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RESEARCH PAPER

Tolerance of native Magellan fungi in peat to anthracene and n-dodecane for potential use in bioremediation

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Abstract

L.A. Rodriguez-Córdova, C.A. Sáez-Navarrete, V. Ishanoglu, L. Herrera, and R. Ginocchio. 2016. Tolerance of Magellan fungi in peat to anthracene and n-dodecane for potential use in bioremediation. Cien. Inv. Agr. 43(1):85-93. The tolerances of filamentous Magellan fungi in peat were analyzed in the presence of structurally different petroleum hydrocarbons to assess their abilities for bioremediation in contaminated soils. Fungi on PDA (potato dextrose agar) and fresh peat plates were cultured and purified. The morphologically identified species were grown in a hydrocarbon adapted-fungi (HAF) medium. Increasing concentrations of anthracene, n-dodecane and furfural were applied to observe tolerances based on the radial growth kinetics of hyphae. The results showed that the radial growths of hyphae in anthracene reached average speeds of 10.95 ± 1.21, 11.03 ± 3.14 and 10.96 ± 4.61 mm h^{-1} in 0.1, 1 and 2 g L^{-1} solutions, respectively. The average growth rates in n-dodecane were 10.52 ± 3.33, 14.67 ± 1.88 and 10.86 ± 3.50 mm h^{-1} in 10, 20 and 40 g L^{-1} solutions, respectively. The growth rate in furfural reached 3.95 ± 1.07 mm/h in a 5 g L^{-1} concentration. The results suggest that the identified filamentous fungi are tolerant to anthracene and n-dodecane, which are the primary components of petroleum fractions. Furfural, a recognized antifungal, limited the growth. The results also indicate hydrocarbon degradation, suggesting that Magellanic peat can be used as a potential inoculum in bioremediation treatment processes associated with petroleum-contaminated soils. The observed filamentous fungi belong to the *Penicillium* genus based on visual identification and 18S rRNA.

Key words: Anthracene, contaminated sites, filamentous fungi, furfural, Magellan peat, n-dodecane

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Introduction

The intensive use of petroleum fuels has been the basis for industrial development since the nineteenth century. Projections for 2035 indicate that fossil fuels may provide 80% of the world energy needs (OPEC Secretariat, 2013). However, massive crude oil extraction processes, the production of hydrocarbon compounds derived from refining operations, oil storage and fuel transportation have resulted in spills and emissions that introduce xenobiotics, toxic and hazardous chemicals into the biosphere. Many of these toxins are highly persistent in aquatic and terrestrial environments (Blinova *et al*., 2012). To reduce these impacts, countries have developed increasingly strict environmental and operational regulations as well as more efficient and effective control strategies for spills and emissions (Deuel *et al*., 2005). These strategies have improved production processes and existing technologies. Additionally, several technologies have been employed to remediate petroleum-contaminated soils. Among these technologies, those based on the use of native microorganisms grown at the impacted site have become particularly popular (Godoy-Faúndez *et al*., 2008; Sáez-Navarrete *et al*., 2008). The idea behind this method is to prevent the introduction of new competitor species, which may jeopardize the biological balance of the soil; therefore, the method uses native species that are adapted to the environmental conditions of the site and, in many cases, to the contaminants (El Fantroussi and Agathos, 2005).

Sources for obtaining these microorganisms are diverse, but organic substrates are preferred due to their ecological diversity (Godoy-Faúndez *et al*. 2008; Sáez-Navarrete *et al*., 2008). In such substrates, complex microecosystems formed by bacteria, fungi, yeast, actinomycetes and microalgae can be identified. These constituents can potentially transform contaminants into less hazardous chemicals or even to carbon dioxide (Beškoski *et al*., 2012). Peatlands are included among these microecosystems. Peat is a spongy, carbon-rich organic matter that is produced by the decomposition and fossilization of plant material (Sáez-Navarrete *et al*., 2008). Its characteristics vary spatially and depend on the local plant species, representing a rich biological stratification (Bragina *et al*., 2014).

In Chile, peatlands are concentrated in the south of the country, encompassing a total area of 10,470 km^2. The most common peatland type is found in Chilean Patagonia, covering a total of 90,000 ha (MMA, 2015). The dominant species is sphagnum moss, which has been previously used as a bulking material and nutrient source for contaminant degraders in remediation studies of petroleum-contaminated soils (Sáez-Navarrete *et al*., 2008). Microorganism studies near Chilean Patagonia and Antarctica have noted that Magellanic peat can potentially be used to treat petroleum hydrocarbon contamination (Vazquez *et al*., 2013) in cold regions and also *in situ*. Some species of filamentous fungi present in Magellanic peat may be able to not only tolerate complex carbon-based compounds but also mineralize and biodegrade them, as has been described for other substrates (Antízar-Ladislao *et al*., 2004).

The purpose of this study is to identify native Magellanic fungi in peat that are tolerant to anthracene, n-dodecane and furfural and possess potential for degradation and bioremediation in contaminated soils.

Materials and methods

Fungal strains and hydrocarbon selection

Fresh peat was extracted from the Magallanes Region and the subantarctic territory (Punta Arenas, 53°09'00" S, 70°55'00" W). The samples were obtained at 20 to 30 cm depth using sterile and airtight plastic bags. The samples were transported in cold medium to later perform growth testing in the presence of the contaminants of interest. Based on the methodology described

by Hughes *et al*. (2007), two peat samples were collected (0.35 g wet weight each) and placed in dextrose saturated water (10 mL) in Petri dishes in triplicate (6 plates). Plates were incubated for 15 days at 28 ± 2 °C, allowing enough time for the growth of filamentous fungi. Incubation was performed in the dark at a relative humidity of 17 ± 2%.

The developed hyphae samples were placed in Petri plates with potato dextrose agar (PDA) and antibiotics. The plates were incubated at 28 ± 2 °C for 20 days. Preliminary identification of the filamentous fungi was performed using a Motic® BA310 optical microscope based on the descriptions of their morphological characteristics.

Anthracene, furfural and n-dodecane were chosen as the selected contaminants. N-dodecane was used as a representative of biodegradable alkanes. Furfural was used as a representative of heterocyclic aromatic hydrocarbons. Anthracene was selected as a representative of low molecular weight PAHs due to its hazardous toxic characteristics (United States Environmental Protection Agency, 2006). Sigma-Aldrich brand reagents were used in the analysis, including furfural CAS No. 98-01-01 (99%), anthracene CAS No. 120-12-7 (99%) and n-dodecane CAS No. 112-40-3 (99%).

Tolerance tests

To establish the tolerance of the fungal species, hydrocarbon-adapted fungi (HAF) (Elshafie *et al*., 2007) were used in sterile Petri dishes. The basal components of the HAF medium consist of 250 mg L^{-1} of KCl, 1 g L^{-1} of NaH_2PO_4, 1 g L^{-1} of NH_4NO_3 and 15 g L^{-1} of agar. A contaminant was then added to each medium. HAF plates without contaminants were used as controls.

All selected hydrocarbons were used at concentrations of 0.1, 1 and 10 g L^{-1}, respectively. Experiments were performed in triplicate. A HAF medium with agar as the sole carbon source was used as a control. Initially, Petri dishes contained the HAF media plus contaminants at the concentrations indicated above. The plates were filled to half their volumetric capacities. Subsequently, 0.35 g of peat was placed on each plate. The plates were developed at 28 ± 2° C for 15 days in the dark to allow fungal growth. A subculture with observable hyphae was then created. To do so, the same HAF media and selected contaminants in the concentrations indicated above were used as purifying mechanisms of tolerant strains. In this subculture, radial growth and morphological characteristics were observed under a microscope.

Tolerant fungal strains

After tolerance testing, hyphae were removed to carry out genetic characterization of those fungal species developed in the experiment, using 18S rRNA (Nishida and Sugiyama, 1993).

Statistical analyses

The differences in the radial growth in each condition studied (treatment) were evaluated by two-way ANOVA using time and treatment as factors (Akbari and Ghoshal, 2014). A statistical analysis was performed using Minitab software with $\alpha = 0.05$. Normality, independence and homoscedasticity of the data were also verified.

Results and discussion

Fungi observations

After the initial incubation, hyphal growth in peat (Figure 1a) was observed. These hyphae quickly colonized the plates where they were inoculated (Figure 1b), covering 100% of the available surface in two days (Figure 1c).

Samples were extracted from the fully colonized Petri dishes to be identified using conventional optical

microscopy. Morphological characterization showed a single type of strain with characteristics similar to those of *Penicillium*. Conidiophores (main stem), metulae (outer branch) and spores were observed, and phialides formed at the end of the conidiophores and metulae (sporulation area shaped like a brush). These observations are consistent with *Penicillium*. Furthermore, a teal color, which is characteristic of this genus, was described. No other strains were observed under the microscope.

After analyzing the characteristics of this filamentous fungus, they were compared to microscopic images of filamentous fungi of *Penicillium* available in the literature. This comparison confirmed the initial hypothesis regarding the presence of this species (Figure 2).

While *Penicillium* is common in temperate environments, it has also adapted to very cold areas. Indeed, samples obtained for this study were obtained from peatlands located in Patagonia and in the subantarctic territory of Chile. This fungal species has also been identified in Magellan soils contaminated with petroleum and petroleum products (Balaji *et al*., 2014), in which its degradative potential has been described. This species produces cellulases, which are nonspecific enzymes capable of degrading complex compounds such as cellulose via osmotrophy (Harms *et al.,* 2011).

Strain tolerance

Controlled fungal cultures and treatments are shown in Figure 3. The availability of the sole carbon source, agar-agar, promoted radial growth at an average of 6.89 ± 2.09 mm h^{-1} (control). The lag phase observed is 15 h.

The experiments using HAF, anthracene, dodecane and furfural exhibited growth rates within

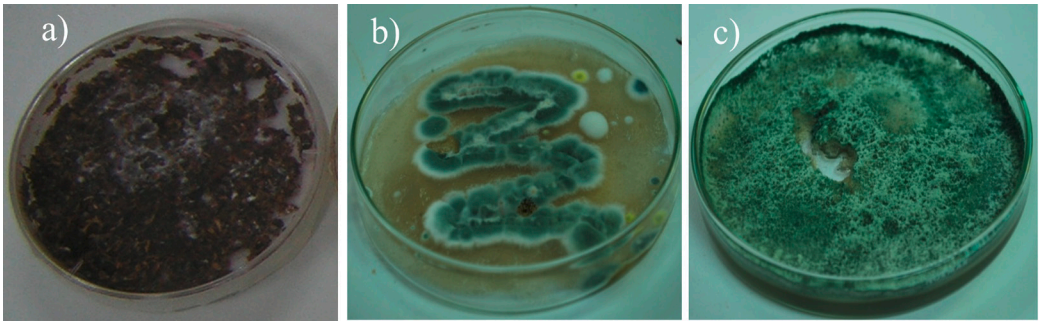

Figure 1. a. Fungal growth in peat. b. Fungi isolated from peat in a PDA medium (Potato Dextrose Agar) with antibiotics. c. Complete fungal colonization of the Petri dish.

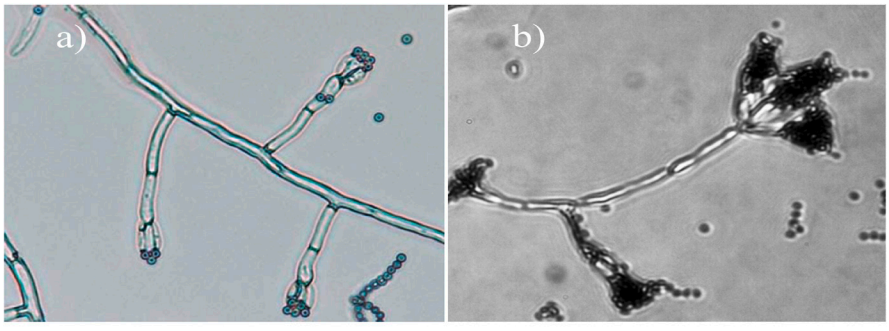

Figure 2. a. Microscopic image of *Penicillium* sp. SA29 (Ohashi *et al*., 2008). b. Microscopic image (40x) of filamentous fungi obtained from Magellan peat.

normal ranges. The radial growth for 5 g L^{-1} of furfural displayed an average speed of 3.95 ± 1.07 mm h^{-1} (Figure 3). Furfural is a known commercial antifungal and antibacterial organic compound that has been associated with a strong antibiotic action and a lesser antifungal action at a concentration of 1 mM (0.096 g L^{-1}) (Kurita *et al*., 1981). These results suggest that a furfural concentration of 10 g L^{-1} was necessary to inhibit fungi growth.

Growth kinetics observed for dodecane (n-dodecane) were significantly higher than those of the control (P=0.009), reaching average growth speeds of 10.52 ± 3.33, 14.67 ± 1.88 and 10.86 ± 3.50 mm h^{-1} for concentrations of 10, 20 and 40 g L^{-1}, respectively. The stereochemical structure of n-dodecane is susceptible to single bond attacks by nonspecific enzymatic activity associated with the fungi, which is able to degrade complex organic compounds such as cellulose, hemicellulose and lignin (Harms *et al.,* 2011). This molecular structure makes it more metabolically assimilable than the polysaccharides present in agar (Figure 3). No significant differences were observed among the three n-dodecane concentrations tested (P=0.186).

The growth kinetics observed for anthracene were significantly higher than those of the control (P=0.003), reaching average growth speeds of 10.95 ± 1.21, 11.03 ± 3.14 and 10.96 ± 4.61 mm h^{-1} for concentrations of 0.1, 1 and 2 g L^{-1}, respectively (Figure 3). Although significant differences among the tested anthracene concentrations (P=0.227) were not observed, the observed kinetics were found to be greater than those of the control.

Neither the growth in plates supplemented with the linear alkane nor in those supplemented with anthracene showed significant differences at the concentrations used (P=0.330). In fact, significant differences were not observed with increases of either organic compound, suggesting that the maximum fungal growth rates were reached for

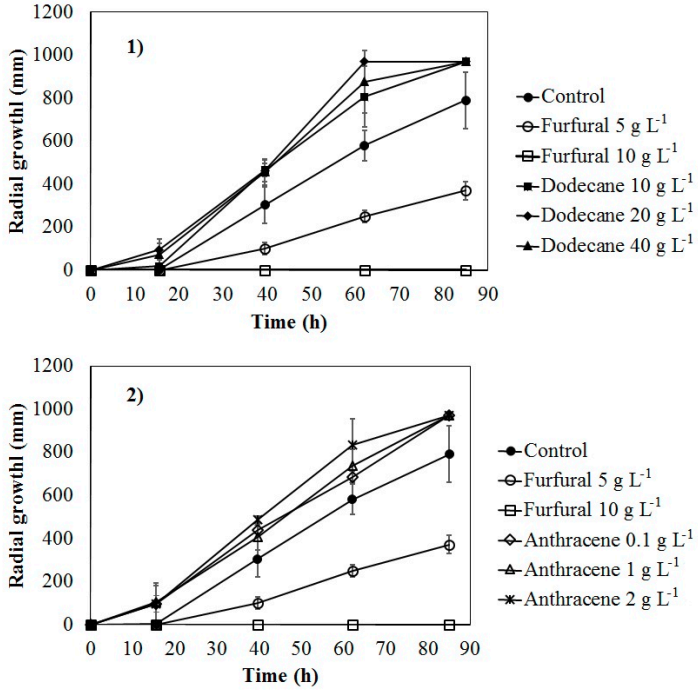

Figure 3. The average radial growth of *Penicillium* on the control and furfural plates (5 and 10 g L^{-1}) compared to: (1) Dodecane plates (10, 20 and 40 g L^{-1}) and (2) Anthracene plates (0.1, 1 and 2 g L^{-1}). Standard deviations of each group of three repetitions are included.

the carbon sources used (n-dodecane and furfural). Toxic and inhibitory effects were not observed at the concentrations tested.

The fungal species tested can tolerate high concentrations of n-dodecane and anthracene, which are both known to be highly toxic. Based on the observed kinetics, it is likely that both hydrocarbons were biodegraded by the fungal species. However, based on the experimental design and focus, the fraction of each hydrocarbon species that was degraded cannot be precisely determined with respect to the carbon source in the HAF medium, which was used as a control.

Identification of tolerant fungal strains

Genetic characterization based on 18S r-RNA identified the fungi as *Penicillium*. Three species of this genus were found. The fungus strains belong to the species *Penicillium purpurogenum*, *Penicillium rugulosum* and *Penicillium variabile* (Figure 4). *Penicillium purpurogenum* has been studied because of its metabolites and potential industrial applications, as its enzymatic process can be used in the production of biodiesel (Gusakov and Sinitsyn, 2012). *Penicillium rugulosum* has been found in arctic soils, and its enzymatic metabolism involves pectinases and cellulases

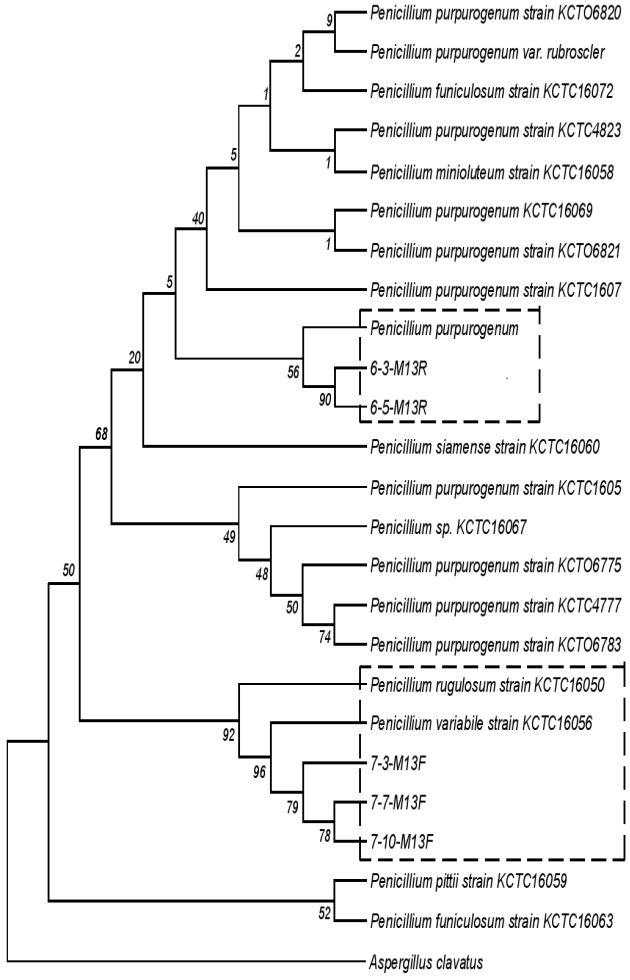

Figure 4. Phylogeny of *Penicillium* identified in Magellan peat.

(Gawas-Sakhalkar and Singh, 2011). *Penicillium variabile* has been studied in the context of phytohormone production (Isa and Don, 2014) and due to its gallotannin metabolism (tannase) (Sharma *et al*., 2008).

The genus *Penicillium* is known for its ability to degrade lignocellulosic materials (Leitão, 2009). Additionally, it is not surprising to find *Penicillium* in Magellan peat in a subantarctic area, as extremophile species of this fungus have been previously identified in cold regions (Gunde-Cimerman *et al*., 2003). The presence of this type of microorganism in a region that has been extensively contaminated by oil extraction since the early twentieth century is beneficial for feasibility programs targeting bioremediation at these contaminated sites.

We conclude that the fungal species identified and tested can tolerate high concentrations of n-dodecane and anthracene, which are known toxins. Additionally, in terms of the observed kinetics, it is likely that both hydrocarbons are biodegraded by the fungal species studied. However, this experimental design focused on determining tolerance. Therefore, the degraded fractions of each hydrocarbon species cannot be precisely determined with respect to the carbon source contained in the HAF medium (control). The identification of these strains as *Penicillium* species demonstrates the degradation potentials of the compounds tested, which should be further studied using Magellan peat as an inoculum.

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Resumen

L.A. Rodríguez-Córdova, C.A. Sáez-Navarrete, V. Ishanoglu, L. Herrera y R. Ginocchio. 2016. Tolerancia a antraceno y n-dodecano de hongos nativos de turba Magallánica para su potencial uso en biorremediación. Cien. Inv. Agr. 43(1):85-93. Se estudia la tolerancia de hongos filamentosos de turba magallánica en presencia de hidrocarburos derivados del petróleo estructuralmente diferentes, con miras a ser empleados como agentes de biorremediación de suelos contaminados. Se cultivaron y purificaron hongos en placas con PDA (Agar de Papa y Dextrosa) y turba fresca. Las especies morfológicamente identificadas se cultivaron en medio HAF (Hidrocarbon Adapted-Fungi) con concentraciones crecientes de antraceno, n-dodecano y furfural para observar su tolerancia a través de las cinéticas de crecimiento radial de hifas. Los resultados muestran que el crecimiento radial de las hifas en antraceno alcanzó una velocidad media de 10.95 ± 1.21, 11.03 ± 3.14, y 10.96 ± 4.61 mm h^{-1} para concentraciones de 0.1, 1 y 2 g L^{-1}; para n-dodecano las velocidades medias alcanzaron los 10.52 ± 3.33, 14.67 ± 1.88 y 10.86 ± 3.50 mm h^{-1} para 10, 20 y 40 g L^{-1}. Ensayos con furfural alcanzaron 3.95 ± 1.07 mm h^{-1} a 5 g L^{-1}. Los resultados muestran que el hongo filamentoso identificado es tolerante a antraceno

y n-dodecano, constituyentes característicos de fracciones del petróleo. Por su parte furfural, reconocido antifúngico, limita el crecimiento. Los resultados también muestran degradación de los compuestos señalados posicionando a la turba Magallánica como potencial contribuyente de inóculos para procesos de biorremediación de suelos contaminados con hidrocarburos del petróleo. Se identificó el género *Penicillium* como el hongo filamentoso desarrollado mediante identificación visual y 18S RNA.

Palabras clave: Antraceno, furfural, hongos filamentosos, n-dodecano, sitios contaminados, turba Magallánica.

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