

# Modeling radial growth of *Amylomyces rouxii* and its tolerance to selected pharmaceutical active and endocrine disruptors compounds

Modelado del crecimiento radial de *Amylomyces rouxii* y su tolerancia a compuestos activos farmacéuticos y disruptores endócrinos seleccionados

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## ABSTRACT

Filamentous fungi that tolerate the presence of toxic compounds has the potential application for their removal. In this work we studied the tolerance of *Amylomyces rouxii* to growth in presence of pharmaceutical active and endocrine disruptors compounds (PhACs and ED). Results showed that radial growth data can be modeled and used to determine the kinetic parameters to quantify fungal growth in the presence of PhACs. The capacity of *A.rouxii* to grow in the presence of 9 PhACs and 3 ED at concentrations between 100 to 5000  $\mu\text{g L}^{-1}$  was evaluated. The studied PhACs were paracetamol, ibuprofen, diclofenac, naproxen, sulfamethoxazole, trimethoprim, ciprofloxacin, ofloxacin, carbamazepine, and endocrine disruptors were  $\beta$ -estradiol, triclosan, and bisphenol-A. The data of *A. rouxii* radial growth was modeled using the logistic equation and linear regression. The  $V_{\max}$ , except in cultures with  $\beta$ -estradiol, and  $\mu_{\max}$  values were not affected by the presence of PhACs. Growth inhibition of fungus was calculated at 24 h. In cultures with diclofenac, triclosan and naproxen, a linear relationship was observed between compound concentration and radial growth inhibition. However, there was no difference in radial growth inhibition at the different assayed concentrations of ibuprofen, trimethoprim, and  $\beta$ -estradiol. In culture with 5000  $\mu\text{g}$  carbamazepine  $\text{L}^{-1}$ , growth of *A. rouxii* was completely inhibited. To the best of our knowledge, this is one the first work reporting PhACs and ED toxicity in zygomycetes.

**Keywords:** fungi, inhibition growth, logistic equation, micro-contaminants, growth modeling.

## RESUMEN

Los hongos filamentosos que toleran la presencia de compuestos tóxicos pueden potencialmente removerlos. En este trabajo se estudió la tolerancia de *Amylomyces rouxii* a crecer en presencia de compuestos activos farmacéuticos (CAF) y disruptores endócrinos (DE). Los resultados mostraron que los datos de crecimiento radial pueden ser modelados y usados para determinar los parámetros cinéticos del crecimiento fúngico en presencia de CAFs y de DE y evaluar la

inhibición del crecimiento por los CAFs. Se evaluó la capacidad de *A. rouxii* para crecer en presencia de 9 CAFs y 3 DE a concentraciones entre 100 a 5000  $\mu\text{g L}^{-1}$ . Los CAFs estudiados fueron paracetamol, ibuprofeno, naproxeno, sulfametoxazol, trimetoprima, ciprofloxacino, ofloxacino, carbamazepina, y los disruptores endócrinos fueron  $\beta$ -estradiol, triclosán y bisfenol A. Los datos del crecimiento radial de *A. rouxii* fueron modelados usando la ecuación logística y se obtuvo la tasa máxima de crecimiento. A 24 h, se calculó la inhibición del crecimiento. Ibuprofeno, trimetoprima, y  $\beta$ -estradiol no causaron inhibición. La carbamazepina, 5000  $\mu\text{g L}^{-1}$ , inhibió completamente el crecimiento de *A. rouxii*. Hasta donde sabemos, este es el primer trabajo que informa de la toxicidad de los CAFs y DE en hongos.

**Palabras clave:** hongos, , inhibición del crecimiento, ecuación logística, microcontaminantes, modelado del crecimiento.

## INTRODUCTION

Filamentous fungi are widely studied due to their role in the production of secondary metabolites and enzymes of industrial importance, as well as for their capacity to remove a great diversity of xenobiotic compounds from the environment. Filamentous fungi have an important role as natural carbon compounds degraders (Tomasini and León-Santiesteban, 2019).

Fungi growth on agar plates simulates the natural growth of fungi more than in the soil. This type of cultures is mainly carried out to isolate fungi and to determine their degree of tolerance to toxic compounds by means of radial growth (Gabiatti *et al.*, 2006; Crane *et al.*, 2010; de Lima Souza *et al.*, 2017).

In bioremediation studies, the isolation of fungi from contaminated sites and the determination of their tolerance, is a strategy that leads to obtain fungal strains with the ability to remove heavy metals and toxic compounds. For example, the effect of heavy metals, like copper, cadmium, lead, arsenic, iron, and mercury, on filamentous fungi was studied using radial growth as a parameter indicating their tolerance

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to the studied metals (Crane *et al.*, 2010; Baldrian, 2003). With respect to toxic compounds, the tolerance to hydrocarbons of selected filamentous fungi was studied through radial growth, finding that *Aspergillus fumigatus* had the highest tolerance to the assayed hydrocarbons (Gabiatti Jr *et al.*, 2006). On the other hand, *Rhizopus nigricans* and *Rhizopus oryzae*, isolated from pentachlorophenol (PCP)-contaminated sites, were cultured in solid medium with agar and PCP to determine their tolerance to the compounds. *R. nigricans* presented radial growth inhibition at 500 mg PCP L<sup>-1</sup> (Tomasini *et al.*, 2001), while *R. oryzae* grew at a concentration of 100 mg PCP L<sup>-1</sup>, but radial growth was slower and 2.6-times less than the growth in cultures without PCP (León-Santiesteban *et al.*, 2008).

The microcontaminants or emergent pollutants are compounds found recently in the environment at concentrations ranging from nanograms to micrograms per liter. This group of pollutants include the pharmaceutical active compounds (PhACs) and personal care products. These compounds are not toxic *per se*, since they are used by humans or animals for a beneficial purpose on health, they discharged into wastewaters through urine and feces. Finally, the PhACs reach natural water sources or the soil. Recent studies have shown that despite their low concentration in the environment, PhACs present toxic effects on some aquatic organisms such as bacteria, algae, invertebrates, and fishes; besides, the group of antibiotics can lead pathogen and non pathogen bacteria to develop resistance (Burkina *et al.*, 2018; Kim *et al.*, 2007; Mehinto *et al.*, 2010; Santos *et al.*, 2010). There are different studies on PhACs degradation by filamentous fungi such as basidiomycetes (Jelic *et al.*, 2010, Cruz-Morató *et al.*, 2013) and some ascomycetes (Wadhah *et al.*, 2017; Olicón-Hernández *et al.*, 2017). Some studies about the tolerance of filamentous fungi has been published, using mainly white-rot fungi or ascomycetes (Argumedo-Delira *et al.*, 2012; Russo *et al.*, 2019), but not zygomycetes.

For this reason, it is important to propose fungi that tolerate the presence of PhACs, to determine, in a future, the ability of these fungi for environmental PhACs removal. There are few publishers about PhACs toxicity to filamentous fungi.

The aim of this work was to evaluate the inhibition of radial growth of *A. rouxii* by 9 selected PhACs, paracetamol (PCT), ibuprofen (IBP), diclofenac (DCF), naproxen (NPX), sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CPX), ofloxacin (OFX), carbamazepine (CBZ), and 3 endocrine disruptors  $\beta$ -estradiol ( $\beta$ EDT), triclosan (TRC), and bisphenol A (BPA). Statistical analysis was made to show the effect of different concentrations of PhACs on radial growth and kinetic parameters of *A. rouxii*.

## MATERIAL AND METHODS

### Strain

*Amylomyces rouxii* CDBB-H-1883, isolated from a PCP-contaminated effluent was used in this study (Montiel *et al.*, 2004). *A. rouxii* spores were produced in 250 mL Erlenmeyer flasks with 25 mL of potato dextrose agar medium (PDA)

during 4 to 5 d at 30°C. Later, spores were harvested using a 1 % Tween 80 solution in water by mechanical agitation. The spore's suspension was then centrifuged at 12,000 *g* for 5 min, the supernatant formed was discarded and replaced by an equal volume of sterile distilled water, this was repeated at least three times.

Cleaned spores (0.5 mL) were utilized to inoculate PDA plates that were incubated at 30°C for 1 day. Once the incubation period had finished, 1-cm cylindrical agar plugs were removed from the center of the fungal colonies and used as inoculum for the PhACs tolerance experiments.

### Target PhACs

All PhACs tested in this investigation were detected in urban and hospital wastewaters from Mexico City (Calderón *et al.*, 2019). Four analgesic/anti-inflammatory compounds (PCT, IBP, DCF, and NPX), four antibiotics (TMP, SMX, OFX, and CPX), one antiepileptic (CBZ) and three endocrine disruptors ( $\beta$ EDT, BPA, and TRC) were assayed. PhACs with a high purity grade (> 98 %) were used, all of them purchased from Sigma-Aldrich, Mexico. A stock solution of 100 mg L<sup>-1</sup> of each PhAC was prepared in a 50 % ethanol solution in water.

### Culture Conditions

The radial growth of *A. rouxii* was assessed in 2X Lee agar plates. The 2X Lee agar medium contained per liter: 5 g glucose, 2.5 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 g KH<sub>2</sub>PO<sub>4</sub>, 4 g MgSO<sub>4</sub>, 10 g NaCl, 15 g agar, with a pH of 5.3. Before gelling, PhAC and ED were added to the culture media at different concentrations (100, 250, 1000, 1500, 2500, and 5000  $\mu$ g L<sup>-1</sup>). Agar plates without PhACs and ED were considered as control cultures. All agar Petri dishes (90 x 15 mm) were inoculated with a 1-cm cylindrical plug containing mycelia and were incubated at 30 °C. The radial growth of *A. rouxii* was measured, in millimeters, at different time intervals until mycelia completely invaded the Petri dishes. All cultures were carried out quadruplicated.

### Kinetic Growth Model

Unstructured models were used to describe the radial growth of *A. rouxii* in the presence and absence of PhACs. Lineal regression was only used to characterize the exponential growth phase (equation 1). In contrast, Logistic equation (equation 2) was applied to model both exponential and stationary phases.

$$\frac{dR}{dt} = V_{max}X \quad (\text{Eq. 1})$$

$$\frac{dR}{dt} = \mu_{max} \left( 1 - \frac{R}{R_{max}} \right) R \quad (\text{Eq. 2})$$

Where,  $dR/dt$  is the radial growth rate (mm h<sup>-1</sup>),  $R$  is the radial growth (mm),  $V_{max}$  is the maximum specific growth rate (mm h<sup>-1</sup>),  $\mu_{max}$  is the maximum specific growth rate (h<sup>-1</sup>), and  $R_{max}$  is the maximum radial growth (mm). Parameters from lineal regression and logistic equations were estimated with the Levenberg-Marquardt method for non-linear regression



with a confidence level of 95 % using the program OriginPro 2017.

### Growth Inhibition

The inhibitory effect of PhACs on the radial growth of *A. rouxii* was calculated at 24 h, during the exponential growth phase. Equation 3, reported by Sagar and Singh (2011), was used to calculate the percent inhibition of *A. rouxii* radial growth.

$$\% \text{ inhibition}_{24h} = \frac{CR_c - CR_f}{CR_c} \cdot 100 \quad (\text{Eq. 3})$$

Where  $CR_c$  is the radial growth in control cultures at 24 h and  $CR_f$  is the radial growth in cultures with PhACs and ED at 24 h.

### Statistical Analysis

A completely randomized design (CRD) was applied to evaluate the effect of PhACs and ED concentrations on the inhibition of the radial growth of *A. rouxii* ( $\alpha = 0.05$ ). Each CRD had seven levels related to the PhAC concentrations (0, 100, 250, 1000, 1500, 2500, and 5000  $\mu\text{g L}^{-1}$ ) and four replicates. The assumption of normal distribution of errors was checked by Kolmogorov-Smirnov and D'Agostino Kurtosis tests ( $p$ -value  $> 0.05$ ), whereas homogeneity of variance was verified by the Levene's test. The assumption of homogeneity of variance was proved in treatments with TRC, NPX, CPX, BPA, SMX, PCT, and TRP ( $p$ -value  $> 0.05$ ), thus, the HDS Tukey post hoc test was used. On the other hand, treatments with DCF, IBP,  $\beta$ ETD, CBZ, and OFX did not meet the assumption of homogeneity of variance ( $p$ -value  $< 0.05$ ), thus, they were analyzed by the Games-Howell post hoc test. All statistical tests were carried out using the software IBM SPSS Statistics 19.0.

## RESULTS AND DISCUSSION

### Radial growth of *A. rouxii* in the presence of different PhACs and ED

The kinetics of *A. rouxii* radial growth in the presence of different PhACs and ED is shown in Figure 1. Growth was measured until the mycelium invaded the Petri dish. In the control culture, the *A. rouxii* mycelium reached 45 mm between 29 to 30 h. In cultures with PhACs and ED, the time to reach 45 mm was between 32 and 40 h, depending on the compound and their concentration. Mycelia of *A. rouxii* cultured with 100  $\mu\text{g L}^{-1}$  of TRC, CPX, OFX, or BPA, invaded the Petri dishes at 29 to 30 h as in the control culture. With the other concentrations of PhACs, fungal mycelium reached 45 mm at 32 h, except with 5 000  $\mu\text{g L}^{-1}$ , which was achieved until 38 h (Figure 1B, D, E, F). In the presence of the other eight PhACs and ED (DCF, NPX, CBZ, SMX, PCT, TMP, IBF,  $\beta$ EDT), regardless of the concentration assayed, the *A. rouxii* mycelium covered the whole Petri dish at approximately 40 h (Figure 1A, C, G, H, I, J, K, L). Each point of Figure 1 is an average of 4 experimental points.

### Modeling of kinetic radial growth

In this work, the  $V_{\max}$  ( $\text{mm h}^{-1}$ ) and  $\mu_{\max}$  ( $\text{h}^{-1}$ ) of *A. rouxii* radial growth were calculated using lineal regression and the logis-

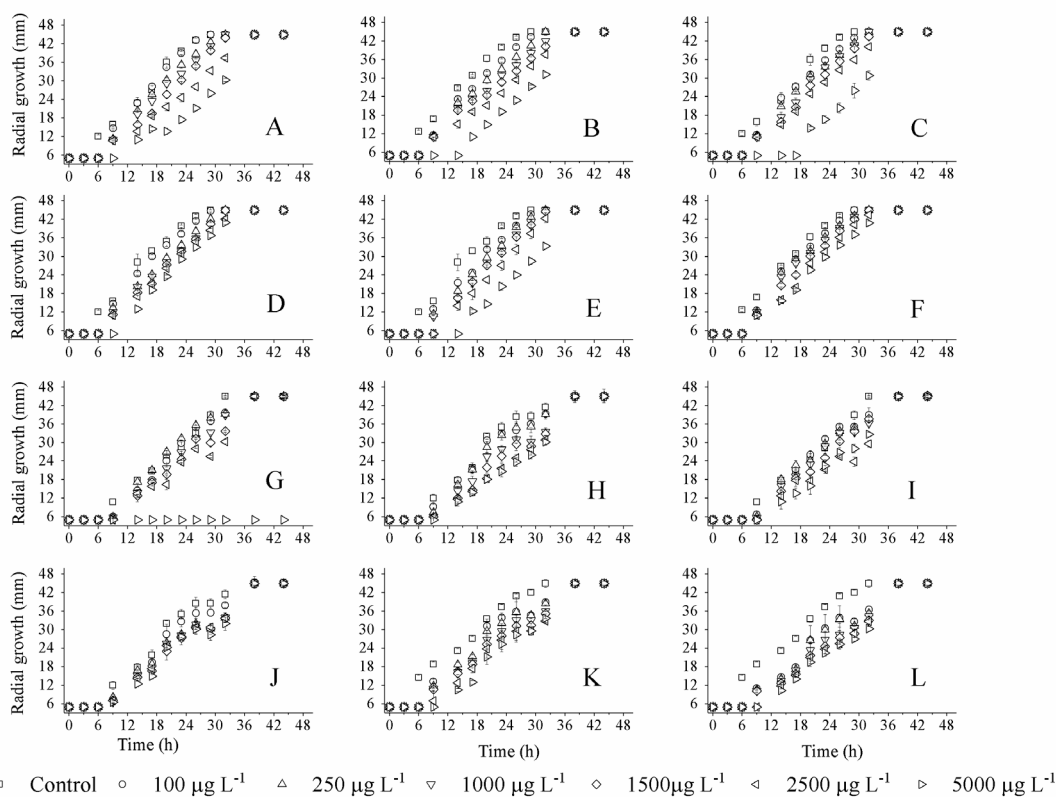
tic equation, to determine if the presence of PhACs affects these kinetic parameters. Results showed that data fitted good with lineal regression as indicated by the  $R^2$  values (0.84 and 0.99). Statistical analysis ( $p$ -value  $> 0.05$ ) showed that the  $V_{\max}$  value (1.56  $\text{mm h}^{-1}$ ) of *A. rouxii* radial growth was not affected by the presence of PhACs and ED, no matter the concentration used in this study. The  $\beta$ ETD was the only active compound that negatively affected the  $V_{\max}$  of *A. rouxii* radial growth, diminishing it 1.4-times.

Data of *A. rouxii* radial growth were also fitted with the logistic equation that allowed modeling the exponential and stationary phases, as well as obtaining the  $\mu_{\max}$  value. Figure 2 shows the data of the control and the cultures with BPA fitted with the logistic equation.

The profile of *A. rouxii* radial growth in control cultures fitted good with the logistic model and showed a sigmoidal growth curve,  $R^2 \geq 0.975$ . In cultures with 100, 250, 1000, 1500, and 2500  $\mu\text{g L}^{-1}$  of PhACs and ED, the growth profile was similar to the control culture and the logistic model fitted good ( $R^2 \geq 0.975$ ). With the exception of cultures with 100  $\mu\text{g L}^{-1}$  of IBP,  $R^2 = 0.966$ , and with 2500  $\mu\text{g L}^{-1}$  of PCT,  $R^2 = 0.943$ , the values may be due to the experimental variation of the radial growth values.

Experimental data from cultures grown in the presence of 5000  $\mu\text{g L}^{-1}$  of BPA, CPX, IBP, PCT, OFX, or TRC, fitted well using the logistic model ( $R^2 \geq 0.975$ ). In contrast, the growth profiles of *A. rouxii* were modified when grown in the presence of 5000  $\mu\text{g L}^{-1}$  of  $\beta$ ETD, TMP, SMX, NPX, or DCF. The sigmoidal curve was not observed because most of the experimental data were mainly grouped in the lag or exponential phases and, less in the stationary phase where few experimental points were found ( $R^2 \leq 0.974$ ), as shown in Figure 3 where it is observed the growth profile in presence of DCF. The modified growth profile of *A. rouxii* observed in the cultures with 5000  $\mu\text{g L}^{-1}$ , indicate the possibility that the primary metabolism of the fungus was affected by the PhACs. That is, probably some proteins of the energy metabolism change their expression due to the presence of these compounds. Recently, it was reported that in *R. oryzae* cultures the expression of some proteins like transaldolase, hydrogenase malate, and ATP synthase, related with energy metabolism, were overexpressed in the presence of PCP (Ruiz-Lara *et al.*, 2020). A hypothesis to explain why *A. rouxii* modify their growth in presence of some PhACs and ED, is that the fungus must produce more proteins for energy metabolism to cope with the effect of these compounds. However, it is necessary to study the growth of *A. rouxii* with PhACs and ED in submerged cultures to provide more information on the effect of these compounds on the metabolism and growth of the fungus.

Experimental data of *A. rouxii* radial growth in the presence of CBZ at 2500  $\mu\text{g L}^{-1}$  was not fitted,  $R^2 = 0.949$ , indicating that at this concentration CBZ affects the growth of *A. rouxii*. At 5000  $\mu\text{g L}^{-1}$  of CBZ, growth of *A. rouxii* was inhibited completely. There are some reports on CBZ toxic effects in some aquatic organisms like fish, algae and crustacean (Hai



**Figure 1.** Kinetics of *A. rouxii* radial growth cultured without micropollutants (control) and with different PhACs and ED concentrations. A) DCF; B) TRC; C) NPX; D) CPX; E) OFX; F) BPA; G) CBZ; H) SMX; I) PCT; J) TMP; K) IBP; L)  $\beta$  ETD. Each point is an average of four experimental values.

**Figura 1.** Cinética de crecimiento radial de *A. rouxii* cultivado sin CAFs (control) y con diferentes concentraciones de CAFs. A) DCF; B) TRC; C) NPX; D) CPX; E) OFX; F) BPA; G) CBZ; H) SMX; I) PCT; J) TMP; K) IBP; L)  $\beta$  ETD. Cada punto es un promedio de cuatro valores experimentales.

*et al.*, 2018; Jos *et al.*, 2003; Santos *et al.*, 2010). The toxicity of CBZ at concentrations of 5 to 30 mg L<sup>-1</sup> has been evaluated in the *Juvenil rainbow* trout, analyzing the antioxidant response in the presence of CBZ (Li *et al.*, 2011). Growth of the algae *Desmodesmus subspicatus* was inhibited with 74 mg CBZ L<sup>-1</sup>. Zhang *et al.* (2012) reported the ecotoxic effect of the CBZ in the algae *Scendesmus obliquus* and *Chlorella pyrenoidosa*, and authors proposed that CBZ could inhibit the growth of these algae. It has been shown that chlorophyll synthesis is inhibited by CBZ. The alga *Raphidocelis subcapitata* was sensitive and its growth inhibited in the presence of 100 mg CBZ L<sup>-1</sup>. Growth inhibition of *Chlorella vulgaris* by CBZ has also been reported (Jos *et al.*, 2003). In this work it was found that of the nine PhACs and three DE tested, CBZ was the most toxic to *A. rouxii* growth. There are some reports on CBZ degradation by basidiomycetes like *Trametes versicolor*, *Pleurotus ostreatus* and *Phanerochaete chrysosporium*, but the authors did not report growth inhibition caused by the compound. This is one of the first works that reported the toxic effect of CBZ for a zigomycete fungus.

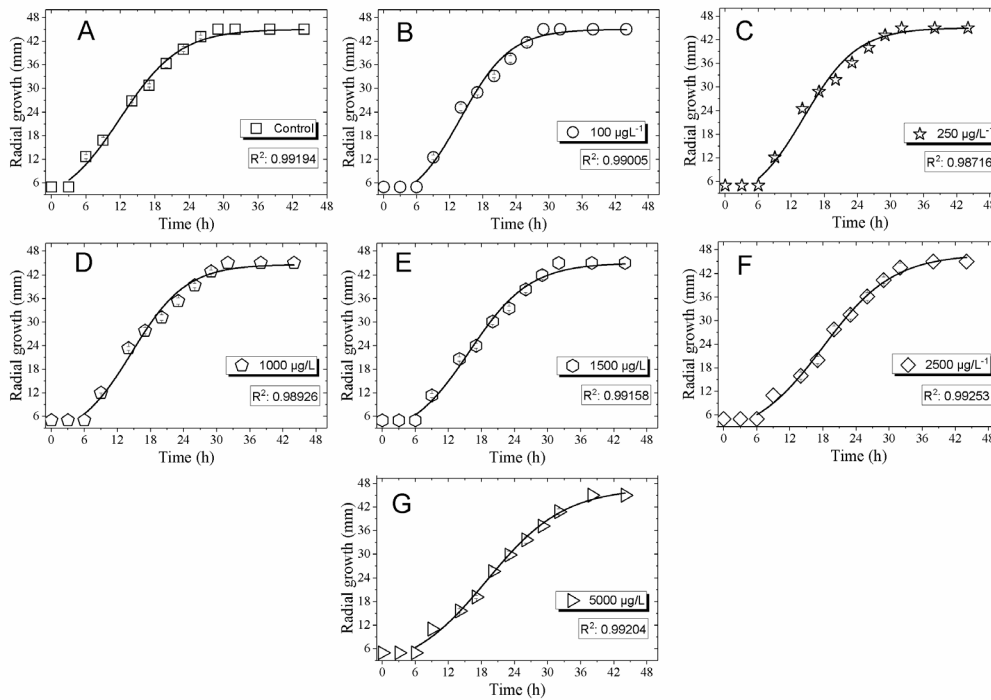
The  $\mu_{\max}$  values of *A. rouxii* radial growth with the logistic equation at different PhACs and ED concentrations, were calculated. Figure 4 shows the  $\mu_{\max}$  values for the cultures with PhACs, ED and the control cultures, without statistical differences ( $p$ -value > 0.05), independently of the concen-

tration of the compounds, with respect to the control cultures. Except the cultures with  $\beta$ ETD, where  $\mu_{\max}$  diminished 1.5-times with respect to the  $\mu_{\max}$  of the control culture, the  $V_{\max}$  value calculated with lineal regression also showed a 1.4-fold decrease. Table 1 show the statistical analysis indicating that the confidence intervals do not touch each other. The effect of dichlorodiphenyltrichloroethane (DDT) on the  $\mu_{\max}$  of the radial growth of *Rhizopus arrhizus* and *Trichoderma hamatum*, has been studied and authors found similar results. *T. hamatum* did not present differences in the growth  $\mu_{\max}$  with DDT as compared to the control culture. However, the  $\mu_{\max}$  for *R. arrhizus* diminished in the presence of DDT (Russo *et al.*, 2019).

The lag phase during *A. rouxii* growth in the control cultures was of 3 h, and of 6 h in cultures with low PhACs concentrations (100  $\mu$ g L<sup>-1</sup>). In cultures with 5000  $\mu$ g L<sup>-1</sup> of OFX, TRC, or NPX, the lag phase was between 14 h and 17 h.

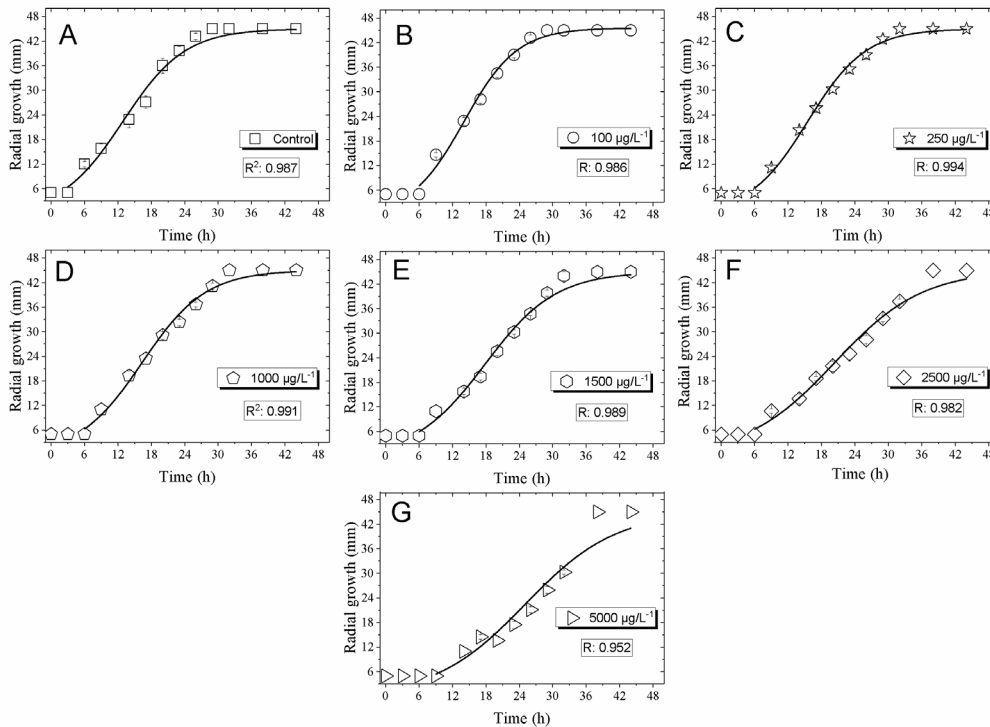
#### Inhibition of *A. rouxii* growth by the PhACs and ED

As was mentioned, radial growth of *A. rouxii* reached 45 mm, at different times within a range from 29 to 40 h. For this reason, the value of radial growth at 24 h in the exponential phase was taken to determine the of radial growth inhibition caused by PhACs and ED. Growth inhibition was calculated using equation 3. The ANOVA results, with four values of ra-



**Figure 2.** Radial growth of *A. rouxii* in control culture and in cultures with different BPA concentrations in  $\mu\text{g L}^{-1}$  (A: 0, B:100, C: 250, D: 1000, E:1500, F: 2500, G: 5000). Symbols represent the experimental data (average from 6 experimental values) and the continuous line corresponds to the fit of the logistic equation.

**Figura 2.** Crecimiento radial de *A. rouxii* en cultivo control y con diferentes concentraciones de BPA en  $\mu\text{g L}^{-1}$  (A: 0, B:100, C: 250, D: 1000, E:1500, F: 2500, G: 5000). Los símbolos representan los datos experimentales (son promedio de 6 valores) y las líneas continuas representan los datos ajustados con la ecuación logística.



**Figure 3.** Radial growth of *A. rouxii* in control culture, and in cultures with different DCF concentrations in  $\mu\text{g L}^{-1}$  (A: 0, B:100, C: 250, D: 1000, E:1500, F: 2500, G: 5000). Symbols represent the experimental data (average from 6 experimental values) and the continuous line corresponds to the fit of the logistic equation.

**Figura 3.** Crecimiento radial de *A. rouxii* en cultivo control y con diferentes concentraciones de DCF en  $\mu\text{g L}^{-1}$  (A: 0, B:100, C: 250, D: 1000, E:1500, F: 2500, G: 5000). Los símbolos representan los datos experimentales (promedio de 6 valores) y las líneas continuas representan los datos ajustados con la ecuación logística.

**Table 1.**  $V_{max}$  ( $\text{mm h}^{-1}$ ) and  $\mu_{max}$  values ( $\text{h}^{-1}$ ), calculated by linear regression and logistic equation respectively, of *A. rouxii* cultured at different concentrations of  $\beta$ ETD, and the confidence intervals  $\alpha = 0.05$  according to ANOVA analysis.

**Tabla 1.** Valores de  $V_{max}$  ( $\text{mm h}^{-1}$ ) y  $\mu_{max}$  ( $\text{h}^{-1}$ ), calculados mediante regresión lineal y ecuación logística, respectivamente, de *A. rouxii* cultivada a diferentes concentraciones de  $\beta$ ETD, e intervalos de confianza  $\alpha = 0.05$  de acuerdo a análisis ANOVA.

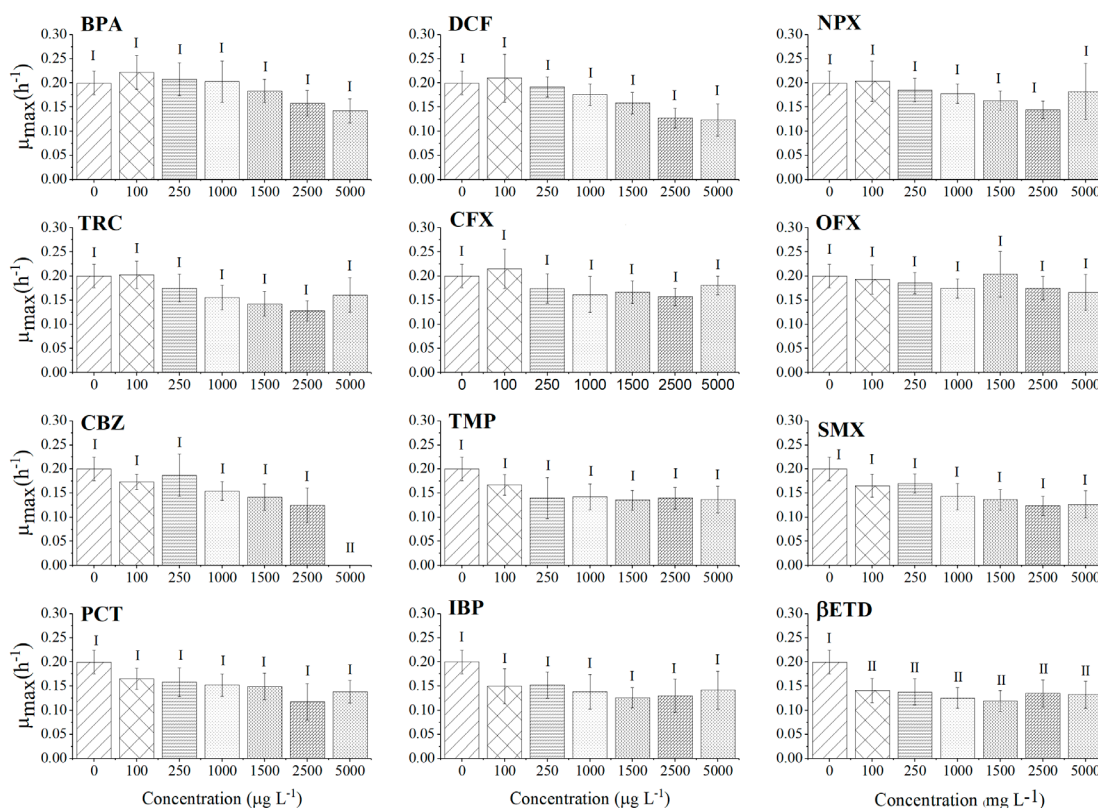
$\beta$ ETD ( $\mu\text{g L}^{-1}$ )	$V_{max}$ ( $\text{mm h}^{-1}$ )	95 % Confidence Interval		$\mu_{max}$ ( $\text{h}^{-1}$ )	95 % Confidence Interval	
		lower	upper		lower	upper
0	1.563	1.405	1.722	0.200	0.175	0.224
100	1.184	1.080	1.288	0.141	0.116	0.166
250	1.168	0.941	1.395	0.138	0.111	0.165
1000	1.063	0.967	1.159	0.125	0.104	0.147
1500	0.980	0.833	1.127	0.119	0.097	0.141
2500	1.070	0.909	1.232	0.135	0.107	0.163
5000	1.116	1.017	1.215	0.132	0.104	0.260

dial growth for each concentration, showed that treatments were statistically different. The 95 % confidence interval for each concentration mean for the nine PhACs and the three ED assayed. The HSD Tukey and Games-Howell Post Hoc tests indicated the differences in radial growth among concentrations of each compound, with a significant mean difference ( $p$ -value < 0.05).

*A. rouxii* was more sensitive to TRC and DCF than to the other compound tested, since radial growth inhibition showed statistical differences among all concentration assayed; six subgroups were found, according to Post Hoc

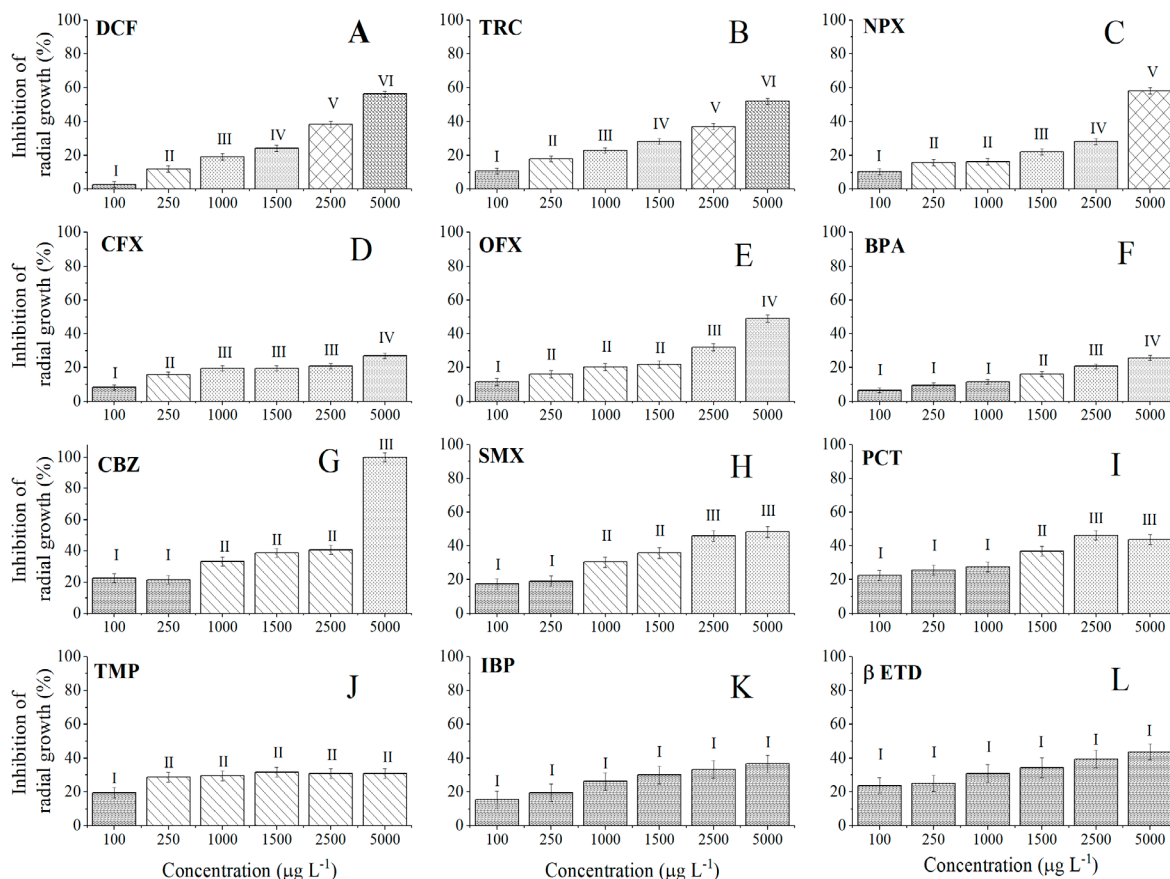
Tests (Figures 5A, B). At the lowest concentration (100  $\mu\text{g L}^{-1}$ ), radial growth inhibition was 2.8 % and 10.8 % in cultures with DCF and TRC respectively. At 5000  $\mu\text{g L}^{-1}$ , inhibition of radial growth increased 56 % and 52 % with DCF and TRC respectively. This means that, in the presence of DCF, the inhibition rate was 19.6-times higher with respect to the lowest concentration and with TCS it was 5.6-times higher.

The effect of NPX concentration on radial growth showed five subgroups according to Post Hoc Tests (Figure 5C). The inhibition rate was 10.4 and 58.3 % for 100 and 5000  $\mu\text{g L}^{-1}$ , respectively, this means 5.6-times higher at 5000  $\mu\text{g L}^{-1}$ .



**Figure 4.** Values of  $\mu_{max}$  ( $\text{h}^{-1}$ ) calculated with the logistic equation from radial growth data of *A. rouxii* cultured with different concentrations of PhACs and ED. Different numbers indicate statistical differences according to the Post Hoc HDS ( $p$ -value > 0.05).

**Figura 4.** Valor de  $\mu_{max}$  ( $\text{h}^{-1}$ ) calculada con la ecuación logística a partir de los datos de crecimiento radial cultivado con diferentes concentraciones de CAFS y ED. Los números diferentes indican las diferencias estadísticas de acuerdo con la prueba de Post Hoc HDS (valor- $p$  > 0.05).



**Figure 5.** *A. rouxii* radial growth inhibition caused by PhACs and ED at different concentrations. Different numbers indicate statistical differences according to the Post Hoc HDS tests ( $p$ -value- $p > 0.05$ ).

**Figura 5.** Inhibición del crecimiento radial de *A. rouxii* causado por las diferentes concentraciones de los CAFs y ED. Los números diferentes indican las diferencias estadísticas de acuerdo con la prueba de Post Hoc HDS (valor- $p > 0.05$ ).

CPX, OFX, and BPA presented four subgroups with significant statistical difference. At 1000, 1500, and 2500  $\mu\text{g L}^{-1}$  of CPX, *A. rouxii* presented the same inhibition rate (Figure 5D). OFX showed similar radial growth inhibition rates at 250, 1000, and 1500  $\mu\text{g L}^{-1}$  (Figure 5E). Radial growth inhibition of *A. rouxii* was not different at 100, 250, and 1000  $\mu\text{g L}^{-1}$  of BPA as shown in Figure 5F. *A. rouxii* was more resistant to CPX and BPA, maximal radial growth inhibition was 27 and 26 %, respectively, at 5000  $\mu\text{g L}^{-1}$ ; approximately 3.5-times higher with respect to the minimal concentrations tested. In cultures with 5000  $\mu\text{g L}^{-1}$  OFX, inhibition rate was 32 %. The HSD Tukey and Games-Howell Post Hoc tests showed that PCT, SMX, and CBZ presented three subgroups. *A. rouxii* was sensitive to CBZ, the inhibition rate was 23 % with the lowest concentrations, 100 and 250  $\mu\text{g L}^{-1}$ . In the cultures with 1000, 1500, and 2500  $\mu\text{g L}^{-1}$  inhibition rates were between 33 and 42 %. *A. rouxii* did not grow in the presence of 5000  $\mu\text{g L}^{-1}$  CBZ. CBZ was the only compound with an inhibition rate of 100 % (Figure 5G). Radial growth inhibition of *A. rouxii* in the presence of SMX was increased at higher concentrations; with 100 and 250  $\mu\text{g L}^{-1}$ , the inhibition rate was 18 %, and 33 % with 1000 and 1500  $\mu\text{g L}^{-1}$ . With the highest SMX concentrations (2500 and 5000  $\mu\text{g L}^{-1}$ ), the inhibition rate was 47 % (Figure 5H). PCT presented

a similar inhibition rate at concentrations of 100, 250, and 1000  $\mu\text{g L}^{-1}$ , around 23 %. With 1500  $\mu\text{g L}^{-1}$ , the inhibition rate was 37 %. *A. rouxii* presented high radial growth inhibition, approximately 45 % in the presence of 2500 and 5000  $\mu\text{g L}^{-1}$  of PCT (Figure 5I).

The last subgroup, according to the Post Hoc HDS Tukey test, practically did not show a difference in the inhibition rate calculated at any tested concentration. The TMP, IBF, and  $\beta$ ETD are in this subgroup. In the cultures with TMP and IBP, the inhibition rate of *A. rouxii* radial growth was between 15 and 36 % at 100 to 5000  $\mu\text{g L}^{-1}$ . With  $\beta$ ETD, the inhibition rate at 100  $\mu\text{g L}^{-1}$  was 24 % and 43 % with 5000  $\mu\text{g L}^{-1}$  (Figure 5J, 5K, 5L). Statistical analysis showed that there are no differences.

Results showed that radial growth inhibition of *A. rouxii* depends on the type of compound and its concentration. CBZ was the only compound that completely inhibited fungal growth at 5000  $\mu\text{g L}^{-1}$ . Radial growth inhibition of *A. rouxii* between 48 and 58 % was determined in the presence of 5000  $\mu\text{g L}^{-1}$  of DCF, TRC, NPX, OFX, or SMX. In the presence of the other six compounds, at the highest concentration, the inhibition rate was between 25 and 43 %. Similar results have been published showing that radial growth inhibition of fungi depends on the type and concentration of toxic compound.

The radial growth inhibition of fungi by the presence of phenanthrene and pyrene has been reported. Results showed that in the presence of phenanthrene, growth inhibition was between 60 and 100 % for the concentration of 240 to 2040  $\mu\text{g L}^{-1}$  for all strains except for *Hypoxylon*. Pyrene, at the same concentrations, showed almost no inhibitory effect on fungal growth, maximal radial growth inhibition was 15 to 20 % at 2040  $\mu\text{g L}^{-1}$  (de Lima Souza *et al.*, 2017). Authors reported that *Trichoderma* species were more resistant to phenanthrene, with radial growth inhibition of around 60 %, and only for one of the strains did the authors reported 100 % of inhibition at the highest concentration (3000  $\text{mg L}^{-1}$ ). In the presence of naphthalene, 100 % of radial growth inhibition was found for concentrations from 250 to 300  $\text{mg L}^{-1}$  (Argumedo-Delira *et al.*, 2012). Hendricks *et al.* (2017) reported radial growth inhibition in *Phyllosticta citricarpa* by the presence of the fungicide, fenbuconazole.

## CONCLUSIONS

The results of this study show that *A. rouxii* radial growth depends on the type and concentration of pharmaceutical compounds. The  $V_{\text{max}}$  (except in cultures with  $\beta\text{ETD}$ ) and  $\mu_{\text{max}}$  values were not affected by the presence of PhACs, indicating that these kinetic parameters were not useful to predicting growth inhibition of *A. rouxii*. In cultures with DCF and TRC, a lineal relationship was found between concentration and percent of radial growth inhibition. The lowest inhibition of growth was observed in cultures with CPX and BPA, showing little toxicity against *A. rouxii*. The different concentrations of TRP, IBP, and  $\beta\text{ETD}$  assayed in this work did not affect the fungus radial growth. The highest toxicity was caused by CBZ, *A. rouxii* did not tolerate concentration of 5000  $\mu\text{g L}^{-1}$ . This work is one of the first reports on fungal radial growth inhibition by the presence of PhACs. More studies, in submerged culture determining the consumption of nutrients and the PhACs concentration during growth kinetic, must be made to understand the toxicity effect caused by the PhACs and the mechanisms of *A. rouxii* to deal with their toxic effect.

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## CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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