Capacidades antagónicas de cepas Trichoderma y su multiplicación en masa usando desechos agrícolas

Antagonistic capacities of Trichoderma species and their mass multiplication with agricultural wastes

Marcela A. Sadañoski, Jimena Gutierrez-Brower, María L. Castrillo, Ana C. López, Paola A. Ojeda, Pedro D. Zapata, Laura L. Villalba, Mónica B. Otegui

Laboratorio de Biotecnología Molecular, Instituto de Biotecnología Misiones, CONICET, Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones, CP3300, Posadas, Misiones, Argentina.

* E-mail: biotecmol2010@gmail.com

Resumen

El objetivo de esta investigación fue aislar y caracterizar cepas de Trichoderma nativas de Misiones (Argentina) explorando sus capacidades antagónicas y su multiplicación masiva utilizando diferentes residuos agroindustriales. Quince cepas nativas de Trichoderma spp. fueron aisladas de muestras de suelo. Estos aislamientos se caracterizaron mediante observaciones morfológicas y moleculares basados en secuencias de ADN de la región espaciadora transcrita interna del ADNr. Las cepas de Trichoderma spp. fueron identificadas como T. koningiopsis, T. harzianum, T. pleuroticola y T. brevicompactum. Estas cepas mostraron actividades antagónicas in vitro contra Alternaria sp., Fusarium sp. y Botrytis sp. T. koningiopsis LBM 090, LBM 091, LBM 092 y LBM 098, T. pleuroticola LBM 097 y T. harzianum LBM 096 presentaron una inhibición del crecimiento micelial mayor del 50% y un índice de antagonismo entre 3 y 4 contra los fitopatógenos ensayados. La cáscara de arroz y el pulido del arroz fueron las combinaciones más adecuadas para la multiplicación de T. harzianum LBM 096.

Palabras clave: Agentes de control biológico; Suelo; Hongos fitopatógenos; Cáscara de arroz; Pulido de arroz.

Abstract

The aim of this research was to isolate and characterize Trichoderma native strains from Misiones (Argentina) exploring their antagonistic capacities to phytopatogens fungi and their mass multiplication using different agricultural wastes. Fifteen native strains of Trichoderma spp. were isolated from soil samples. These isolates were characterized via morphological observations and molecular phylogenetic analysis based on DNA sequences of the rDNA internal transcribed spacer region. The Trichoderma native strains were identified as T. koningiopsis, T. harzianum, T. pleuroticola and T. brevicompactum. All strains showed antagonistic activities in vitro against Alternaria sp., Fusarium sp. and Botrytis sp. T. koningiopsis LBM 090, LBM 091, LBM 092 and LBM 098, T. pleuroticola LBM 097 and T. harzianum LBM 096 presented radial mycelial growth inhibition higher than 50% and antagonism index between 3 and 4 against the phytopathogens assayed. Among the different substrate sources evaluated, rice husk and rice polishing were the most suitable combination for mass multiplication of T. harzianum LBM 096.

Keywords: Biological control agent; Soil; Phytopatogens fungi; Rice husk; Rice polishing.

INTRODUCTION

The genus Trichoderma comprises a great number of fungal strains that act as biological control agents (BCAs), which antagonistic properties are based on the activation of multiple mechanisms [1, 2, 3]. Antagonists of phytopathogenic fungi have been used to control plant diseases, and 90% of such applications were carried out with different strains of the genus Trichoderma [3]. These properties made Trichoderma a ubiquitous genus present in any habitat and with high population densities. Trichoderma strains are always, or frequently associated with plant roots and root ecosystems [4]. Some authors have defined Trichoderma strains as plant symbiotic opportunist avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms [5].

It is suitable to know the Trichoderma genus when it is introduced as a biocontrol agent into the rhizosphere of a given ecosystem for both control efficiency and environmental conservation reasons [6]. Therefore, it should be
considered morphological and molecular data from DNA sequencing to identify and characterize *Trichoderma* spp. [7]. One of the most reliable analyses for identifying a strain at the species level is the internal transcribed spacer (ITS) region [8].

Some of the phytopathogens that affect regional horticulture and plantation crops of Misiones province species are *Fusarium* sp., *Alternaria* sp. and *Botritis* sp. *Fusarium* sp. is reported as a soil pathogen that affects yerba mate nurseries [9]. *Alternaria* sp. and *Botritis* sp. are responsible for diseases in ornamental flowers and citrus plants [10, 11].

One of the widely used approaches to improve the agriculture production is the application of chemical pesticides. However, the degradation in the environment of such compounds is very difficult and the concentration and/or accumulation of them in food chains lead to high toxicity levels in animals [12]. Nevertheless, intensive agriculture needs continuous research for the development of new fertilizers and plagues controllers that care about environmental pollution and human health hazards. Biological methods would be a preferable alternative for controlling regional plant diseases using endogenous and domestic microorganisms. The potential of *Trichoderma* strains as control plant pathogens embodies an economically attractive choice [13, 14, and 15]. In recent years, the search for *Trichoderma* isolates with a high antagonistic potential has increased [5, 2]. The application of strains with biological control capacities can help to reduce the input of chemical pesticides in agriculture, where it is necessary to introduce high levels of spores to the field. Therefore, agro industrial wastes can provide and economical and suitable source of substrates to produce significant amount of spores of *Trichoderma* [16, 17].

The primary aim of the present study was to isolate *Trichoderma* strains from soil samples of ecosystems without human impact in Misiones (Argentina). The native *Trichoderma* isolates were characterized by morphological observation and quick molecular identification. The strains were evaluated for their antagonist capacity against *Fusarium* sp., *Alternaria* spp. (strains 1 and 2), and *Botrytis* sp., which are phytopathogens that affect agriculture crops. Finally, agricultural wastes were assessed for their suitability as substrates for *Trichoderma* mass multiplication pursuing an economically and effective production methodology.

**MATERIALS AND METHODS**

**Plant pathogens**

*Fusarium* sp., *Alternaria* spp. (strains 1 and 2) and *Botrytis* sp., isolated from Argentina, were kindly provided by the Culture Collection of the Buenos Aires University (BAFC). These phytopathogens are the main responsible for plant diseases.

**Soil sampling and fungal isolation**

Soil samples were collected from "Ñacanguazu" creek in Gobernador Roca (GPS coordinates 27°06' S; 55°22' W), “Teyú Cuaré” Provincial Park in San Ignacio (GPS coordinates 27°16' S; 55°35' W) and “Profundidad” Provincial Park in Candelaria (GPS coordinates 27°33' S; 55°42' W), all in Misiones, Argentina. The soil samples were collected at 15 cm of depth in an area of 1 m² with 5 repetitions. The temperature and humidity of each collected place were recorded. The samples were immediately transported to the laboratory for pH and water content determination. The samples were diluted in sterile distilled water (1:1000 and 1:10000) and cultured using potato dextrose agar (PDA 3.9%), pH 6.5. After 10 days of incubation at 27±1°C, single colonies with morphology characteristic of *Trichoderma* sp. were selected for sub-culture and for further morphological and molecular identification. All strains were stored at -80°C in their respective liquid media with 15% glycerol.

**DNA extraction and quick molecular identification**

Fungal genomic DNA was extracted following a modified method described by Doyle [18]. Mycelia for DNA extraction were grown in liquid cultures at 27±1°C in malt extract broth (MEB) for 3-5 days in the dark. Mycelia was filtered and was washed with 0.1 M Tris-HCl (pH 8) and 0.02 M EDTA (pH 8). DNA extraction was carried out with buffer solution (0.1 M Tris-HCl pH 8, 1.5 M NaCl, 0.05 M EDTA pH 8) at 60°C, containing 0.1 mg/ml Proteinase K, 0.01 M β-mercaptoethanol and 2% SDS. DNA was purified with chloroform:isoamilyc alcohol (24:1) and 3M potassium acetate and then was precipitated with isopropyl alcohol. Pure DNAs extracted from the isolates were amplified at the ITS region and sequenced for phylogenetic analyses.

ITS1-5.8S-ITS2 region sequences of the ribosomal gene were used for fungi classification as described previously [19]. For the amplification, reaction primers ITS1 5’ TTCGTAGGTGAACCTGCGG and ITS4 5’ TCCTCCGCTTATTGATATGC [20] were used. The amplification reactions were prepared in a final volume of 20 μL containing 1X KCl buffer, 2.5 mM MgCl₂, 200 μM dNTPs for each 10 pmol primer [20], 0.5 U of Taq polymerase and template 5 ng/μl DNA. PCR amplification conditions were 94°C for 40 s, 50°C for 40 s and 72°C for 40 s for 35 cycles, with 10 min extension at 72°C used for the final cycle. Agarose gels at 1% and 2% were carried out to visualize genomic DNA and PCR products, respectively. The purification and sequencing procedures were performed by Macrogen Korea.

The ITS gene sequences of the isolated strains were compared with those deposited in the National Center for Biotechnology Information Database (NCBI, www.ncbi.nlm.nih.gov) with the special alignment search tool TrichoKey 2.0 and TrichoBLAST 2.0 to confirm *Trichoderma*
species [19] and with Database Fungal barcoding (www.fungalbarcoding.org/). The isolates had significant hits to the genus that owed the lowest e-value in the results of BLAST. Alignments of nucleotide sequences were carried out with the Clustal W software [21]. To allow an appropriate phylogenetic analyses, sequences of another strains of Trichoderma were included in the present study (accession numbers: JQ040311, EU280132, EU280136, EU280105, KP898755, FJ430784, JX125615, HQ596981, KF294838, KJ767092, KC478546, HQ637329, HQ260623, EU280095, DQ200259, HM461859, KC582841, FJ459964, JN943376, JN943375, JN943374, AY737767, FJ860752, AF275322, YJ737762, HM142362, GU934533, JX069200, JX069201, EU280087, KC884785, KC561076, JX908732). The parsimony analyses were performed with Bootstrap methods [22] included 1000 replications. In the analyses, Hypomyces subiculosus (EU280093) were used as an out-group.

Fungal inhibition assays

Nine cm Petri plates containing potato dextrose agar (PDA) were inoculated with 7 mm mycelial discs of either Fusarium sp., Alternaria sp. or Botrytis sp., and Trichoderma isolates 10 mm away from the edge of the plate opposite to each other. Plates inoculated with the Trichoderma strains and pathogens strains alone served as control. Plates were incubated at 27±1°C for Alternaria sp. and Fusarium sp. and 14±1°C for Botrytis sp. Three replicate plates were done for each treatment. The radial growth was measured progressively. The efficacy of the antagonist in inhibiting the pathogen growth was evaluated quantitatively by the inhibition grade formula. The percentage of growth inhibition was calculated using the equation RI=100 x (R2 - R1)/R2 when the contact started, where RI was the percentage of reduction in mycelial growth, R1 was the averaged growth of pathogen in treated plates and R2 was the averaged growth of pathogen in control plates [23]. An effective antagonist strain is capable of inhibiting 50% or more the pathogen growth. The antagonism index was calculated after ten days of the starting day with Bell scale modified by Calistru [24, 25]: 4 corresponded to 100% Trichoderma sp. coverage on the phytopathogen and 0 corresponded to 100% phytopathogen coverage over Trichoderma sp. Three replicate plates were done for each treatment. The radial growth was measured progressively. The efficacy of the antagonist in inhibiting the pathogen growth was evaluated quantitatively by the inhibition grade formula. The percentage of growth inhibition was calculated using the equation RI=100 x (R2 - R1)/R2 when the contact started, where RI was the percentage of reduction in mycelial growth, R1 was the averaged growth of pathogen in treated plates and R2 was the averaged growth of pathogen in control plates [23]. An effective antagonist strain is capable of inhibiting 50% or more the pathogen growth. The antagonism index was calculated after ten days of the starting day with Bell scale modified by Calistru [24, 25]: 4 corresponded to 100% Trichoderma sp. coverage on the phytopathogen and 0 corresponded to 100% phytopathogen coverage over Trichoderma strains. An index of 3 or 4 it is considered an effective antagonist strain.

Culture substrates of Trichoderma strains for mass production

The best sporulation condition of T. harzianum LBM 096 using agro industrial wastes as solid substrate for microbial cultivation was assayed. Rice was used as control. The substrates consisted of sawdust, rice polishings, rice flour and rice husk with different moisture content depending on the treatment (Table 1). As primary inoculum the strain was grown on 2 g/ml rice substrate. Fermentation assays were carried out without external nutrient addition. All substrates were autoclaved for 15 min at 121°C, inoculated with 3x10⁸ spores and incubated at 27±1°C in natural light for 15 days. Spores were removed by washing with distilled water containing 0.5% Tween 20 and were counted following the Neubauer method.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Substrate</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40 g</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20 g</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>20 g</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>20 g</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>10 g</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>10 g</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>40 g</td>
</tr>
</tbody>
</table>

Table 1: Media formulations with different local agro industrial wastes.

Statistical analysis

The data obtained in all experiments were subjected to analysis of variance (ANOVA) with Turkey’s test using the SPSS software, version 22.0 (IBM Corp, Armonk, NY, USA), at a significance level of 5%.

RESULTS

Soil sampling and fungal isolation

Out of 20 colonies per plate obtained from soil samples, 15 fungal isolates were selected as probable Trichoderma strains (Table 2). Aerial mycelium of the colonies was initially thick and whitish. When the sporulation started, the colony turned green and the periphery of the colonies remained white. At a microscopical level, we identified phialides with a flask-shaped formed at wide angles to the conidiophore, and with conidia clustered together at the end of each phialide.

<table>
<thead>
<tr>
<th>Soil Sample</th>
<th>Location</th>
<th>Isolate</th>
<th>RH %</th>
<th>T °C</th>
<th>pH</th>
<th>WC %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nancanguazu stream</td>
<td>LBM 090</td>
<td>70</td>
<td>17.8</td>
<td>7.60</td>
<td>36.4</td>
</tr>
<tr>
<td>2</td>
<td>Nancanguazu stream</td>
<td>LBM 091</td>
<td>70</td>
<td>17.1</td>
<td>7.28</td>
<td>44.6</td>
</tr>
<tr>
<td>3</td>
<td>TeyúCuaré</td>
<td>LBM 103</td>
<td>65</td>
<td>17.2</td>
<td>7.86</td>
<td>45.8</td>
</tr>
<tr>
<td>4</td>
<td>TeyúCuaré</td>
<td>LBM 104</td>
<td>66</td>
<td>16.5</td>
<td>7.56</td>
<td>37.8</td>
</tr>
<tr>
<td>5</td>
<td>Profundidad</td>
<td>LBM 095</td>
<td>55</td>
<td>15.0</td>
<td>7.60</td>
<td>36.9</td>
</tr>
</tbody>
</table>

Table 2: Locations and soil conditions of fungal isolates

Note: RH: relative humidity, WC: water content.
DNA extraction and quick molecular identification

We were able to accurately classify at genus level the isolated *Trichoderma* strains by applying morphological criteria.

The ITS1-5.8S-ITS2 gene sequences of the 15 isolates were compared with those sequences deposited in the three database utilized, indicating that they had significant hits to the genera *Trichoderma*. Seven isolates were identified as *Trichoderma harzianum* (LBM094, accession number of NCBI: JX069200, LBM096, accession number of NCBI: JX069196, LBM097, accession number of NCBI: JX069197, LBM100, accession number of NCBI: JX069198, LBM101, accession number of NCBI: JX069199, LBM103, accession number of NCBI: JX069201, LBM104, accession number of NCBI: JX069195), six as *Trichoderma koningiopsis* (LBM090, accession number of NCBI: JX069205, LBM091, accession number of NCBI: JX069206, LBM092, accession number of NCBI: JX069207, LBM098, accession number of NCBI: JX069202, LBM099, accession number of NCBI: JX069203, LBM102, accession number of NCBI: JX069204), one as *Trichoderma brevicompactum* (LBM095, accession number of NCBI: JX069193), and one as *Trichoderma pleuroticola* (LBM093, accession number of NCBI: JX069194). All strains, in all database consulted, had 97% to 100% of similarity with the reference sequence of each species.

A phylogenetic tree using the fifteen *Trichoderma* spp. isolated ITS sequences [19] was constructed based on parsimony analyses (Figure 1).

The phylogenetic analysis revealed close positioning of seven isolates of *T. harzianum* (LBM094, LBM096, LBM097, LBM100, LBM101, LBM103, LBM104) and *T. pleuroticola* LBM093 in a closely related group, in concordance with actual taxonomic classification of clade Harzianum, Pachybasium Section (accessible at http://www.isth.info/biodiversity/index.php). The six isolates corresponding to *T. koningiopsis* (LBM090, LBM091, LBM092, LBM098, LBM099, LBM102) revealed close related positioning in the same group, in *Trichoderma* Section. *T. koningiopsis* LBM091 and LBM092 corresponded to the same strain because they were no genetic distance between them and were isolated from the same place (Nancanguaçu creek). Finally, *T. brevicompactum* LBM 095 showed high genetic distance to the other strains and belongs to Lutea clade, Lone Lineages Section (Figure 1).

Figure 1: Parsimony analysis based on ITS sequences from all isolates of Trichoderma. Group support with 1000 Bootstrap.
Fungal inhibition assays

On dual confronted cultures, Trichoderma strains reached Fusarium sp. and Alternaria sp. (strain 1) in 3 days, Alternaria sp. (strain 2) in 4 days and Botrytis sp. in 10 days (Table 3). The strains Ta koningiopsis LBM 090 and LBM 091, LBM 098, LBM 092, T. pleuroticola LBM 097, T. harzianum LBM 096 and Trichoderma koningiopsis presented %RI higher than 50% indicating that strains are effective antagonists. Among the isolates, T. koningiopsis LBM 090 and T. koningiopsis LBM 098 showed the highest percentage of growth inhibition (60-70%) against Botrytis sp. (Table 3).

From all strains studied, inhibition by the Trichoderma strains in dual cultures did not show statistically significant differences with Alternaria sp. (strain 1), Alternaria sp. (strain 2) and Fusarium sp. In contrast, Trichoderma strains showed significant differences with Botrytis sp. Trichoderma koningiopsis LBM090 showed the best %RI in dual confronted assays towards the four phytopathogens strains studied (Table 3). Based on the result obtained with Bell scale modified, all strains resulted effective antagonists with a value of 3 and 4 in this scale indicating 100% coverage of the pathogen colonies. Trichoderma koningiopsis LBM090, LBM091 and LBM098 presented a value of 4 to all strains of pathogens. These strains exerted antagonist and parasitism capacities against regional phytopathogens and would be potential biological controls.

Table 3: Mycelial growth inhibition percentage (%RI) of Trichoderma strains versus phytopathogenic fungal strains.

<table>
<thead>
<tr>
<th></th>
<th>Alternaria sp.</th>
<th>Alternaria sp.</th>
<th>Fusarium sp.</th>
<th>Botrytis sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(strain 1)</td>
<td>(strain 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%RI on day 3</td>
<td>%RI on day 4</td>
<td>%RI on day 3</td>
<td>%RI on day 10</td>
<td></td>
</tr>
<tr>
<td>T. koningiopsis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBM 090</td>
<td>56.51 ± 5.50a</td>
<td>39.73 ± 5.48a</td>
<td>51.44 ± 1.87a</td>
<td>63.11 ± 5.99a</td>
</tr>
<tr>
<td>T. koningiopsis</td>
<td>28.57 ± 4.76a</td>
<td>15.98 ± 3.16a</td>
<td>46.63 ± 5.46a</td>
<td>49.67 ± 1.65ab</td>
</tr>
<tr>
<td>LBM 092</td>
<td>28.57 ± 4.76a</td>
<td>28.77 ± 5.48a</td>
<td>46.63 ± 1.52a</td>
<td>45.19 ± 7.00ab</td>
</tr>
<tr>
<td>T. koningiopsis</td>
<td>33.33 ± 9.52a</td>
<td>34.25 ± 7.25a</td>
<td>51.44 ± 5.72a</td>
<td>58.93 ± 5.74ab</td>
</tr>
<tr>
<td>LBM 091</td>
<td>25.40 ± 5.50a</td>
<td>30.60 ± 6.33a</td>
<td>41.38 ± 5.46a</td>
<td>43.08 ± 2.74ab</td>
</tr>
<tr>
<td>T. koningiopsis</td>
<td>16.66 ± 3.37a</td>
<td>30.59 ± 4.18a</td>
<td>45.76 ± 1.52a</td>
<td>36.76 ± 3.16ab</td>
</tr>
<tr>
<td>LBM 093</td>
<td>17.86 ± 1.69a</td>
<td>23.29 ± 5.48a</td>
<td>39.63 ± 6.94a</td>
<td>nd</td>
</tr>
<tr>
<td>T. harzianum</td>
<td>30.16 ± 7.27a</td>
<td>26.94 ± 8.37a</td>
<td>45.76 ± 6.06a</td>
<td>45.19 ± 2.41ab</td>
</tr>
<tr>
<td>LBM 094</td>
<td>30.16 ± 7.27a</td>
<td>32.74 ± 7.85a</td>
<td>42.26 ± 10.50a</td>
<td>45.19 ± 5.30ab</td>
</tr>
<tr>
<td>T. harzianum</td>
<td>18.25 ± 3.64a</td>
<td>28.77 ± 5.48a</td>
<td>40.51 ± 1.52a</td>
<td>37.55 ± 1.12ab</td>
</tr>
<tr>
<td>LBM 096</td>
<td>20.63 ± 2.75a</td>
<td>24.20 ± 6.90a</td>
<td>40.07 ± 4.97a</td>
<td>32.02 ± 7.91ab</td>
</tr>
<tr>
<td>T. harzianum</td>
<td>25.40 ± 5.50a</td>
<td>23.30 ± 0.01a</td>
<td>43.57 ± 3.47a</td>
<td>42.55 ± 1.82ab</td>
</tr>
<tr>
<td>LBM 097</td>
<td>26.42 ± 3.55a</td>
<td>26.30 ± 1.51a</td>
<td>42.67 ± 4.74a</td>
<td>39.65 ± 3.21ab</td>
</tr>
<tr>
<td>T. harzianum</td>
<td>29.36 ± 3.64a</td>
<td>25.11 ± 6.33a</td>
<td>37.88 ± 1.52a</td>
<td>39.92 ± 5.47ab</td>
</tr>
<tr>
<td>LBM 098</td>
<td>25.40 ± 2.75a</td>
<td>23.29 ± 7.75a</td>
<td>43.13 ± 1.52a</td>
<td>48.88 ± 7.80ab</td>
</tr>
<tr>
<td>T. pleuroticola</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBM 093</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Means followed by the same letter in each column do not differ significantly by Tukey test at 5% probability. nd: not determined.

Culture substrates of Trichoderma strains for mass production

The suitable condition for mass multiplication of T. harzianum LBM096 was evaluated. Among the substrates evaluated, the mix of rice husk and rice polishing yielded the highest sporulation (1.96x10^10 spores/g substrate) followed by rice husk and rice flour (1.45x10^10 spores/g substrate), sawdust and rice polishing (1.12x10^10 spores/g substrate) and sawdust and rice flour (6.4x10^9 spores/g substrate). Results indicated that the best spore production was found in substrates with rice flour and rice polishing. Significantly lower spore concentration was recorded in 1, 3, 4 and control treatments (Figure 2).

Figure 2: Evaluation of substrates for mass production. Identical number means no statistically significant differences between these treatments (P > 0.05). 1: Sawdust; 2: Sawdust and Rice polishings; 3: Sawdust and Rice flour; 4: Rice husk; 5: Rice polishings and Rice husk; 6: Rice flour and Rice husk; 7: Rice.

DISCUSSION

The fifteen Trichoderma strains were isolated from low human impacted ecosystems because the expected diversity, as it is documented by other authors [26, 27, 28].

The correct identification at species level is highly desirable because some of them can be possible risks to human health [29]. Systematic studies of microorganisms were based almost exclusively on morphological criteria (classical microbiological classification). Currently, the tools used in systematic studies have increased, and not only the morphological criteria, but also molecular criteria are considered. However, the number of tools used in systematic studies has increased. Nowadays, both morphological and molecular criteria need to be taken into account [30, 31]. Since the primary aim of the present study was to isolate Trichoderma strains from soil samples from low human impacted ecosystems, in this work it was done a rough molecular identification of the Trichoderma strains using only ITS Barcodes. We considered the classical microbiological classification by morphological criteria and
molecular techniques by amplification of ITS1, 5.8S and ITS2 regions. The ITS1-5.8S-ITS2-28S regions can have nucleotide variations since their transcripts are excised from the final rRNA fragments. Therefore, the ITS sequence includes both ITS1 and ITS2, which are separated by the conserved short 5.8S rRNA. They are usually used to infer phylogenetic relationships of closely related species as well as to assess the variability of a population, e.g. of geographically distant isolate (ecotypes) and recommended by Druzhinina et al. [19] as Hypocre/Trichoderma barcode. These regions are appropriate for detecting differences between conspecific individuals and hence, are potentially useful markers to study the relationships of populations and closely related species in fungal, plant, and animal taxa due to their relatively rapid evolutionary rates [32, 33]. The ITS was chosen as the official barcode for fungi by a consortium of mycologists and it is among the markers with highest probability of correct identification for a very broad group of sampled fungi [34, 35]. Recently, there are many researches that used tef-1 and RPB2 as complementary barcodes for Trichoderma species identifications [36].

We isolated 15 native strains of Trichoderma genus from Misiones, Argentina to study their antagonist capacity. T. koningiopsis LBM 090 was the most promissory strain; however, T. koningiopsis LBM 090, LBM 091 and LBM 098 showed some interesting aspects as potential biological control agents. Two strains of T. koningiopsis (LBM090 and LB098) showed the best results in the antagonism tests. However, using qualitative analyses, all strains were capable to reduce and invade more than 75% of the phytopatogen fungi growth after ten days. These results indicate the potential of antagonistic strain to reduce diseases in field conditions. Larralde-Corona et al. [37], selected new T. koningiopsis strains as biological control agents to sorghum phytopathogens. There are other reports showing the inhibitory effect of other Trichoderma species on the same phytopathogens used in this work. T. harzianum on Alternaria alternata, for tobacco [1]; T. harzianum on Fusarium oxysporum with similar %RI [38]; T. harzianum on Fusarium solani [39]; T. hamatum and T. atroviride for Pinus radiata [40], Trichoderma asperellum strain CCTCC-RW0014 showed to have good biocontrol potential with disease reduction of 71.67% against Fusarium oxysporum f. sp. cucumerinum [41]. Pugliese et al. [42] selected antagonistic fungi from compost as a promising strategy for the development of new biological control agents against soil-borne pathogens.

One important aspect to biotechnological prospection is a high production of spores in an adequate substrate. Forestry is one of the principal activities of Misiones province, Several candidate strains were identified to act against Alternaria sp., Fusarium sp. and Botrytis sp. The formulation of rice husk and rice polishing resulted as the best suitable combination of substrates to increase the mass production. Our results are the starting point for future studies on the utilization of native strains with antagonist properties in our region. It is still needed to study deeply the biological control mechanisms in field experiments with these strains.

Acknowledgements

The authors thank the Biofábrica de Misiones SA (BIOMISA). Part of the experimental work was funded by BIOMISA. JGB had a fellowship for postgraduate studies of CEDIT, Misiones, Argentina. MAS, MLC and ACL has a fellowship of CONICET.

REFERENCES

9. Pérez, ML; Collavino, MM; Sansberro, PA; Mroginski, LA; Galdeano,


28. Adetutu, EM; Thorpe, K; Bourne, S; Cao, X; Shahsavari, E; Kirby, G; Ball, AS. Phylogenetic diversity of fungal communities in areas accessible and not accessible to tourists in Naracoorte Caves. Mycologia. 103: p. 959-968. 2011.

29. Fontenelle, ADB; Guzzo, SD; Lucon, CMM; Harakava, R. Growth promotion and induction of resistance in tomato plant against Xanthomonas euvesicatoria and Alternaria solani by Trichoderma spp. Crop Prot. 30: p. 1492-1500. 2011

30. Pérez Valencia, L; Santerre, A; Villalobos Arámbula, AR; Galván Corona, A; Torres-Torres, MG; Rodríguez Contreras, A; Guzmán Dávalos, L. Extracción de DNA y amplificación de secuencias del ITS del DNAr de Ganoderma (Fungi, Basidiomycetes) para su uso en el análisis filogenético. CUCBA. 8. 2005.


37. Larralde-Corona, CP; Santiago-Mena, MR; Sifuentes-Rincon, AM; Rodríguez-Luna, IC; Rodríguez-Perez, MA; Shirai, K; Narvaez-Zapata, JA. Biocatalyst potential and polyphasic characteriz


41. Saravana Kumar, K; Yu, C; Dou, K; Wang, M; Li, Y; Chen, J. Synergistic effect of Trichoderma-derived antifungal metabolites and cell wall degrading enzymes on enhanced biocontrol of Fusarium oxysporum f. sp. cucumerinum. Biol Control. 94: p. 37-46. 2016.


Recibido: 05/12/17.
Aprobado: 24/04/18.