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Original article

Residue decomposition of sugar cane harvest by an autochthonous fungal strain of *Trichocladium pyriforme*

Descomposición del residuo de cosecha de la caña de azúcar por una cepa fúngica autóctona de *Trichocladium pyriforme*

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ABSTRACT: During the green harvesting system of sugarcane, a large amount of agricultural harvest residue is produced that can be left as mulch on the soil, removed from the field or incorporated into the profile, depending on the agroecological characteristics of each area. It is important to rapidly decompose the cover crop residue in areas where it is detrimental to sugarcane production. One of the alternatives to accelerate the natural decomposition of the residue is the use of lignocellulolytic fungi. The objective of this work was to isolate autochthonous fungal strains from sugarcane ARH, select and characterize culturally, morphologically and molecularly those with the greatest potential to accelerate the decomposition of the green harvest residue of the sugarcane field. Five autochthonous fungal strains were isolated from fragments of freshly harvested residue. Cellulolytic and ligninolytic activity was evaluated *in vitro*, using carboxymethylcellulose and guaiacol as substrates, respectively. Strain HR5E3 was the only strain able to decompose cellulose and lignin. This strain was culturally, morphologically and molecularly characterized as *Trichocladium pyriforme* and produced enzymes of the lignin peroxidase group, polyphenol oxidases and laccases. In solid substrate fermentation bioassays, this strain accelerated the decomposition of the residue by di-auxic growth with glucose. *Trichocladium pyriforme* HR5E3 could be used as a bioinoculant capable of degrading lignocellulose, and avoid the detrimental effects that the unaltered cover of the agricultural residue could have on the development of sugarcane.

Key words: biodegradation, fermentation, lignocellulolytic enzymes.

RESUMEN: Durante el sistema de cosecha en verde de la caña de azúcar, se produce una gran cantidad de residuo agrícola de cosecha que puede dejarse como cobertura sobre el suelo, retirarse del campo o incorporarse en el perfil, según las características agroecológicas de cada área. Es importante descomponer rápidamente la cobertura con residuo en zonas donde resulta perjudicial para la producción del cañaveral. Una de las alternativas para acelerar la descomposición natural del residuo es la utilización de hongos lignocelulolíticos. El objetivo de este trabajo fue aislar cepas fúngicas autóctonas, a partir de la conservación del residuo agrícola de cosecha (RAC) de la caña de azúcar, seleccionar y caracterizar en forma cultural, morfológica y molecular aquellas que presenten mayor potencial para acelerar la descomposición del residuo de la cosecha en verde del cañaveral. A partir de fragmentos de residuo recién cosechado, se aislaron cinco cepas fúngicas autóctonas. Se evaluó la actividad celulolítica y ligninolítica *in vitro*, utilizando carboximetilcelulosa y guaiacol, como sustratos, respectivamente. La cepa HR5E3 fue la única capaz de descomponer la celulosa y la lignina. Esta cepa, se caracterizó en forma cultural, morfológica y molecular como *Trichocladium pyriforme* y produjo enzimas del grupo de la lignina peroxidasa, polifenol oxidasas y lacasas. En bioensayos de fermentación en sustrato sólido, dicha cepa aceleró la descomposición del residuo mediante crecimiento diáuxico con glucosa. *Trichocladium pyriforme*, cepa HR5E3 podría utilizarse como un bioinoculante capaz de degradar la lignocelulosa, y evitar los efectos perjudiciales que la cobertura inalterada del residuo agrícola podría tener sobre el desarrollo del cañaveral.

Palabras clave: biodegradación, fermentación, enzimas, lignocelulosa.

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INTRODUCTION

Sugarcane is one of the most important regional crops in northwestern Argentina; its production is concentrated in Tucumán province, with a cultivable area of 276.880 ha for the 2020 harvest (1). In search of improvements in production strategies, Tucumán has implemented sugarcane harvesting without burning, known as green harvesting. This change in the system prompted the study of sugarcane field management through the conservation of the agricultural residue of the harvest (ARH) as a cover crop. In this new production scheme, a significant amount of residue (leaves and trimmings) remains on the soil surface, which, for Tucumán conditions, has been estimated at between 7 and 16 t ha^{-1} of dry matter (2). Different studies have shown that the conservation of ARH on the soil surface provides organic matter and improves its structural stability, enhances moisture conservation, reduces evaporation and improves water infiltration, reduces erosion, allows the recycling of nutrients and reduces weed infestation (2,3). However, the mineralization of sugarcane ARH is a slow process, since it is a residue with a high C N-1 ratio (2), which represents a difficulty in areas with excess moisture due to drainage problems and the presence of a water table close to the surface. In these regions, maintaining the ARH as a cover negatively affects the growth of the sugarcane field (4); for this reason, achieving its rapid decomposition represents a productive advantage. The use of decomposing microorganisms is an alternative to accelerate the natural decomposition of agricultural residues and thus reduce their possible negative effects (5-7). Fungi are the predominant soil organisms responsible for the degradation of lignocellulose present in plant residues (8,9), due to the presence of different enzymatic hydrolytic systems consisting of cellulases and hemicellulases, and an extracellular oxidative ligninolytic system consisting of enzymes such as laccases, phenol oxidases, lignin peroxidases and manganese peroxidases (6,10,11). Based on these considerations, the objective of this work was to isolate autochthonous fungal strains from sugarcane ARH, select and characterize culturally, morphologically and molecularly those with the greatest potential in the decomposition of the residue from the green harvesting of sugarcane.

MATERIALS AND METHODS

Isolation of fungal strains from ARH

ARH of the LCP 85-384 variety was collected from sugarcane fields located in the Department of Simoca, Tucumán province, Argentina. This commercial line is the most cultivated with 76 % of the sugarcane area of the province (12). The ARH was collected in July 2018, immediately after harvest, the samples were transferred in closed plastic bags to the Microbiology laboratory of the Obispo Colombres Agroindustrial Experimental Station (EEAOC) and stored at room temperature (25 ºC). For processing, the residue was cut into fragments of ≈ 2 cm in length, which were washed three times with sterile distilled water, disinfested with 70 % ethanol (v v v-1) for 1 min and then with $NaClO_3$ % (v v v-1) for 3 min. Five successive washes were performed with sterile distilled water to remove traces of the disinfectant. The pieces were dried with sterile absorbent paper and placed in Petri dishes containing potato glucose agar culture medium (APG, Britania). Plates were incubated for 7 to 10 d at 35 °C. Once developed, the fungi were purified by successive plating on malt extract agar (EMA) culture medium (maltose 12.75 g L^{-1}; dextrose 2.75 g L^{-1}; glycerol 2.35 g L^{-1}; bacterial peptone 0.78 g L^{-1}) and the enzyme activities associated with residue decomposition were evaluated.

Determination of enzymatic activities

Ligninolytic activity was evaluated by two different techniques: 1) EMA culture medium with 1 mM guaiacol (Sigma Aldrich, USA) at 0.01 % (p v-1) was used. Plates were inoculated from a solid culture of the fungal strains on EMA, and incubated for 7 d at 35 °C. Ligninolytic activity was assessed by the appearance of a dark brown growth halo (13); 2) alkaline lignin agar medium including 1 mM guaiacol 0.01 % (p v-1) was used. Plates were inoculated from a solid culture of the fungal strains on EMA and incubated under the same conditions as in technique 1. The appearance of a dark brown pigmentation; indicates oxidation of guaiacol in the presence of the enzymes manganese and lignin peroxidases (13). Cellulolytic activity was evaluated in minimal salt medium (MMS) with carboxymethylcellulose (CMC) as the sole carbon source (6,14).

Plates with CMC were inoculated from a solid culture of the fungal strains grown on MMS and incubated for 7 d at 35 °C. Subsequently, plates were stained with a 0.1 % (p v-1) Congo Red (CR) solution for 15 min and washed three times with 1 M NaCl. Cellulolytic activity was determined by the appearance of clear halos around the colonies. The technique consisted in the ability of the CR dye to adhere to the CMC, thus the Petri dish acquires a red color. When the CMC is degraded by the cellulase enzyme complex, the hydrolyzed area acquires a light yellow color indicating that the dye could not adhere to the polymer that has been degraded as a consequence of the cellulolytic activity of the fungus (14). The fungal strain that showed positive ligninolytic and cellulolytic activities was characterized culturally, morphologically and molecularly.

Characterization of the selected fungal strain

Cultural and morphological characterization was performed by micro and macroscopic observations. For molecular characterization, the fungus was plated in Petri dishes with EMA solid culture medium. Prior to inoculation, a disc of sterile cellophane paper was placed on the plates to facilitate the subsequent extraction of the mycelium. The plates were incubated in an oven for 10 d at 35 °C. DNA extraction was performed using the phenol-chloroform technique (15) and electrophoretic analysis was carried out

on a 1 % agarose gel (p v^{-1}). PCR was performed with ITS1/ITS4 primers that are designed in these same internal regions and amplify 600 bp. The amplicons were verified by 1 % agarose gel electrophoresis (p v^{-1}) and sent for sequencing to the Sequencing Laboratory of the National Institute of Agricultural Technology (INTA) Castelar, Buenos Aires, Argentina. The sequences obtained were analyzed with the BLAST (Basic Local Alignment Search Tool) program of NCBI (2020).

Ligninolytic enzyme production profile

Ligninolytic enzyme production was evaluated on solid basal ligninase (MBL) medium (KH_2PO_4 1 g L-1; yeast extract 0.01 g L_{-1}; $C_4H_{12}N_2O_6$ 0.5 g L^{-1}; $CuSO_4$. $5H_2O$ 0.001 g L^{-1}; $MgSO_4.7H_2O$ 0.5 g L^{-1}; $Fe_2(SO_4)_3$ 0.001 g L^{-1}; $CaC_{l2}.2H_2O$ 0.01 g L^{-1}; $MnSO_4.H_2O$ 0.001 g L^{-1}), supplemented with specific substrates, according to the enzyme to be evaluated. The *Pycnoporus* sp. P6 strain with ligninolytic activity was used as a control (16). The solid MBL medium was inoculated with mycelial discs of 0.5 cm in diameter, obtained from the periphery of a fungal colony that developed in EMA medium for 7 d at 35 ºC.

The enzymatic activities evaluated were as follows: 1) Lignin peroxidase (LiP). The MBL medium was supplemented with a solution of Azure B dye at a final concentration of 0.01 % (v v^{-1}). LiP production is evidenced by the appearance of a halo of discoloration around the fungal colony (17); 2) Polyphenoloxidases (POx). MBL medium was supplemented with 0.5 % tannic acid solution (p v^{-1}). The formation of a brown halo around the fungal colony indicates a positive reaction (17); 3) Lacase (Lac). Two methodologies were carried out for their evaluation. The MBL medium was supplemented with the ABTS substrate [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid], at a final concentration of 0.1 % (p v^{-1}). The appearance of dark green halos indicates Lac activity (17). On the other hand, fungal strains were grown on MBL medium without addition of substrates. After incubating the plates for 10 d at 35 ºC, punctures were made with a sterile punch in the peripheral zone of mycelial growth, in which different substrates were added, in order to confirm Lac activity. Syringaldazine (3,5-dimethoxy-4-hydroxybenzaldehyde) 0.5 mM solution, dissolved in dimethyl sulfoxide (DMSO), was added to one of the punctures in the presence and absence of 50 mM phosphate buffer, pH 6. The formation of a pink quinone product indicates a positive reaction (17). In another borehole, 0.1 % (p v-1) ABTS was added, and in another 0.8 g L^{-1} of 2,6- dimethoxyphenol (DMP). In the latter case, Lac activity was demonstrated by the appearance of orange halos, due to oxidation of the substrate (18).

ARH decomposition bioassay

The decomposition of ARH was evaluated by a fermentation assay on solid substrate, performed under controlled conditions (25 °C and 90 % relative humidity). As a substrate, ARH of the LCP 85-384 variety, collected immediately after harvest (July, 2019) and autoclaved (20 min, 121 °C), was used. In 500 mL plastic trays, 4 g of sterilized dry residue were placed and the following treatments were evaluated: 1) ARH inoculated with three 0.5 cm2 fragments of the agarized medium containing fungal mycelium; 2) ARH inoculated with three 0.5 cm2 fragments of the agarized medium including fungal mycelium and 850 µL of 8 % (p v^{-1}) glucose sterilized by filtration; 3) ARH noculated with 5 mL of a suspension of 109 conidia mL^{-1}; 4) ARH inoculated with 5 mL of a suspension of 10^9 conidia mL^{-1} and 850 µL of 8 % (p v^{-1}) glucose sterilized by filtration and 5) ARH uninoculated and moistened with 850 µL of sterile distilled water as a control. Treatments were distributed in a completely randomized experimental design with three replicates per treatment, and five individuals per replicate. At the beginning of the trial (t0) and at 65 days post-inoculation (t65), lignin content was determined using the lignin detergent acid (LDA) technique (19). Data were analyzed by analysis of variance (ANOVA) and the LSD Fisher test with the InfoStat program (Statistical Software, 2010) for Windows.

RESULTS AND DISCUSSION

The use of native fungal strains adapted to the agroecological conditions of sugarcane fields in the region, and with the capacity to decompose the ARH, constitutes one of the most promising alternatives to accelerate the decomposition of the residue and avoid the harmful effects generated by the accumulation of biomass on the soil surface. Five fungal strains with different cultural and morphological characteristics were isolated from freshly harvested RAC of the LCP 85-384 variety. Of these, two showed neither cellulolytic nor ligninolytic activity, three showed cellulolytic activity when incubated in the presence of CMC, and one had both activities (Table 1). This strain (HR5E3) was selected for its ligninolytic and cellulolytic activity for molecular characterization.

The cultural and morphological characterization of strain HR5E3 was carried out in EMA medium. The colony, circular, developed irregular edges with relief, velvety texture and grayish black coloration, due to the abundant production of chlamydospores (Figure 1a) (20). The morphology of the hyphae and spores was observed under the optical microscope (Nikon, model E200). The hyphae, septate, presented rounded swellings close to the septa (Figure 1b). The average size of the conidia was 8-10 × 4-5 µm, with a pyriform or "pear" shape (Figure 1b).

Molecular identification of the fungus was performed by PCR with ITS1/ITS4 primers that amplify a 600 bp fragment of the 16S rDNA gene. Sequencing confirmed that the selected fungal strain HR5E3 corresponds to the species *Trichocladium pyriforme* (20) with 98.75 % identity. The morphological and cultural characteristics of *T. pyriforme* (20) coincided with those observed in these determinations. The presence of this species was previously reported as part of the fungal community associated with decaying

Table 1. Cellulolytic and ligninolytic activities in fungal strains isolated from sugarcane ARH

Fungal strains	Ligninolytic activity	Cellulolytic activity
HR5	ND 	1,1 ± 0,17 cm
HRE2	ND 	0,4 ± 0,0 cm
HR5E3	+ 	0,77 ± 0,15 cm
HR2E2	ND 	ND
HRE1	ND 	ND

ND indicates no enzyme activity detected.

sugarcane leaves (21), in spite of the variations that exist in the microbial populations associated with the tissues of the crop in question (22).

The growth and production of extracellular ligninolytic enzymes by *T. pyriforme* strain HR5E3 was evaluated using various solid culture media containing substrates or indicators that allow direct visualization of enzyme production (Table 2) (16). *Pycnoporus* sp. strain P6, with ligninolytic activity (16), was used as a positive control.

The results showed that the selected fungal strain produced extracellular ligninolytic enzymes from the LiP, Lac, and POx group, which are involved in lignin decomposition (18). However, the production potential for the three ligninolytic enzymes evaluated was lower than that of the control strain. In this work, MBL medium supplemented with tannic acid was used to detect the production of extracellular oxidases (16), despite the fact that only some fungi are able to grow in the presence of this compound, due to its high toxicity (23). In this sense, *T. pyriforme* HR5E3 was not only able to grow in the presence of tannic acid at 0.5% (p v-1), but also showed moderate

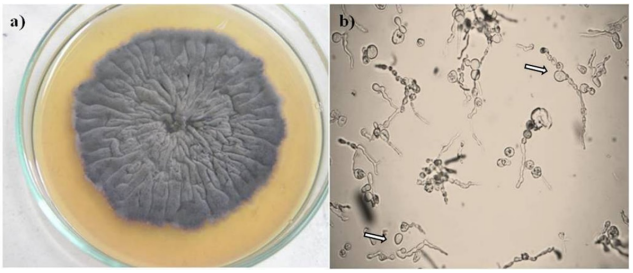

Figure 1. Morphology of Trichocladium pyriforme HR5E3. a) Appearance of the colony from the front with circular edges and grayish coloration; b) Microscopic appearance of hyphae and conidia. Arrows indicate pyriform or "pear-shaped" conidia

POx activity, so it would be interesting to study the possible use of this microorganism, not only to accelerate the decomposition of sugarcane ARH, but also in bioremediation processes of soils contaminated with this group of phenolic compounds (24).

Because Lac has the capacity to differentially oxidize different substrates according to their redox potential (16), the activity of this strain enzyme under study was evaluated in MBL medium supplemented with different substrates: syringaldazine with and without buffer P, DMP, and ABTS. *Pycnoporus* sp. P6 was used as a positive control. The extracellular Lac enzymes produced by *T. pyriforme* HR5E3 have affinity and activity for substrates such as ABTS and DMP, whereas they were not able to oxidize syringaldazine (Table 3). The differences observed between the substrates used could be attributed to the fact that they present chemical groups in different positions, which generates different affinities of Lacs for each compound (11).

The ability of *T. pyriforme* HR5E3 to decompose ARH was evaluated in solid substrate fermentation bioassays under controlled conditions. At the beginning of the trial (t0), the lignin content of the ARH, prior to inoculation, was 16.74 % (p p p-1). As shown in Table 4, at 65 d post-inoculation (t65), the lignin content of the ARH of treatments 2, 3 and 4 was significantly reduced, while no differences were observed in treatment 1, when the inoculation of the ARH was performed only with the mycelium of the fungus.

Table 2. Ligninolytic enzyme activities of *Trichocladium pyriforme* HR5E3 in solid MBL medium

Fungal strains	LiP (sulphur B)	Lac (ABTS)	POx (tannic acid)
T. pyriforme HR5E3	**+ C**	**+ C**	**++ C**
Pycnoporus sp. P6	**+++ C**	**+++ C**	**+++ C**

The number of crosses indicates the intensity of positive reaction [weak (+) to very strong (+++)]; (C) no growth restriction; (LiP) Lignin peroxidase; (Lac) Lacase; (POx) Polyphenoloxidase; (ABTS) [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]

Table 3. Lac activity of *Trichocladium pyriforme* HR5E3 in the presence of different substrates

Fungical strains	Lac (syringaldazine+buffer P)	Lac (syringaldazine+buffer P	Lac (DMP)	Lac (ABTS)
T. pyriforme HR5E3	-	-	++	++
Pycnoporus sp. P6	+	+	+++	+++

The number of crosses indicates the intensity of positive reaction [weak (+) to very strong (+++)]; (C) no growth restriction; (LiP) Lignin peroxidase; (Lac) Lacase; (POx) Polyphenoloxidase; (ABTS) [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]

Table 4. Percentage of lignin in the ARH in the different treatments evaluated

Treatment	Description	Lignin		Lignin reduction (%)
		t0 (% p p^{-1})	t65 (% p p^{-1})	
1	ARH inoculated with mycelium	16.74 a	15.27 a	8.78
2	ARH inoculated with mycelium + glucose 8 %.	16.74 a	11.05 b	33.99
3	ARH inoculated with conidia suspension	16.74 a	12.06 b	27.95
4	ARH inoculated with conidia suspension + glucose 8 %.	16.74 a	7.98 b	52.33
5	ARH without inoculation	16.74 a	16.74 a	0

(t0) initial time; (t65) indicates 65 d postinoculation. Different letters, per row, indicate significant differences (LSD test, Fisher) with p≤ 0.05

As expected, the lignin content of the uninoculated ARH (treatment 5) remained constant until the end of the trial.

Inoculation with conidia on the ARH reduced the lignin content by 27.95 % with respect to the initial value, probably due to the increase in the contact surface between the fungus and the residue. In the two treatments supplemented with 8 % glucose (p v-1), the final lignin content of the ARH was lower with respect to the control and lower with respect to the same treatment without the addition of glucose (Table 4). The decrease in lignin content, when the fermentation was carried out by inoculating the ARH with an agarized mycelium fragment supplemented with glucose, was 33.99 %, in relation to the initial lignin content; whereas, when it was carried out by inoculation with conidia and supplemented with glucose, the greatest reduction in lignin content was reached with a value of 52.33 % (Table 4). This would indicate that fungal decomposition of lignin by strain *T. pyriforme* HR5E3 is carried out with a di-auxic growth kinetics, as two different carbon (C) sources are present. It was reported that a critical factor for decomposing sugarcane harvest residue using fungal strains is the addition of a readily assimilable C source for the fungi to initiate biomass development (25). According to what was observed in this work, and in relation to what was reported by Chu and Barnes (26), in a first stage the fungus grows at the expense of glucose and, once this source of C is exhausted, it continues its growth by degrading lignin.

The capacity of various species of *Trichocladium* to decompose lignocellulolytic residues was reported (27,28), when these fungi were cultivated on sugarcane bagasse and sawdust, the degradation of lignocellulosic material was promoted. Some species of the genus in question produce endo- and exoglucanases, xylanases and β-glucosidases capable of decomposing cellulose under aerobic, microaerophilic and anaerobic conditions. This capacity, together with the production of Lacs, both in aerobic and microaerophilic conditions, gives this group of fungi great versatility to digest the structural polysaccharides of the cell wall of plants, regardless of the level of oxygen found in the environment, giving them a competitive advantage in natural environments over other decomposing microorganisms (27).

If studies on the molecular basis associated with the production of these biotechnologically relevant enzymes are deepened, the strain *T. pyriforme* HR5E3 could be used on a large scale to promote the rapid release of fermentable sugars from RAC for subsequent transformation into alcohol or other compounds. In this way, it would be possible to take advantage of a crop residue that is produced in large quantities, converting it into raw material for obtaining compounds of interest, and achieve its revaluation.

CONCLUSIONS

- Strain HR5E3, identified as *Trichocladium pyriforme* and isolated from ARH of LCP 85-384 sugarcane in Tucumán (Argentina), showed cellulolytic and ligninolytic activity *in vitro*, and accelerated the decomposition of ARH in solid substrate fermentation tests, by diauxic growth, in the presence of glucose.
- The selected strain could be used as a potential bioinoculant to accelerate the decomposition of ARH that remains as a mulch on the soil surface after green harvesting of sugarcane. This would contribute to avoid the detrimental effects that, in some areas, the maintenance of the unaltered ARH cover could have on the growth and development of the sugarcane field.

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