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RESEARCH PAPER

Antifungal evaluation of TiO_2 nanoparticles that inhibit *Fusarium solani* in African oil palm

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Abstract

S.A. Monclou-Salcedo, S.N. Correa-Torres, M.I. Kopytko, C. Santoyo-Muñóz, D.M. Vesga-Guzmán, R. Castellares-Lozano, M. López Amaris, A.D. Saavedra-Mancera, and A.P. Herrera-Barros. 2020. Antifungal evaluation of TiO_2 nanoparticles that inhibit *Fusarium solani* in African oil palm. Int. J. Agric. Nat. Resour. 126-133. The ability of titanium dioxide nanoparticles (TiO_2 NPs) to attenuate and control several microorganisms was studied, especially for a disease that affects the health and productiveness of crops. For this purpose, the antifungal capacity of TiO_2 nanoparticles obtained from two synthesis procedures was evaluated: the first procedure involved green chemistry using an aqueous extract prepared from African palm oil (*Elaeis guineensis Jacq*.) leaves; the second procedure followed the Pechini sol-gel method. The nanoparticles obtained by the green chemistry method and the Pechini sol-gel technique had average sizes of 14.60±0.44 nm and 12.30±0.54 nm as determined by scanning electron microscopy (SEM), respectively. The antifungal properties of TiO_2 NPs were evaluated on the fungus *Fusarium solani* Mart. isolated on Sabouraud culture medium. A factorial experimental design was implemented with two variables: (1) exposure time ranging from 24, 48, and 72 h; (2) nanoparticle concentration between 100 and 400 mg L^{-1}. All measurements were performed in quintuplicate. The results show that the TiO_2 nanoparticles synthesized by the Pechini method inhibited the fungus by 96.16±0.85% with a nanoparticle concentration of 400 mg L^{-1}, while the TiO_2 nanoparticles obtained from the green chemistry method generated a high inhibitory activity of approximately 98.51±0.02% at the four experimental concentrations.

Keywords: Antifungal property, biotechnology, synthesis of nanoparticles, titanium dioxide nanoparticles.

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Introduction

Bud rot disease (BRD), which is mostly caused by the fungus *Fusarium solani*, has been considered the most critical problem in the palm oil crop areas of Colombia. In 2011, it was reported that 100,000 hectares of palm oil crops were affected by these fungi, mainly in Puerto Wilches municipality in Santander, Colombia. Several attempts to eradicate BRD have been implemented; for example, the complete elimination of affected crops as well as the usage of heavy-metal high-content fertilizers and pesticides, which cause damage to the soils and underground and surface water contamination (Drenth *et al.*, 2013).

Nanoparticles (NPs) such as Cu, Zn, and Ag have been gradually incorporated in agriculture for fungal control (Pariona *et al.*, 2018). Esparza and coworkers observed that nanoparticles have excellent potential against phytopathogenic fungi in nanoparticle concentration-dependent manner (Esparza, 2015). There are several methods to synthesize nanoparticles (Medina *et al.*, 2015) and (Naranjo *et al.*, 2017); green chemistry is one of the most environmentally friendly methods and involves phytochemical components from vegetable extracts. In this work, we report the green synthesis of TiO_2 nanoparticles using natural plant extracts with a composition of terpene and flavonoids. Furthermore, TiO_2 nanoparticles were obtained through the Pechini method (Ochoa *et al.*, 2009), and their ability to inhibit the fungus *F. solani* was evaluated to provide alternatives to control bud rot disease (BRD) in African oil palms.

Materials and methods

Phylogenetic analysis of Fusarium solani

Infected tissues were seeded in selective culture medium; to accurately identify the inoculated organisms in the samples, the fungus was analyzed at the Center for Research and Biotechnology - Corpogen Corporation through an isolation and purification procedure. DNA was isolated followed by PCR amplification of the genetic region called the ITS (internal transcribed spacer) of the fungal ribosomal DNA with the initiators ITS4 and ITS5. Then, the PCR fragments were purified and sequenced with the primers ITS1 and ITS. A taxonomic analysis of the assembled sequence was performed by comparison against the database available from the NCBI (National Center for Biotechnology Information), UNITE (Unified System for the DNA-based fungal species linked to the classification), and the Warcup Fungal ITS, and the taxonomic classification of the consensus sequence was generated.

F. solani *samples*

Fungal isolation was initiated with sampling; leaves and buds from African oil palm (*Elaeis guineensis*) were gathered in a palm farm from Puerto Wilches municipality, Santander, Colombia, at 7° 27’ 081’’ N, -73° 580’ 57’’ W, over 75 m.a.s.l. and were characterized by applying a punctual selection method for sick plants with bud rot disease (BRD). Samples were stored and then analyzed in laboratories at the University Pontificia Bolivariana, Sectional Bucaramanga, Colombia.

Bioassays and fungus isolation

The leaves with infected tissue were washed with distilled water and sodium hypochlorite solution (NaClO) at 6% (v/v) and pH 12 (1.22 – 1.25 g cm^{-3} at 20 °C) (Merck). Later, the leaves were washed with ethanol at 96% for 5 min. Then, they were washed with sterile distilled water and dried with Whatman paper filters (Vásquez Cruz *et al.*, 2016). Sowing and analysis of fungal cultures were performed through a DNA isolation and purification process followed by PCR amplification. For inhibition tests, the fungal radial growth percentage was calculated considering the variable fungal radius developed in the presence of TiO_2 NPs and developed in the absence of titanium dioxide nanoparticles (control sample).

African palm (E. guineensis) *leaf extract*

The extraction of organic compounds present in the African oil palm leaves was performed by the Soxhlet technique, where 6 g of cut leaves were weighed (Mora Cruz, 2011). Then, 150 mL of hexane (0.66 g cm^{-3} at 20 °C) was added. The system was operated for 3 h at 70 °C. The obtained extract was concentrated in a rotary evaporator. The results of vegetable extraction were analyzed through solid phase microextraction (SPME) with gas chromatography coupled to a selective detector for mass spectrometry, model AT 6890 (GC/MS). The fiber used for extract absorption was fused silica covered with PDSM/DVB (Pino, 2017).

TiO_2 nanoparticle synthesis

Titanium dioxide nanoparticles (TiO_2 NPs) were synthesized by the green chemistry method. For this, 15 mL of the plant extract was used to reduce the inorganic salt precursor. A solution of titanium tetra-iso propoxide (TTIP) was prepared at a concentration of 5 mM and centrifuged at 5000 rpm for 15 min. Finally, the supernatant was removed, and three washes with ethanol, distilled water, and ethanol were performed. The nanoparticles were dried at room temperature and calcinated at 550 °C for 4 h.

Another chemical synthesis was carried out using the Pechini method (Vargas Urbano *et al.*, 2011) modified by using the alkoxide compound TTIP as the precursor solution. For the preparation, 21 mL of polyethylene glycol ($C_{2n}H_{4n+2}O_{n+1}$) (pH 5 - 7 at a concentration of 100 g L^{-1} and 20 °C) and 17.5 g of citric acid (pH 1.7 at 100 g L^{-1} and 20 °C) were mixed in proportions of 4:1 at 70 °C for 20 min. Afterward, 5 mL of TTIP was added under continuous stirring by applying ultrasound. Then, 23 mL of ammonium hydroxide (25% v/v at pH 12) was added to ensure that the mixture was transparent and to maintain a basic pH (8.3– 9). The mixture was heated for 40 min at 120 °C with constant agitation to perceive black resin formation. Finally, the resin was precalculated for 3 h at 350 °C and then calcined for 7 h at 450 °C, changing into a white powder as evidence of TiO_2 nanoparticle formation.

Nanoparticle characterization

After TiO_2 NP synthesis, the presence and size of the nanoparticles were determined through scanning electron microscopy (SEM) (Odziomek *et al.*, 2017). This process was carried out in an Electronic Microscope MIRA3 FEG 650 Tesco brand Electronic Microscope MIRA3 FEG 650, which provided the data with "high vacuum mode" using a backscattered electron detector (BSED).

Statistical analysis

To evaluate the inhibition of the fungus *F. solani*, a multifactorial design was applied, and two factors were established: (1) exposure time of TiO_2 NPs with the fungus, ranging from 24, 48, and 72 h; and (2) nanoparticle concentration of 100, 200, 300, and 400 mg L^{-1}, where the response was fungal radial growth. All tests were performed in quintuplicate. With the obtained results, an-ANOVA statistical analysis was performed on the dependent variables.

Fungus tolerance

Analysis of variance multifactorial ANOVA was performed to corroborate the proposed hypotheses during experimentation. The results showed that if $F(3.7587)>F\text{-critical}(2.4844)$ and $p\geq0.05$, the increments in TiO_2 NP concentrations directly affected the fungal radial growth rate.

Results and discussion

Phylogenetic identification of Fusarium solani

Figure 1 shows the fungal characterization results, including the molecular typing and identification of the sample, including the species name, percentage of identity, and NCBI (National Center for Biotechnology Information) search.

Gas chromatography coupled to mass spectrometry (GC/MS):

The African-oil-palm leaf extract was analyzed using GC/MS operated in full scan mode. The chromatograms displayed three higher abundance peaks, which corresponded to retention times of 20.003, 21.607, and 28.392 min. These peaks were related to the chemical species β-myrcene, p-cymene, and methyl salicylate, respectively. These chemical compounds form part of the phytocomponents present in the natural extract, including terpenes and flavonoids, as reported by Esequiel and coworkers, who indicated the possibility of using them for medicinal treatments (Esequiel *et al.*, 2018). These phytocomponents can work as reducing agents in the formation of TiO_2 nanoparticles, as described by Solano and coworkers, who synthesized TiO_2 NPs using an aqueous lemongrass extract with a high concentration of terpenes and flavonoids (Solano *et al.*, 2019).

Characterization of synthesized nanoparticles:

TiO_2 nanoparticles obtained from green chemistry and the Pechini method were analyzed using scanning electron microscopy (SEM). Figure 2 shows the images of these nanoparticles; for both samples, a highly agglomerated hemispherical form was observed. Based on these measurements, the TiO_2 nanoparticles prepared via green chemistry had an average size of 14.60 ± 0.39 nm (Figure 2a), which agrees with the results reported by Solano and coworkers (Solano *et al.*, 2019). Moreover, the TiO_2 nanoparticles prepared by the Pechini method had a size distribution of 12.30 ± 0.54 nm, indicating the ability of this technique to synthesize nanomaterials.

A normal distribution was observed, corroborating the size of the synthesized TiO_2 NPs; a standard

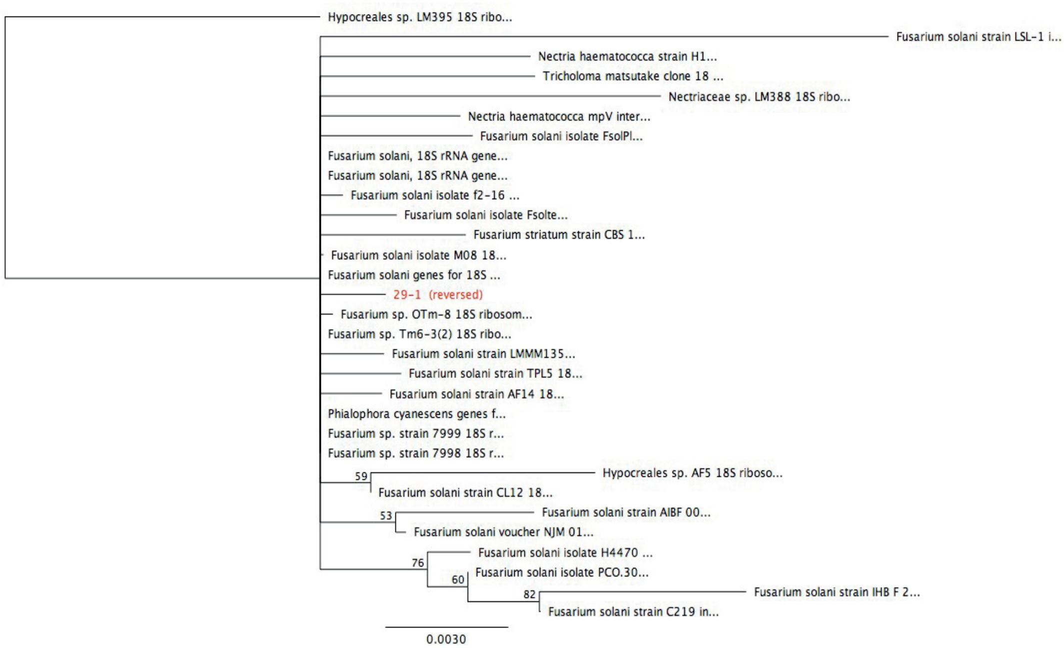

Figure 1. Phylogenetic tree of the fungus *F. solani* identified in African palm oil leaves (*Elaeis guineensis*) affected by BRD.

deviation of 4.4% for the nanoparticles prepared by green chemistry and 2.7% for those synthesized through the Pechini method was obtained. The small size and sharp size distribution observed for these nanomaterials suggest that both methods are suitable to render nanomaterials with high surface area, and these nanomaterials can be applied for antifungal treatments.

Inhibitory capacity of TiO_2 *nanoparticles:*

It has been reported in the literature that the antimicrobial and antifungal properties of titanium dioxide nanoparticles that allow the photodegradation of bacteria and fungi are caused by the photoactivation of the TiO_2 anatase phase under visible light (Betancur *et al.*, 2016), (Vargas & Rodríguez, 2013). In the present study, after 48 h of exposure, TiO_2 nanoparticles prepared by the Pechini method caused 100% inhibition of the growth of the fungus *F. solani*. However, after 72 h of exposure, it was noted that the growth of the fungus started to decrease at a TiO_2 NP concentration of 400 mg L^{-1}; the average *F. solani* inhibition was estimated to be on the order of 96.16 ± 0.85%

A similar behavior was observed for the nanoparticles synthesized by the green chemistry method, which also inhibited 100% of the growth of *F. solani* during the first 48 h of exposure. After 72 h of exposure with a low nanoparticle concentration of 100 mg L^{-1}, a slight decrease in inhibition was detected, showing an average inhibition of over 98.51 ± 0.09%; this result suggests the versatility of the eco-friendly TiO_2 nanoparticles, which exhibit a better inhibition of the fungus *F. solani*. Table 1 shows the results obtained during the experimentation process. In addition, Figure 3 shows images for the growth of the fungus *F. solani* after 72 h of exposure to the synthesized TiO_2 nanoparticles in Sabouraud culture medium.

The main conclusions are as follows. Phylogenetic analysis of a sample isolated from an African palm oil leaf affected by bud rot disease (BRD) revealed that the sequence of the isolated fungus had 99% similarity with the ITS sequences belonging to the species *F. solani, Fusarium* sp., or *Phialophora cyanescens*. Scanning electron microscopy (SEM) analysis demonstrated the possibility of synthesizing titanium dioxide nanoparticles using the phytocomponents present in a natural extract prepared with healthy African palm oil leaves (*Elaeis guineensis*)

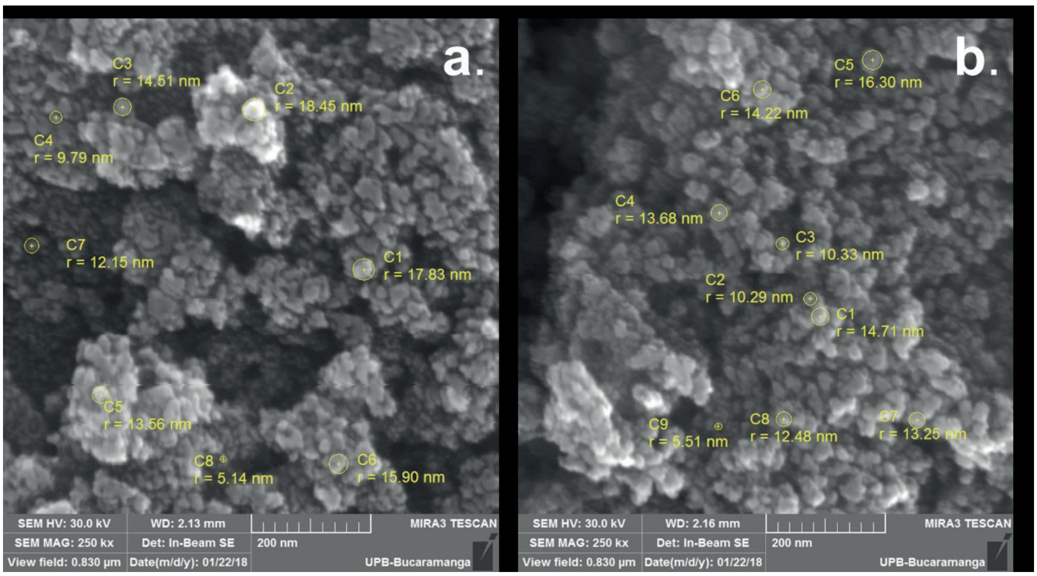

Figure 2. SEM images of TiO_2 nanoparticles synthesized by (a) green chemistry and (b) the Pechini method.

as a reducing agent for the alkoxide precursor TTIP Ti[OCH(CH_3)]$_4$, and nanoparticles with an average size of 14.60 ± 0.39 nm were obtained. Moreover, it was possible to obtain TiO_2 NPs by applying the Pechini method (sol-gel), and nanomaterials with an average size of 12.30 ± 0.54 nm were obtained. In general, TiO_2 nanoparticles decelerated the growth of the fungus *F. solani,* generating an average inhibition of 96.16 ± 0.85% after 72 h for nanoparticles prepared with the Pechini method with the highest evaluated nanoparticle concentration (400 ppm). Similar results were observed on the inhibition of the fungus *F. solani* after 72 h of exposure with TiO_2 nanoparticles prepared from the green chemistry method, showing an inhibition of 98.51 ± 0.09%; however, with a lower nanoparticle concentration (100 ppm), the applied TiO_2 nanoparticles could control pests in African palm crops depending on the nanoparticle concentration, environmental conditions, and microbiological composition present in the palm.

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Table 1. Evaluation of the growth of *F. solani* in Sabouraud culture medium and exposed to TiO2 nanoparticles as a function of exposure time and nanoparticle concentration.

NP Concentration (mg L^{-1})	Growth of the fungus *Fusarium solani* after exposure to TiO_2 NPs (%)								
	24 h			48 h			72 h		
	Control	Green Chemistry TiO_2 NPs	The Pechini Method TiO_2 NPs	Control	Green Chemistry TiO_2 NPs	The Pechini Method TiO_2 NPs	Control	Green Chemistry TiO_2 NPs	The Pechini Method TiO_2 NPs
100	67	0	0	89	0	0	93	1,42	4,01
200		0	0		0	0		1,50	8,6
300		0	0		0	0		4,42	1,14
400		0	0		0	0		1,61	1,63

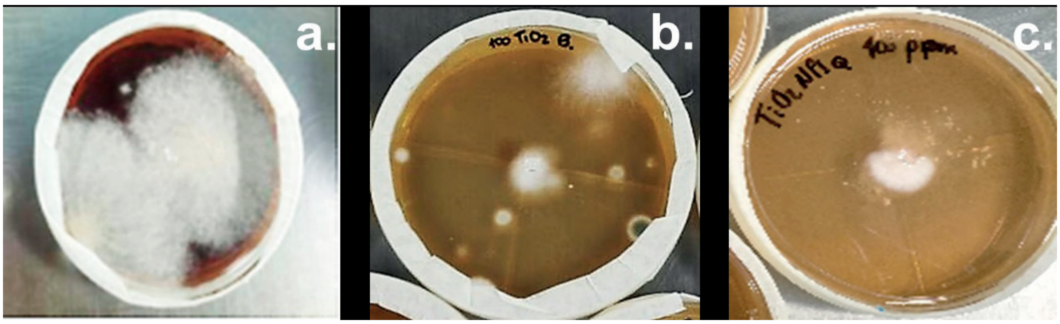

Figure 3. Growth of the fungus F. solani after 72 h in Sabouraud culture medium. (a) Control sample, (b) sample exposed to 100 mg L^{-1} TiO_2 NPs synthesized by green chemistry, and (c) sample exposed to 400 mg L^{-1} TiO_2 NPs obtained by the Pechini method.

Resumen

S.A. Monclou-Salcedo, S.N. Correa-Torres, M.I. Kopytko, C. Santoyo-Muñóz, D.M. Vesga-Guzmán, R. Castellares-Lozano, M. López Amaris, A.D. Saavedra-Mancera, y A.P. Herrera-Barros. 2020. Evaluación antifúngica de nanopartículas de TiO_2 para inhibición de *Fusarium solani* en Palma Africana. Int. J. Agric. Nat. Resour. 126-133. La efectividad de nanopartículas de dióxido de Titanio ($NPsTiO_2$) en la atenuación y control de varios microorganismos ha sido material de estudio en especial para control de enfermedades que atentan contra la salud y productividad en cultivos agrícolas. El presente estudió evaluó la propiedad antimicrobial de nanopartículas de dióxido de Titanio ($NPsTiO_2$) obtenidas a partir de dos métodos de síntesis: uno vía química verde, mediante la utilización de extractos naturales de Palma de Aceite Africana (*Elaeis guineensis);* otro por mecanismo sol-gel, por medio de la metodología Pechini. Por medio de la técnica microscopía electrónica de barrido (SEM) se determinó tamaños promedio de partícula de 14.60 ± 0.44 nm y 13.16 ± 0.26 nm respectivamente a cada método. Las propiedades antimicrobiales de las $NPsTiO_2$ fueron evaluadas sobre el hongo *Fusarium solani*, aislado en medio de cultivo Sabouraud, en un diseño experimental multifactorial con dos variables: el tiempo de contacto en un rango de 24, 48,72 h y concentraciones entre 100 a 400 mg L^{-1} en muestras por quintuplicado. Los resultados evidencian que las concentraciones de 400 mg L^{-1} de nanopartículas sintetizadas por método Pechini promueven una inhibición de 96.16 ± 0.85 %, mientras que las obtenidas a partir de química verde generaron una gran actividad inhibitoria de 98.51 ± 0.09 % constante a lo largo de las cuatro concentraciones experimentadas.

Palabras clave: Biotecnología, nanopartículas de dióxido de titanio, propiedad antimicrobial, síntesis de nanopartículas.

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