

## ORIGINAL ARTICLE

# Antioxidant activity of exo-metabolites produced by *Fusarium oxysporum*: An endophytic fungus isolated from leaves of *Otoba gracilipes*

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## Abstract

Tropical ecosystems hold an extremely diverse array of endophytic fungi, but their potential use still remains to be explored. In this study, we isolated an endophytic fungus from the leaves of *Otoba gracilipes*, a medicinal tree from a tropical rainforest in Colombia. Following isolation and cultivation, we evaluated its extracellular crude extract for antioxidant activity. Using traditional and molecular methods (ITS1, NL1 regions), the endophyte was identified as *Fusarium oxysporum*. Fresh spores from the fungal isolate were inoculated in liquid media (potato dextrose broth [PDB] and potato dextrose–yeast extract broth [PDYB]) and centrifuged for recovering extracellular polysaccharides from the exhausted medium after 30 days of cultivation. Crude extracts were recovered, purified, lyophilized, and evaluated for their ability to inactivate the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The extracts obtained from PDB culture media had a 51.5% of scavenging effect on DPPH after 5 min of reaction compared with the extracts from PDYB (26.4%), which suggests a high antioxidant potential of these fungal extracts. Thus, our results suggest other fungi from tropical ecosystems should be explored as potential sources of novel enzymes and other metabolites with bioactivity.

## KEYWORDS

antioxidant activity, crude extract, endophytic fungi, *Fusarium oxysporum*, *Otoba gracilipes*, tropical forest

## 1 | INTRODUCTION

Endophytic fungi are microorganisms that grow inside plant tissues without causing any adverse effects (Meenatchi, Ramesh, Bagyalakshmi, Shanmugaiah, & Rajendran, 2016). The identity and potential role of biologically active metabolites they produce as a result of their association with host plants have been investigated before (Bhardwaj, Sharma, & Agrawal, 2014; Meng et al., 2011;

Schulz, Boyle, Draeger, Römmert, & Krohn, 2002). In particular, given that to colonize their host endophytes produce exoenzymes, they can be easily isolated, for they grow well in the host apoplastic washing fluid. Thus, previous studies have found that different endophytes produce secondary metabolites that mimic their host plant, with some showing a potential for pharmaceutical and medicinal applications (Hussain et al., 2014; Sharma, Pramanik, & Kumar, 2016; Strobel, 2003). For instance, several studies have

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shown that extracellular polysaccharides of endophytic fungi present antioxidant activity (Guo et al., 2013; Khiralla et al., 2015; Prihantini & Tachibana, 2017; Yadav, Yadav, & Yadav, 2014; Yan et al., 2016; Ye et al., 2013). On the other hand, endophytic microorganisms can be rich sources of novel enzymes and other metabolites with bioactivity as well (Hussain et al., 2014), although their potential function in biocatalysis has been poorly explored (Rodríguez, González, & Rodríguez, 2016).

Most antioxidants known today are industrially synthesized although being accounted for causing liver damage and carcinogenesis (Yuan, Zhang, Fan, & Yang, 2008). In contrast, natural-derived antioxidants, like those produced by endophytes, have not been found to be harmful. In particular, due to a high biological diversity and biochemical evolution (Fernandes et al., 2009), endophytes have the ability of using several substrates, producing a wide array of secondary metabolites (Guanatilaka, 2006). These comprise a large but little explored proportion of fungal diversity (Perottoab et al., 2013; Yuanab, Chena, & Mac, 2011). For example, since the discovery of paclitaxel, a potent anticancer agent isolated from endophytic fungi such as *Taxomyces andreanae* and *Pestalotia* spp., endophytes have been recognized as potential new sources of anticancer, antimicrobial, and antimalarial bioactive metabolites, attracting much more attention from researchers (Cui, Guo, Ren, Zhang, & Wang, 2015). These metabolites include steroids, xanthines, phenols, isocoumarins, quinones, and terpenoids (Schulz et al., 2002), among others.

It is relevant to mention that the biotechnological use of endophyte metabolites for pharmaceutical or agrochemical products is still in the developmental stage. For example, rugulosin, a mycotoxin produced by a spruce endophyte, has been shown to be effective against pine worm (Miller, Mackenzie, Foto, Adams, & Findlay, 2002), but is still not commercially produced. In this study, we explored secondary metabolites produced by fungal endophytes of *Otoba gracilipes* (family Myristicaceae), a tropical medicinal tree not previously explored for potential bioactive metabolites (Bernal, García, & Quevedo, 2011). Since previous studies have shown that the leaves of other trees such as *Quercus ilex* and *Nothapodytes foetida* contain a high diversity and abundance of fungal endophytic strains (Fisher et al., 1994; Fhatima et al., 2013; Musavi & Balakrishnan, 2015), we predicted that leaves of *O. gracilipes* would contain a high diversity of endophytic fungi, with a high potential for producing secondary metabolites. For this purpose, we isolated, cultivated, and molecularly characterized a leaf endophyte of *O. gracilipes*. In addition, crude extracts composed mainly of polysaccharides were evaluated for antioxidant activity by a DPPH free radical test.

## 2 | MATERIAL AND METHODS

### 2.1 | Study Area and collection of plant material

Fresh and healthy leaves of two young trees of *Otoba gracilipes* were collected during the dry season in "La Carolina" (3°24'10.662"N,

76°36'52.774"W), Cali, Colombia, and a humid low montane forest at 1,600 m.a.s.l. The leaves were cut with a sterile scalpel and stored at 4°C in a sterile polyethylene bag until being used.

### 2.2 | Isolation of the fungal strain

The isolated fungus was obtained following a modified method described by 1993). Leaves were surface-sterilized by a thorough wash of sterile demineralized water followed by 70% ethanol for 1–2 min and 3% sodium hypochlorite for 15 min. Small pieces of plant tissue were then placed on potato dextrose agar (PDA, Merck®) medium pH 4.5 supplemented with antibiotic clindamycin (0.2 ml/100 ml) in Petri dishes and incubated at 28 ± 2°C until the fungus started to grow.

### 2.3 | Crude extracts obtained from the endophytic fungal isolate grown on different culture media

Fresh fungal spores were suspended on a Tween-80 sterile solution (0,01% v/v), from which 3 ml was inoculated on Erlenmeyer flasks containing 200 ml of potato dextrose broth (PDB) or potato dextrose broth and 0,5% yeast extract broth (PDYB). These were grown at a pH of 6 and incubated for 30 days at 29°C. After removal of mycelium by filtration, the supernatants were collected and concentrated (KNF® RC 900 vacuum rotary evaporator) at 40°C to a final volume of 20 ml.

The recovered fraction was added to two volumes of 96% (v/v) ethanol to precipitate the exopolysaccharides (EPS), which were recovered by centrifugation (1,440 g, 20 min) and resuspended in 25 ml of distilled water type I. This solution was then dialyzed (SnakeSkin® membrane of 3,500 molecular weight cutoff (MWCO)) against double-distilled water for three days to obtain the crude extracellular polysaccharides and freeze-dried (FDU-1110.2110 EYELA® lyophilizer).

### 2.4 | Phenotypic and molecular identification of the endophytic fungus

The isolated fungus was grown on Sabouraud dextrose agar (Merck®) and incubated during 5–7 days at 29°C. Fungal colonies were macroscopically characterized, and reproductive and vegetative structures were microscopically analyzed with lactophenol cotton blue (Merck®) and KOH (Merck®). Microscope slides were observed and photographed using a light microscope (Leica® DM500, coupled with a camera Leica® ICC50).

For molecular identification, fungal DNA was extracted using EZNA® Tissue DNA Kit (Omega Bio-Tek), and total DNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop, Wilmington, DE). Universal fungal primers were used to amplify the internal transcribed spacer (ITS1) ribosomal DNA region (forward 5'-AGA GTT TGA TCH YTY AGA TGG-3' and reverse 5'-TTG TTA ACC ACG GYT CGA CTT-3'), as well as the D1-D2 region of

the large-subunit RNA gene (28S rDNA) using primers forward: D1/D2-NL-GGTCCGTGTTTCAAGACGG and reverse: D1/D2-NL1-GCATATCAATAAGCGGAGGAAAAG, by PCR and sequenced using automated dye termination sequencing. PCR amplification, PCR product purification, and sequencing of PCR products were the same for the ITS and D1-D2 regions as follows. PCRs were run at 94°C for 1 min, and 35 cycles of 94°C: 30 s, specific primer Tm 60°C for 30 s, and 72°C for 5 min, using the Taq DNA polymerase (Thermo Fisher Scientific) in a Swift™ MiniPro Thermal Cycler (ESCO). PCR products were visualized in a 1% agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega, USA) before being sequenced in an ABI prism 3500 sequencer (Applied Biosystems). DNA sequences were analyzed and compared with those available in GenBank via BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Results were deposited in GenBank with accession number MN038127.1 (<https://www.ncbi.nlm.nih.gov/nuccore/1679350429>).

## 2.5 | Antioxidant activity assay of fungal crude extracts

The antioxidant potential of EPS' crude extracts was assessed by free radical scavenging using a DPPH assay following Prihantini and Tachibana (2017). Correspondingly, we used 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable synthetic free radical widely used to evaluate the ability of compounds to act as free radical scavengers or radical hydrogen donors. To do this, crude extracts (2 mg/ml) were dissolved in a solution of water: methanol (3:1), and a volume of 50 µl was added to 100 µl of DPPH (100 µM in methanol) solution and mixed in a 96-well plate at room temperature. The absorbance at 515 nm (A<sub>515</sub>) was measured spectrophotometrically (Synergy™ H1 Multi-Mode Reader microplate) following a kinetic reaction of 30 min in quadruplicate every five minutes. Gallic acid (0.5 to 1.5 µM) was used as a positive control. The DPPH scavenging percentage (%) was calculated after García et al., 2012, as follows:

$$\% \text{ scavenging} = 100 - \left[ \frac{[(A_{\text{sample}} - A_{\text{blank}}) - A_{\text{sample blank}}] \times 100}{(A_{\text{control DPPH}} - A_{\text{blank}})} \right] \quad (1)$$

where  $A_{\text{Blank}}$  is the absorbance of the blank (150 µl methanol without DPPH),  $A_{\text{control DPPH}}$  is the absorbance of the control (100 µl methanol solution of DPPH and 50 ml methanol),  $A_{\text{sample}}$  is the absorbance of the sample and DPPH, and  $A_{\text{sample blank}}$  is the absorbance of 100 µl methanol and 50 µl sample with no DPPH.

## 2.6 | Statistical analyses

In order to estimate the DPPH scavenging percentage, we monitored the absorbance values of five minutes of kinetic activity. Given that the data followed a normal distribution, we ran an analysis of variance (ANOVA) and a Tukey ( $p \leq 0.05$ ) test to compare the difference in the bioactivity of the crude media extracts from the two fungal strain cultures. Statistical analyses were performed using the Minitab18 software (Minitab Inc., State College, PA, USA).

## 3 | RESULTS AND DISCUSSION

Several studies have addressed endophytic fungal diversity and fungi-host interactions across different ecosystems (Bandra et al., 2001). However, fewer studies have explored the potential of endophytic fungi as secondary metabolite producers. According to Schulz and Boyle (2005), natural products continue to be an important source of new pharmaceutical products. Taking into account that six of the twenty drugs mostly prescribed in the United States are of fungal origin, and only 5% of the world's fungi have been described (Gloer, 1997; Hawksworth, 1991, 2001), there is an enormous potential for finding new pharmaceutical products in fungi. However, many studies have shown that the fungus-plant symbiosis is labile and varies from antagonistic, to neutral or mutualistic, depending on the stage of life of the host and the fungus, the genotype and abiotic and biotic conditions (Hamilton, Gundel, Helander, & Saikkonen, 2012). Hence, it is important to mention that the benefit that endophytic fungi provide in a diverse group of host plants has been mostly observed in nutrient-poor environments or when plants are under stress such as drought, flood, or plant competition, or under herbivore and/or pathogen attack. Thus, in order to optimize the search for fungal endophyte secondary metabolites, it is necessary to consider that these metabolites may correspond to the fungal respective taxon and ecological niche and that only particular metabolic interactions can trigger the synthesis of secondary metabolites.

Tropical forests are known for holding a huge diversity of plants (Bernal, Gradstein, & Celis, 2016) and for having great structural complexity comprising different microclimates and both horizontal and vertical microhabitats (Gamboa-Gaitán, 2006a, 2006b). Correspondingly, there is a huge diversity and abundance of endophytic fungi (Gamboa-Gaitán, Laureano, & Bayman, 2005; Gamboa-Gaitán, 2013), and a high probability of finding metabolites with interesting pharmacological function.

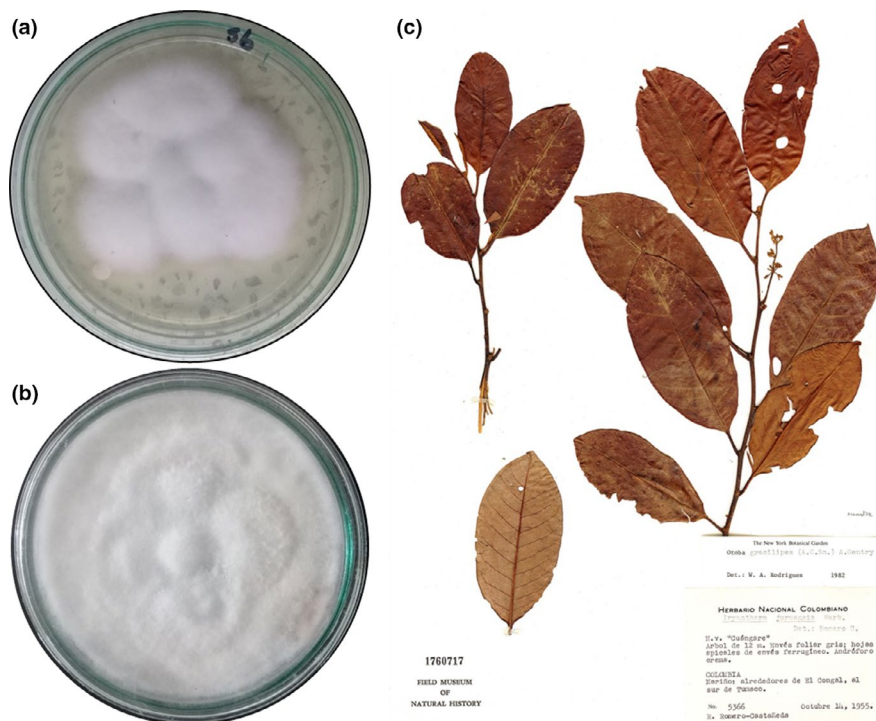
### 3.1 | Sample collection and isolation of endophytic fungi

A filamentous endophytic fungal strain was isolated from the leaves of *Otoba gracilipes* (Figure 1c) and grown in potato dextrose agar (PDA) (Figure 1a) and Sabouraud agar (Figure 1b).

The isolate was coded as EBB-ET-01 (ecology, bioprocess, bioprospecting—tropical endophytes) and stored at -20°C in a glycerol solution (20%) in the strain collection of Universidad Icesi.

### 3.2 | Phenotypic and molecular identification of the isolated endophytic fungus

After incubation on Sabouraud dextrose agar (SAB-Merck®), we observed white-pinkish (surface and reverse), cottony or woolly textured colonies of 5 cm in diameter, with no exudate. Septate, hyaline, and branching hyphae were microscopically (10× y 40×) observed, as well as hyaline, fusiform, curved, and septate macroconidia (Figure 2a),



**FIGURE 1** *Fusarium oxysporum* isolated from leaves of *Otoba gracilipes* in (a) PDA media, (b) Sabouraud dextrose agar media, and (c) *Otoba gracilipes* ([https://fm-digit.al-assets.fieldmuseum.org/176/073/MYRI\\_otob\\_grac\\_col\\_1760717.jpg](https://fm-digit.al-assets.fieldmuseum.org/176/073/MYRI_otob_grac_col_1760717.jpg))

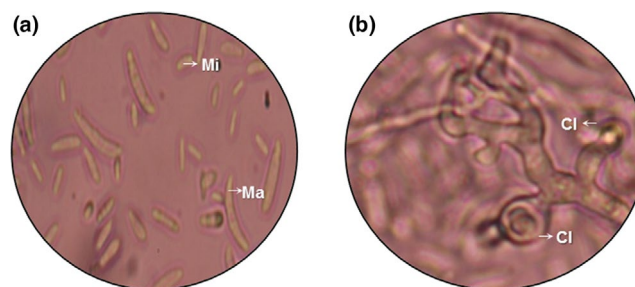
and ovoid to cylindrical microconidia (Figure 2a). Additionally, we found globular chlamydospores (Figure 2b) formed individually or in pairs. The identification of EBB-ET-01 based on morphological and microscopic features revealed the fungus was *Fusarium* sp.

Molecular identification was done using the amplified fungal sequences (ITS and Di-D2 regions) as BLAST queries against the NCBI database in GenBank (Table 1), with a 99% match with related fungi. Sequence was deposited in GenBank with accession number MN038127.1 (<https://www.ncbi.nlm.nih.gov/nuccore/1679350429>).

### 3.3 | Antioxidant activity assay of fungal crude extracts

The DPPH free radical scavenging assay is widely used for testing antioxidant activity because it changes in coloration from intense violet to bright yellow when reacting with antioxidant compounds (García et al., 2012). Correspondingly, a higher percentage of scavenging corresponds to a higher antioxidant activity of the isolate being tested. In this study, the crude extract from the PDB culture had a scavenging of 51.5%, which was significantly higher than the PDYB culture (26.4%) ( $F_2 = 2,299.7$ ;  $p < 0.001$ ). Moreover, the fungal isolates from the PBD culture had the highest antioxidant activity because the DPPH free radical was inactivated in more than 50% after 5 min of reaction (Figure 3). These results suggest that PDB presumably induced a higher synthesis of secondary metabolites responsible for the observed bioactivity.

On the other hand, it was impossible to compare the equivalent  $IC_{50}$  for gallic acid as DPPH radical inhibitor (0.85  $\mu$ M) with the same values used for the crude extract, because these contained a no purified mixture of polysaccharides.

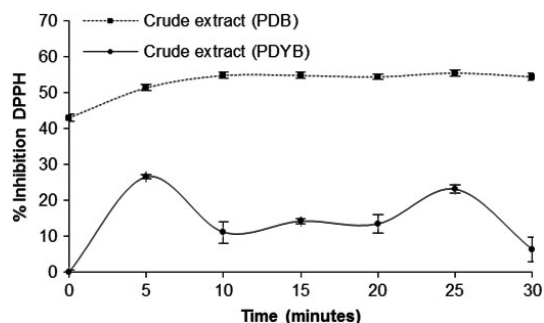


**FIGURE 2** Conidia of the endophytic fungus *Fusarium oxysporum* (EBB-ET-01). (a) Macroconidia (Ma) and microconidia (Mi). (b) Chlamydospores (Cl)

Recent studies have shown that endophytic fungi synthesize EPS involved in plant-endophyte interactions and that such biopolymers are characterized by structures that exhibit antioxidant activity (Chen et al., 2011; Liu et al., 2017; Orlandelli et al., 2017). In this study, an endophyte crude extract obtained from the leaves of *Otoba gracilipes* showed a 51.5% of antioxidant activity in 5 min. This is much higher than the 20% scavenging activity of DPPH reported by 2011) after 30 min of an EPS obtained from the culture of the strain *Fusarium oxysporum* *Dzf17* and with a 10-fold lower concentration (200  $\mu$ g/ml). However, in Li et al., 2011, crude extracts were subjected to deproteination and decolorization processes. Using a different approach, 2017) isolated two endophytes (*Aspergillus* sp. y *Fusarium* sp.) from roots of a close relative of *Otoba gracilipes*, a *Virola* sp. tree, and found an  $EC_{50}$  of 17.4  $\mu$ g/ml for antioxidant activity for the crude extracts of *Aspergillus* sp. Such activity was also measured on DPPH and found to be related to the presence of secondary metabolites such as flavonoids. However,

**TABLE 1** Identification of fungal isolates from *Otoba gracilipes* by sequencing of the internal transcribed spacer 1 (ITS1) and the D1-D2 region of rRNA gene compared with sequences listed in GenBank

Region amplified by PCR from cultured fungus	GeneBank information						Accession N in GenBank
	Sequences producing significant alignments	Max score	Total score	Query cover	E value	Ident	
ITS1	<i>Fusarium oxysporum</i> isolate MC-26-F internal transcribed spacer 1.	962	962	100%	0.0	99%	KU527806.1
D1-D2	<i>Fusarium oxysporum</i> genomic DNA sequence contains 18S rRNA gene	1,078	1,078	98%	0.0	99%	LT746254.1

**FIGURE 3** DPPH radical scavenging activity of crude extract obtained from *Fusarium oxysporum* strain EBB-ET-01 growing in potato dextrose broth (PDB) or potato dextrose broth and 0.5% yeast extract (PDYB) at pH 6.0 and 29°C for 30 days

no antioxidant activity was detected for crude extracts obtained from *Fusarium* sp. Thus, the high performance of culture media extracts of *Fusarium oxysporum* in this study suggests that the novel fungal strain we describe here has a greater potential for producing exopolysaccharides with antioxidant activity. Given that in our study the fungus was isolated from plant leaves and not from roots, it is possible that the production of secondary metabolites with antioxidant activity is tissue-dependent. In addition, it is important to mention that exopolysaccharides with antioxidant activity were detected as secondary metabolites; therefore, the production of these compounds should be measured at different developmental stages of the fungal endophyte.

Considering that the antioxidant mechanisms of EPS are complex (Freitas, Torres, & Reis, 2016; Li et al., 2011), additional methods should be used in further investigations to re-evaluate and compare the percentage of scavenging such as OH free radical, ABTS (Pan, Su, Cai, & Wei, 2017), and reducing powder. Simultaneously, studies on the chemical structure of EPS should be performed considering *Fusarium oxysporum* (EBB-ET-01) as a potential candidate for the production of bioactive compounds.

Finally, it is important to note that we found a high antioxidant activity of the crude extracts of only one endophytic fungus isolated from a single plant species (*Otoba gracilipes*) from a particular tropical forest in Colombia. Thus, there is still much to explore in the potentially hugely diverse array of fungal endophytes present across tropical ecosystems.

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## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

N.H.C., A.F.D, and P.A.C conceived and designed experiments and contributed to the writing of the manuscript. P.A.P and A.Y.R conducted experiments.

## ETHICS STATEMENT

Protocols and procedures employed in this investigation were reviewed and approved by the appropriate institutional review committee. *Otoba gracilipes* and *Fusarium oxysporum* were kept and handled in compliance with the guidelines of the "Autoridad Nacional de Licencias Ambientales (ANLA)-Colombia" through permission "Permiso Marco de Recolección de Especímenes Silvestres de la Diversidad Biológica con fines de Investigación Científica No Comercial-Resolution 00,272, 14 March 2017." For molecular identification of *Fusarium oxysporum*, The Universidad Icesi have the permission "Contrato de Acceso a Recursos Genéticos y sus Productos Derivados" from "Ministerio de ambiente y desarrollo sostenible, Colombia-Resolution 0,763, 9 May 2018."

## DATA AVAILABILITY STATEMENT

All data are provided fully in the Results section of this paper. The DNA sequence of ITS region from *Fusarium oxysporum* was

submitted to NCBI with accession number MN038127.1 and is available at (<https://www.ncbi.nlm.nih.gov/nuccore/1679350429>).

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