High tolerant and degrader *Actinomucor elegans* to fungicides isolated from contaminated soils

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High tolerance and degradation of fungicides by fungal strains isolated from contaminated soils

The aim of this work was to isolate fungal strains from phytotoxic agricultural soils and study their bioremediation potential by degrading three fungicides. In this study, 28 fungal strains were isolated from phytotoxic agricultural soil with intensive use of pesticides. An exploratory multivariate analysis of degradation experiments by the fungal strains showed the capacity of fungi to resist and degrade different concentrations of carbendazim, captan and zineb. Actinomucor elegans LBM 239 were identified as the most tolerant fungi to these pollutants. *A. elegans* LBM 239 removed 63.8% of the carbendazim in the culture medium after 7 days of treatment. In conclusions, we found two fungal strains able to tolerate and biodegrade the three fungicides studied in this work, particularly, the carbendazim. The capability of these fungi, *A. elegans* LBM 239, to biodegrade high doses of fungicides make them suitable for bioremediation of contaminated soils with carbendazim, captan or zineb.

Keywords: *Actinomucor elegans*; mycoremediation, phytotoxic soil, biodegradation

Introduction

Microorganisms play an important role in the decomposition and nutrient cycling in the soil. The addition of pesticides to the soil produces different effects on microorganisms that differ according to each species. Some microorganisms can be intoxicated while others are tolerant and may increase their biomass due to decreased competition (Eman et.al. 2013).

The resistance mechanism of some fungi to chemical fungicides is due to genetic mutations, which reduces the susceptibility to the fungicides and decreases their efficacy (Chaparro et.al. 2011). From the microorganisms of the soil, fungi are among the main agents of transformation and degradation of agrochemicals. These transform

pesticides and other xenobiotics through the generation of small structural changes that favor subsequent biodegradation by bacteria (Gianfreda and Rao 2004; Ortiz-Hernández 2013).

Bioassays are useful tools for detecting the toxicity of various compounds, using living organisms and biological processes to measure the short and long-term effects of exposure to chemicals (Foti et al. 2005; Wieczerzak et al. 2016).

The phytotoxicity of a substance can be determined by evaluating the germination of seeds and the development of seedlings, since these stages are highly sensitive to external adverse factors (Sobrero 2010). Regarding the technique used, various methodologies considered as diagnostic criteria have been proposed to determine the effect and tolerance of a contaminant on a particular species. Within standardized methodologies, *Lactuca sativa* (lettuce) seeds have been recommended among the most sensitive species for phytotoxicity tests (USEPA 1996; IRAM 2008) and were applied by different environmental protection organisms for the ecotoxicological evaluation of environmental samples. At the end of the tests, the germination of the seeds and the development of the seedlings during the first days of growth are evaluated. The inhibition of elongation in the radicle and hypocotyl allows to observe the toxic effect of soluble compounds present at even low concentration levels that are not sufficient to inhibit germination. In this way, they are constituted as very sensitive sublethal indicators for the evaluation of biological effects in vegetables, providing complementary information (Pentreath et.al. 2015; Hernández-Valencia 2017). In this regard, various authors have used this methodology (Tiquia 2000; Emino and Warman 2004; Varnero et.al. 2007; Charles et.al. 2011; Mañas et.al. 2018) and consider that this Germination Index (GI) is the most complete indicator to describe the phytotoxic potential of an organic material.

Carbendazim is a systemic fungicide having wide applications for controlling fungal diseases in agriculture, forestry and veterinary medicines. Carbendazim is a major pollutant detectable in food, soil and water. Carbendazim extensive and repeated use induces acute and delayed toxic effects on humans, invertebrates, aquatic life forms and soil microorganisms (Goodson et al. 2015; Singh et.al. 2016; Panda et.al. 2017).

Zineb (Zinc ethylene-bis dithiocarbamate) is a contact and preventive fungicide, hence it is important to do the treatments before diseases appear. It has a broad spectrum of control, formulated based on the zinc ions. They react in a general way with -SH groups, inhibit enzymes, and interfere with energy production within the cell (Ombroni 2010).

Captan (N- (trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide) is a non-systemic, broad spectrum, multisite contact fungicide that acts immediately by penetrating fungal spores. Captan reaction with thiol groups is the main mode of action on phytopathogenic fungi, being responsible for the reduction of enzymatic activities, respiration, physiological changes, and fungal death (Arce *et al.* 2010; Scariot *et al.* 2016).

Our goal was to search for fungal isolates from agricultural soils with intensive use of fungicides for future biotechnological application on mycoremediation. For that, the aim of this study was to screen the fungal isolates, categorize the most tolerants to carbendazim, captan and zineb by a multivariate analysis and identify them by a molecular approach.

Materials and methods

Chemicals

All chemicals were reagent grade and were obtained from Sigma (St Louis, MO, USA) unless otherwise noted and used as received.

Three pesticides were selected: carbendazim 50 WP (2-methoxycarbamoyl-benzimidazole), captan 80 WP (N- (trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide) and zineb 70 WP (dithiocarbamate, zinc). The different concentrations of these pesticides were prepared by dissolving them in sterile distilled water (Liu et al. 2010; Mohiddin and Khan 2013).

Soil sampling

The soil samples were collected in a vegetable nursery with intensive use of fungicides, in the locality of Gobernador Roca (Misiones - Argentina). Three *Lactuca sativa* (lettuce) field plots were randomly chosen (identified as A, B and C) and five samples were taken at equidistant points, to cover the entire field. From each sample was taken three subsamples collected at 15 cm depth (Fuentes 2011; Sousa Ramos 2014).

2.3 Soil toxicity

Soil phytotoxicity was performed to confirm the extensive use of pesticides on it. It was determined measuring lettuce (*Lactuca sativa*) seeds germination and root elongation. Twenty lettuce seeds were placed in Petri plates containing 40 g of soil samples by triplicated. The plates were incubated at 22 ± 2 °C in darkness during 120 h. Soils samples without contamination were used as controls. At the end of the incubation period, the number of germinated seeds was registered, and the length of roots and

hypocotyls was measured (Sadañoski et al. 2020). Root growth was measured using the Miyoshi 0-150 mm digital caliper.

At the end of the trial, the Germination Index (GI) that integrates the relative percentage of germination (RPG) and the relative root growth (RRG) was calculated using the following formula according to the methodology described by Tiquia (2000).

$$GI=RPG*RRG/100 \quad (1)$$

where, RPG is the number of germinated seeds in the samples/number of germinated seeds in the control; RRG is the elongation of the roots in the samples/elongation of the roots in the control.

The results obtained from GI meet in three phytotoxic categories: severe, moderate and mild according to the criteria established by Zucconi et al. (1981). GI values $\leq 50\%$ would indicate that there is a strong presence of phytotoxic substances; if a value between 50 and 80% is obtained, it would be interpreted as the moderate presence of these substances and slight if the GI $\geq 80\%$, which would indicate that there are no phytotoxic substances or they are in a very low concentration.

2.4 Fungal isolation

Fungal isolates were obtained from adding 25 g of contaminated soil to 250 mL of sterile water. After stirring, aliquots of the suspension were serially diluted and spread onto Petri dishes containing potato dextrose agar medium (PDA) 39 gl^{-1} supplemented with chloramphenicol antibiotic 10 mg/L, to prevent the bacterial growth (Dritsa et.al. 2007). Cultures were incubated at 28 ± 2°C for 7 days. To obtain pure cultures, mycelium was repeatedly transferred onto new plates. The isolated isolates were stored at 4 °C in test tubes containing the PDA (Colla et.al. 2008).

2.5 Tolerance of fungal isolates to fungicides

Three pesticides were selected: carbendazim 50 WP (2-methoxycarbamoyl-benzimidazole), captan 80 WP (N- (trichloromethylthio) cyclohex-4-ene-1,2-dicarboximide) and zineb 70 WP (dithiocarbamate, zinc). The different concentrations of these pesticides were prepared by dissolving them in sterile distilled water (Liu et.al. 2010; Mohiddin and Khan 2013).

Growth rate of fungal isolates was tested in solid media containing PDA and different systemic and contact fungicides: carbendazim, captan and zineb at different concentrations (1, 10, 100, 1000 y 10,000 ppm) (Muhammad and Shahzad 2007). A 5 mm inoculum disc of fungal isolate was cut from the margin of the growing colony and placed in the centre of each Petri plate. Plates containing PDA without fungicide were used as control. The plates were incubated at 28 ± 2 °C for 7 days. The experiments were carried out by triplicate. The radial growth of the colony in each treatment was measured and the percent inhibition of growth was calculated using the following equation:

$$TR\%=FGR/GRC*100 \quad (2)$$

where, FGR is the fungal growth rate and GRC is the growth rate of a control culture. Both FGR and GRC were determined by measuring the diameters of the expanding colonies, and TR is the tolerance rate (Lee *et al.* 2014).

2.6 Identification of most tolerant fungus

2.6.1 Morphological characteristics

The most tolerant isolate, LBM 239, was characterized by morphological criteria and identified according to the general principle of fungal classification (Smith 1963; Kendrick 2000).

2.6.2 Molecular identification

Total genomic DNA was extracted from axenic isolate LBM 239 grown for 24 h on 15 gl^{-1} yeast extract 30 g/L sucrose (YES) medium, following the protocols of Fonseca *et al.* (2015). Partial gene sequences were determined for the ITS1-5,8S gene-ITS2 region, using primers ITS1 and ITS4 (White et al. 1990), the large subunit 28S of rDNA gene (D1/D2) using primers NL1 and NL4 (Kurtzman and Robnett 1997), and small subunit 18S of rDNA gene using primers NS1 and NS4 (White et al. 1990). Amplicons were sequenced in both directions and consensus sequences were determined using the software Chromas v2.6.5 and BioEdit Sequence Alignment Editor v7.0.5. Subsequent alignments were carried out using 18S, ITS and 28S sequences belonging to type strains of *Actinomucor* genus (less the isolate LBM 239) of culture collection CBS-KNAW referenced by Nguyen et al. (2017). Alignments were generated for each individual locus and then assembled using MEGA v.7.0 and manually corrected if necessary. Concatenated sequences were included in the phylogenetic inference based on neighbour joining (NJ) applying substitution Kimura's model and a bootstrap with 1 000 replicates. *Umbelopsis nana* NRRL 22420 was used as external group. The phylogenetic inference was carried out with software MEGA version 7.0.

2.7 Degradation of carbendazim in liquid media

The most tolerant fungus to the evaluated fungicides was selected to continue the degradation assays in liquid media supplemented with Carbendazim. This fungicide was chosen for its high persistence and acute toxicity for organisms in the soil (Supplementary table 1). In this study, we also applied a fast diagnostic method by an inquest to agricultural producers; they preferred the study of Carbendazim due to its high use and effectiveness (data not shown).

The liquid media degradation experiments were performed as static cultures, incubated in 100 ml Erlenmeyer flasks. The culture medium (malt extract, 12.7 gl^{-1} and corn steep liquor, 50 gl^{-1}) were autoclaved at 105°C for 20 min to prevent microbial contamination (Fonseca et al. 2010). The cultures containing 50 ml of media were spiked with a solution of the carbendazim in water reaching 10 gl^{-1} per flask. Each Erlenmeyer flask was inoculated with 1 agar plug (5 mm diameter) of the fungus grown for 7 days on PDA plates. Unspiked, heat-killed and abiotic cultures were used as controls. The heat-killed controls were performed with 4 days growth of fungal cultures, which were killed in an autoclave before addition of the carbendazim solution. All of the cultures were incubated in the darkness at 28 °C in three replicates for 7 days. At the end of the test, the samples were harvested and the mycelium was separated from the supernatant by centrifugation at 8000 rpm for 10 min.

2.8 Carbendazim determination

To study the action of the most tolerant fungus on the degradation of carbendazim, the concentration of this fungicide was determined in liquid media after the fungal growth by HPLC. The fungus was incubated in liquid media containing an initial concentration of 100 ppm of carbendazim. Also, the fungus was incubated in control media without

the addition of fungicide. Quantitative analyses of carbendazim were performed using a Agilent model 1100 coupled to a column of C18, X-Select CSH of 3.5 microns of particle, 75 mm long and 4.6 mm in diameter, operating at a constant flow rate of 0.5 ml min^{-1} with a mobile consistent phase in a mixture of methanol and water (70:30) both added with 0.1% formic acid in isocratic mode. The molecular identity of the fungicide in samples was carried out using an agilent brand diode array detector with fixed 230 and 254 nm and UV-VIS between 190 to 700 nm. The samples were diluted in nanopure water with the same treatment of the standards, filtered by 0.22 μm systems prior to injection into the HPLC.

Removal percentage was calculated as:

$$\text{Removal rate (\%)} = (1 - A/B) \times 100 \quad (3)$$

Where A is the residual fraction of carbendazim after remediation and B is the total amount of carbendazim before remediation.

2.9 Statistical analysis

All experiments were carried out in duplicate. The data were analyzed using the software Statgraphics Centurion (StatPoint, Inc. version 15.2.05). An analysis of variance (ANOVA), followed by the least significant difference (LSD) post-hoc test at $p<0.05$ were used to determine the significance of tolerance of fungi strains to the three fungicides concentrations.

To identify the most tolerant fungi to fungicides and their correlation with the biodegradation of these fungicides, an exploratory analysis was carried out. A principal component analysis (PCA) and a conglomerate analysis were carried out using the software InfoStat 2017 version.

3. Results

3.1 Fungal isolation

In this work 28 fungi strains were isolated and deposited in the Culture Collection of the Laboratory of Molecular Biotechnology (LBM, from Spanish Laboratorio de Biotecnología Molecular) of the Institute of Biotechnology Misiones and summarized in **Table 1**.

3.2 Soil toxicity

The soil sample from field plot B was the most sensitive to the presence of contaminants in the average with a germination percentage of 71.67% while the samples from field plot A (93.33%) and C (86.67%) obtained values similar to the initial germination power of the seeds.

The differences observed in root length directly influenced the results of the GI as can be seen in **Fig. 1**. According to the criterion proposed by Zucconi et al. (1981), it is realized that field plot B was located in the severe phytotoxic category, while the samples from field plot A and C were located in the moderate category, this was reflected in the germination of the seeds and in the length of the radicles.

3.3 Tolerance of fungal isolates to fungicides

We investigated the tolerance of 28 fungal strains on agar plates supplemented with carbendazim, captan, and zineb in increasing concentrations. Radial growth rate was measured after incubation at 28 ± 1 ° C for 7 days. The concentrations of the pesticides have had different effects on the mycelial development of the strains. In some cases, there is an inhibition of development at minimum concentrations, while in others, there

is a mycelial decrease as the concentration increases. All fungi were inhibited by the concentrations of 10,000 ppm.

The tolerance of fungi to the pesticides carbendazim, captan and zineb was evaluated by a principal component analysis (**Fig. 2**). **Fig. 2a** shows the score plot, that is a map of the isolates (observations) and how they are situated with respect to each other based on the variables in the loading plot (**Fig. 2b**). The loading plot shows the influence of the variables (pesticides at different concentrations) on fungal isolates and displays the correlation structure of the variables. The statistical analysis of the main components on the tolerance of fungi to fungicides showed that the first principal component (PC1) was responsible for explaining 61.51% of the results' variability, and the second main component (PC2) was responsible for 21.5% variation, resulting in 83% of total variation of the experimental results. Regarding PC1 analysis, the different concentrations of pesticides presented a positive correlation between them, particularly carbendazim at 1 ppm, captan at 1 and 10 ppm and zineb at 1 ppm. Respect on variables according to PC2, the other concentrations of pesticides had more influence on fungal tolerance. From this multivariate analysis, we observed that the isolates LBM 230 and 239 showed a differential behavior compared with the other fungal isolates. The position of both isolates, in the right lower quadrant, showed their tolerance to high concentrations of pesticides since in this quadrant, the concentrations of carbendazim (10 and 100 ppm) zineb (10 and 1000 ppm), captan (10 and 100 ppm) are the most influential variables. The other isolates positioned together from those that showed nule tolerance to pesticides (left lower quadrant) to those that showed moderate tolerance (left upper quadrant).

With the aim of classifying the isolates and forming homogeneous groups based on their tolerance to different concentrations of pesticides, we carried out an analysis of

conglomerates. The dendrogram formed is shown in **Fig. 3** with a cophenetic correlation of 0.945. Isolates LBM 230 and 239 were separated from the other isolates which formed two groups. Both isolates LBM 230 and LBM 239 showed tolerance to all pesticides, being the isolate LBM 239 the highest tolerant. Regarding the groups formed, the group formed by the isolates LBM 222, LBM 224, LBM 228, LBM 229, LBM 244, LBM 245 and LBM 246 showed moderate tolerance to pesticides. The rest of the isolates, LBM 223, LBM 225, LBM 226, LBM 227, LBM 231, LBM 232, LBM 233, LBM 234, LBM 235, LBM 236, LBM 237, LBM 238, LBM 240, LBM 241, LBM 242, LBM 243, LBM 247, LBM 248 and LBM 249 formed the group that had no tolerance to any fungicide concentration.

The data from the ANOVA and LSD analysis showed that the three variables (pesticides, concentrations and types of fungal strains) had significant effects on the development of the mycelium (p values <0.05). It was observed that of the 28 strains tested, only the strain encoded as LBM 239 presented a high tolerance to the three pesticides tested, showing a statistically significant difference with the other strains (Supplementary Table 2).

We found that isolates LBM 239 demonstrated a high tolerance to zineb with a TR 78.00% at 1000 ppm, 72.35% at 100 ppm of captan and 67.35% at 100 ppm of carbendazim. Furthermore, no variable morphological changes were observed compared to the control. This high tolerance probably occurred because the strain was isolated from a contaminated soil, thus developing a remarkable tolerance to these compounds.

3.5 Degradation of carbendazim in liquid media

LBM 239 removed 63.8% of the carbendazim in the culture medium after 7 days of treatment.

3.4 Identification of the most tolerant fungus to fungicides

Characterization of the most tolerant fungus, isolate LBM 239, from the contaminated soil was carried out by morphological criteria and molecular approach (**Fig. 4**). The isolate LBM 239 rapidly grew on PDA at 28 °C, filling the entire 90 mm Petri dish within 4 days of incubation. The colony appeared white to cream-colored, loosely floccose forming abundant aerial mycelium, reaching the lid of the Petri dish (**Fig. 4a**). The colony reverse was yellow (**Fig. 4b**). On PDA, a profuse mycelial growth with numerous branched rhizoids was produced (**Fig. 4c**). The sporangiophores were hyaline, extremely variable in length and width, arising from stolons, mostly without opposite rhizoids, branched, terminating in sporangia. Whorls of branched sporangiophores, terminating with formation of subglobose to dome-shaped columellae were common (**Fig. 4d**). The sporangi were globose to subglobose, slightly buff-colored, thick-walled, smooth or spiny, produced on branched and unbranched sporangiophores. For molecular identification, a phylogenetic analysis was performed by adding the reference sequences available in the GenBank database. In this work we amplified for 5.8S, ITS and 28S and the consensus sequences were deposited in the GenBank of the National Center for Biotechnology Information (NCBI) under the numbers 2527626, MW015099 and MW015105, respectively. ITS sequences of other fungal strains were used for the alignment that is deposited in the website TreeBASE under the submission number: TB2: S29094 (http://purl.org/phylo/treebase/phylows/study/TB2:S29094?x-access-code=44d2f49f67e1fb11b604b4f64ab64d62&format=html). The CBS type strains grouped into the expected phylogenetic tree using NJ method and showed that the isolate LBM 239 belongs to the species *A. elegans* (**Fig. 4e**).

Discussion

The aim of this study was to screen the fungal isolates, categorize the most tolerants to carbendazim, captan and zineb and identify them by a molecular approach. A total of 28 fungal strains were isolated from agricultural soil with intensive use of pesticides. The tolerance test was carried out at different concentrations of pesticides. From the 28 strains tested, the strain LBM 239 presented a high tolerance to the three pesticides tested, showing a statistically significant difference with the other strains. Strain LBM 239 was molecularly identified as *Actinomucor elegans*.

To our knowledge, tolerance studies of these fungi *A. elegans* to carbendazim, captan and zineb fungicides have not been reported. Furthermore, very few studies are reported on the tolerance of other fungal strains to captan and zineb although these fungicides are commonly used in agricultural practices. The concentrations of the pesticides have had different effects on the mycelial development of the strains. In some cases, development inhibition was observed at minimum concentrations, while in others, mycelial decrease was observed as the concentration increased, being totally inhibited at the maximum concentration (10,000 ppm).

Resistance to benzimidazole fungicides such as carbendazim in this work has been detected in many species of fungi (Liu, et al 2010). These results are in agreement with that reported by other authors (Shah et al. 2006; Muhammad and Shahzad 2007; Mohiddin and Khan 2013) who indicated that the strains belonging to the Trichoderma or Fusarium genera have resulted tolerant and demonstrated their remarkable degradation capacity towards carbendazim. Fungicides exert selection pressure on microbial populations; often, the more frequently a fungicide is used during a season, the higher the frequency of detection of fungicide-resistant isolates (Ma and Michailides 2005; Ying Yang et.al. 2018).

We used GI to determine the phytotoxicity of the soil and we observed differences in seed germination and radicle length between samples belonging to a severe phytotoxic field (plot B) from moderate phytotoxic field (plots A and C). These results are in agreement with that reported by other authors (Tiquia 2000; Varnero et.al. 2007; Pentreath et.al. 2015; Hernández Valencia 2017) who associated the differences in CRR with the presence of moderate phytotoxic metabolites, that did not prevent the germination of all the seeds, but did limit the development of the radicle. Considering the chemical characteristics of the soil, the phytotoxicity observed in this work could respond to the constant applications of agrochemicals in each harvest cycle.

The tolerance of the isolated fungal strains to the different fungicide concentrations were studied by two multivariate analyses, a PCA and a conglomerate analysis. The PCA showed a map of the strains and how they are situated with respect to each other based on the variables Nilsson et.al. (2016) in the loading plot. The loading plot shows the influence of the variables on fungal strains and displays the correlation structure of the variables. Two plots help to analyze the correlation between the observations and the variables (Bohacz 2017; Díaz *et al*. 2019). The statistical analysis of the main components showed 83% of total variation of the experimental results, indicating that PCA adequately represents the relationships between the strains and the variables (Balzarini *et al*. 2015). The strain LBM 239 was the most tolerant to fungicides. The conglomerate analysis improved the interpretation of principal component analysis showing 4 clades with a cophenetic correlation of 0.945. Also, LBM 239 was positioned separately in the dendrogram as it was the most tolerant to fungicides. *A. elegans* LBM 239 showed mycelial development and the highest tolerance to zineb, captan and carbendazim. The behavior of this strain was probably due to the fact that it was isolated from pesticide contaminated soil, thus developing a

remarkable resistance to these compounds. The maximum tolerance concentration for *A. elegans* LBM 239 showed a higher tolerance to carbendazim (100 ppm). It is important to consider that the most tolerant fungi tested in this study, *A. elegans* LBM 239 was isolated from the severe phytotoxic field (plot B). Probably the fungi that grew in this soil developed the ability to tolerate these agrochemicals. In most cases, resistance was correlated with point mutations in the β-tubulin gene that encodes the target to which the fungicide binds (Ying Yang et.al. 2018).

The biodegradation ability of *A. elegans* LBM 239 that showed high resistance to carbendazim was examined. The results showed that LBM 239 removed 63.8% of the carbendazim in the culture medium after 7 days of treatment. Similar results were reported by Raheem (2021); the authors studied the degradation potential of *A. niger* and found that the fungus degraded 69.66% and 99.96% of total carbendazim fungicide after 10 and 20 days of incubation, respectively.

Based on these results, this autochthonous pesticide-tolerant fungi *A. elegans* LBM 239 is an excellent organism to carry out soil bioremediation strategies which is a process that uses live microorganisms and their products to remove contaminants from the soil. Native soil microorganisms play a key role in bioremediation as biogeochemical agents to transform complex organic compounds into simple inorganic compounds or their constituent elements (Megharaj et al. 2011; Adams *et al*. 2015). Bioremediation can be carried out with two approaches: bioaugmentation that is carried out with external addition of degrading microorganisms and the biostimulation that is carried out with the addition of nutrients to promote the growth of microorganisms and thus increase the rate of degradation (Pino Rodriguez et.al. 2012).

In conclusion, 28 fungal strains were isolated from agricultural soil with intensive use of pesticides and two of them demonstrated the highest tolerance to the

three fungicides tested. These strains were molecularly identified as *Actinomucor elegans* LBM 239. The capability of this species to survive and grow in the presence of a high concentration of these fungicides is an adaptive feature that makes them very promising candidates for biotechnological applications and bioremediation.

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LEGENDS OF TABLES AND FIGURES

Table 1. Fungi isolated from contaminated soils of Misiones rainforest used in this study.

Figure 1. Soil phytotoxicity was determined by the L. sativa test measuring RPG, RRG and GI. Blue horizontal line shows a moderate phytotoxicity; red horizontal line shows a severe phytotoxicity. Means with different letters are significantly different ($p < 0.05$). RPG, relative percentage of germination; RRG, relative root growth and GI, germination index.

Figure 2 Differential tolerance of soil fungi to three fungicides evaluated by a principal component analysis. a) Scores of fungal isolates according to their tolerance to the fungicides in the plane formed by the first and the second principal components. The isolates located in the same part of the loading plot means the higher impact of the variables (tolerance to fungicides) on the observations. b) Loading plot of the first and second principal components for tolerance to carbendazim, captan and zineb at different concentrations (0 ppm, 10 ppm, 100 ppm and 1000 ppm). PC, principal component; C, carbendazim; CP, captan; Z, zineb.

Figure 3 Groups of fungal isolates formed by the Conglomerate analysis according to their tolerance to the fungicides zinec, carbendazim and captan. The classification was based on their enzymatic levels and six clusters with different specificities were formed. Cophenetic correlation: 0.945.

Figure 4 Morphology and molecular identification of *A. elegans* LBM 239. (a) Colony on PDA, observed view; (b) Colony on PDA, reverse view; (c) Young sporangia on sporangiophores, 10X; (d) Young sporania, 40X; (e) Phylogenetic tree obtained by the

neighbour joining method, showing placement of the isolate LBM 239 among species of *Actinomucor* genus and reference species *Umbelopsis nana* NRRL 22420 (the outgroup species in the analysis) as presented by the bootstrap consensus tree inferred from 1 000 replicates derived from analysis of 18S-ITS-28S. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1 000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as used for evolutionary distances used to infer the phylogenetic tree. PDA, potato dextrose agar.

Supplementary material

Supplementary table 1. Datasheet of the fungicides used in this study.

Supplementary table 2. Analysis of variance of percentage of tolerance of the fungal isolates based on different concentrations of the fungicides tested in this study.