungal pathogens like *Fusarium*, *Phytophthora capsici*, and *Colletotrichum gloeosporioides* (**De la Cruz-Quiroz *et al*., 2018; Nawaz *et al*., 2018; Adnan *et al*., 2019; Saravanakumar and Wang, 2020**). The achieved inhibition percentages for the five FocR1 isolates are similar to those reported by **Bunbury-Blanchette *et al*. (2019)** who evaluated the antagonism of five native strains of *Trichoderma* against *F. oxysporum* f. sp. *cepae*, in which *T. harzianum* was the most prominent antagonist with 51 % of inhibition.

It is possible to correlate the observed antagonism of *T. harzianum* EMV6SIC5 with its capability to degrade cellulose and chitin, as detected in our previous experimental stages with complex substrates (Figure 2). Figure 2A shows visual effects of the fungal confrontation between *T. harzianum* EMV6SIC5 and the five isolates of FocR1, in which the growth inhibition of the pathogen CNRF-MIC17190 was of 52.2 %. Moreover, this antagonism is associated to the enzyme production (FPase, CMCase, chitinase) by the *Trichoderma* strain (Figure 2B) which was dependent on the type of substrate, at days 1, 3, 7, 9 and 14.

In terms of fungal antagonism, the growth inhibition observed for FocR1 may be due to the expression of a mycoparasitic mechanism (**Saravanakumar *et al*., 2018; Silva *et al*., 2019**) in which *Trichoderma* may produce hydrolytic enzymes like cellulase, chitinase, and glucanase, that act on the target fungal cell wall (**Garcia-Espejo *et al*., 2016; Adnan *et al*., 2019**). These enzymes are induced or regulated by the presence of simple (sugars) or complex (sugarcane bagasse, lignocellulosic residues) carbon sources used for enhancing the fungal biomass (**Wang *et al*., 2017; De la Cruz-Quiroz *et al*., 2018; Weiss *et al*., 2020**). The later denotes the relevance of assessing the capability of fungal biocontrollers for producing enzymes that contribute on the lysis of cell walls of fungal phytopathogens, which in general, are constituted by chitin, excepting for Oomycetes such as *Phytophthora* whose cell wall is constituted by cellulose (**Lees *et al*., 2012; Saravanakumar *et al*., 2016**).

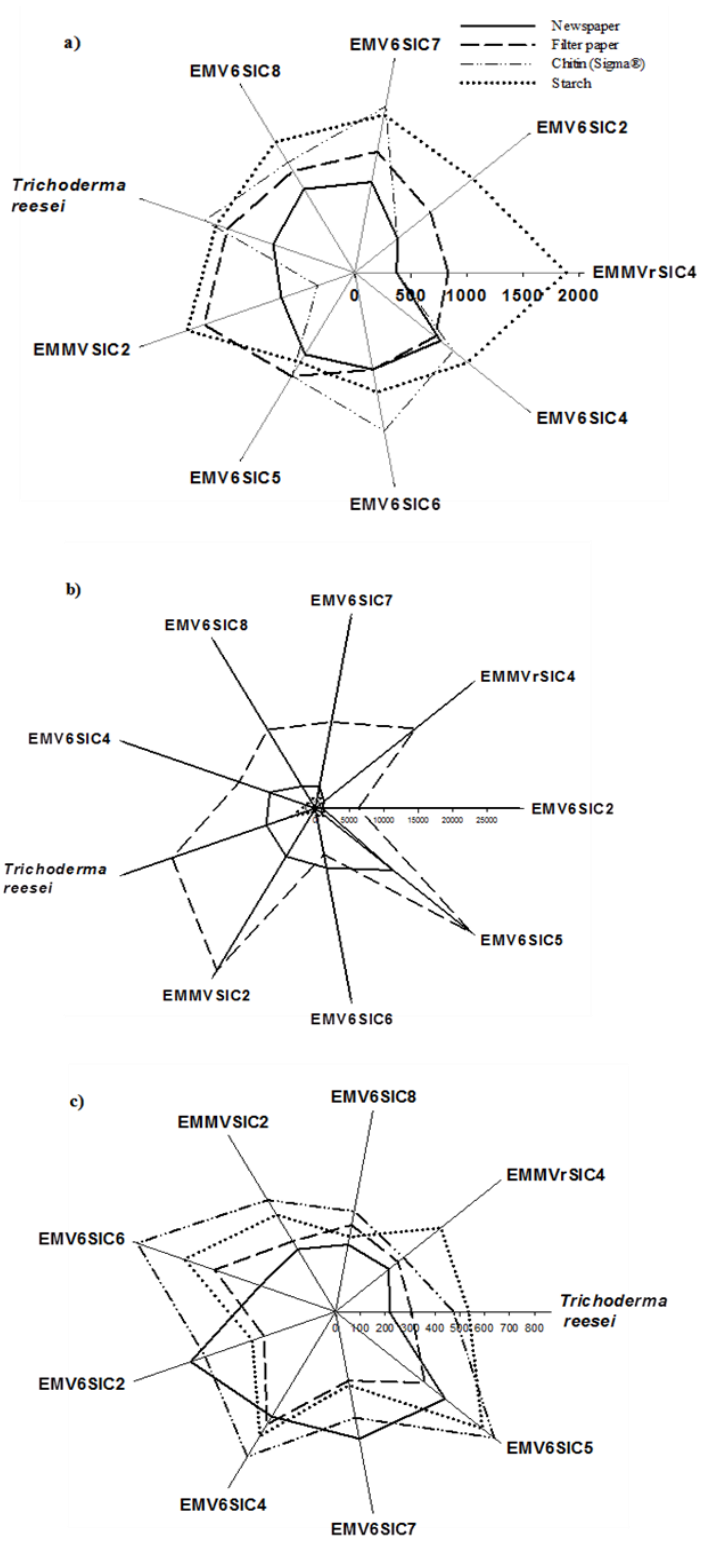

Figure 1 Radar plotting describing enzyme activities in respect to sampling time (14th day), determined by nine *Trichoderma* fungal strains acting on four target substrates. a) Cellulase activity on filter paper (FPase) (IU/L), b) Carboxymethyl cellulase (CMCase) (IU/L), and c) Chitinase activity (IU/L).

Based on the last discussion, the cellulolytic and chitinolytic activities detected for our native *Trichoderma* strains, represent a good characterization for selecting prominent fungal strains directed to the biocontrol of several fungal phytopathogens as reported for *Gibberella fujikuroi* (**Watanabe *et al*., 2007**), *Phytophthora capsici*, *Colletotrichum gloeosporioides* (**De la Cruz-Quiroz *et al*., 2018**), and *Sclerotinia sclerotiorum* (**Ordóñez-Valencia *et al*., 2015; da Silva *et al*., 2020**), *Macrophomina phaseolina*, *Fusarium graminearum*, *Botrytis cinerea* (**Saravanakumar and Wang, 2020**), among others.

From all native *Trichoderma* strains assessed in the present research, the strain *T. harzianum* EMV6SIC5 showed better biotechnological features based on its enzymatic potential, and on its antagonism to five FocR1 isolates. The later allows the possibility for developing a feasible biotechnology directed to agricultural and environmental purposes, by designing efficient bioprocesses for either producing high fungal biomass or assuring the benefits of *Trichoderma*-based formulations.

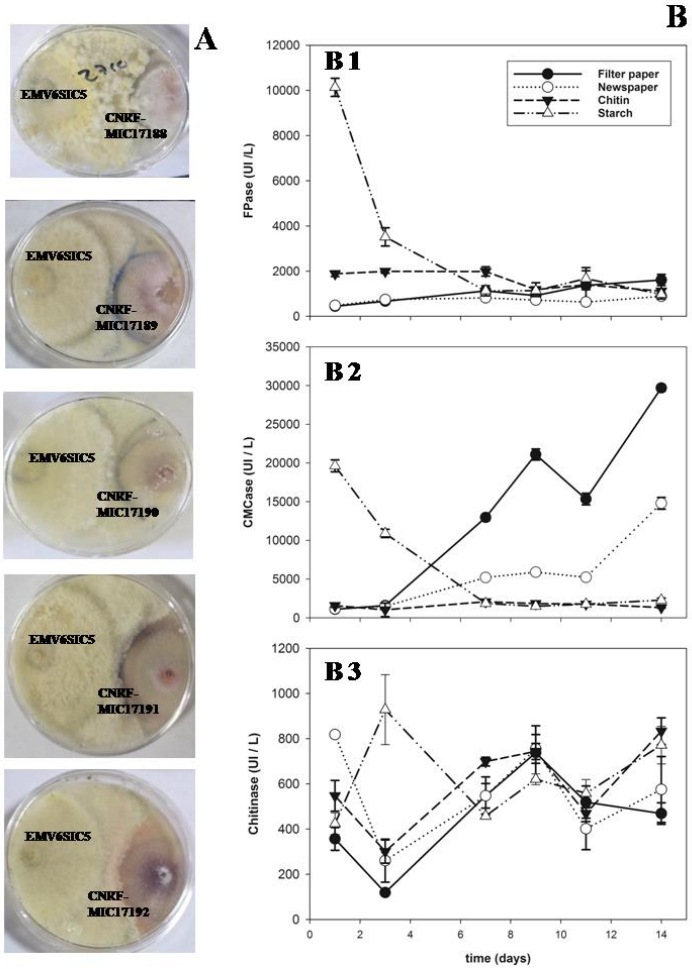

Figure 2 (A) Fungal confrontation and biocontrol activity of *Trichoderma harzianum* EMV6SIC5 against to five isolates of *Fusarium oxysporum f.sp.cubense* Race 1. (B) Cellulase (Filter paper and carboxymethyl cellulase -CMCase-) and chitinase activities of *T. harzianum* EMV6SIC5 grown in liquid media added with four complex carbon sources, n=3.

CONCLUSION

All *Trichoderma* strains isolated from the maize rhizosphere showed positive qualitative and quantitative activity of enzymes like cellulase, chitinase and pectinase. Also, the radar plot of the Tukey multiple-comparison test showed the potential capability of these strains to produce enzymes during the degradation of filter paper, newspaper, colloidal chitin and starch as carbon sources, let us to observe the best results for *T. harzianum* EMV6SIC5. In addition, this fungal strain inhibited the growth of FocR1 in about 19 to 52 %.

The enzyme activity and antagonism of the eight selected *Trichoderma* strains contributed on the degradation of complex substrates, and on the biocontrol of FocR1. Particularly, the strain *T. harzianum* EMV6SIC5 has special relevance for being utilized in biotechnological processes directed to agricultural purposes like the degradation of cellulose-rich organic compounds or the preparation of bioformulations directed for the biocontrol of fungal phytopathogens. Thus, *Trichoderma* species are susceptible for being massively propagated in bioreactors for developing agricultural inoculants.

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