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Characterization and *in vitro* Application of Aqueous Extracts from Nance Fruit for the Control of *Colletotrichum asianum*

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ABSTRACT

The nance (*Byrsonima crassifolia*) is renowned for its diverse applications in food, medicine, and pharmaceuticals. However, due to limited industrialization and distribution, around 60% of total production is lost. This study aims to utilize nance fruit as a valuable source of aqueous extracts for controlling *Colletotrichum asianum*. The research involves quantifying and identifying phenolic compounds (PC) in the extracts and conducting *in vitro* tests to evaluate mycelial growth, sporulation, and germination. Key PC identified in the extracts include gallic acid, catechin, and galocatechin. Four treatments with varying aqueous extract concentrations (0%, 1%, 0.5%, and 0.1%) were used. The most notable mycelial growth inhibition occurred at 0.1%, with a $34.7\% \pm 1.60$. In the sporulation test, the 1% concentration showed the lowest spore count at 2.5×10^5 . The 1% concentration also exhibited the highest germination inhibition 89%. The assessment of antifungal potential in bioactive compounds from nance (*Byrsonima crassifolia*) exhibited significant *in vitro* antifungal efficacy against *Colletotrichum asianum*, a critical pathogen in mango cultivation causing anthracnose. Nance aqueous extracts show promise as an eco-friendly alternative for anthracnose treatment in mango cultivation.

Keywords: bioactive compounds, inhibition, fruit pathogen, fruit bioactives.

Caracterización y aplicación *in vitro* de extractos acuosos de fruto de nance para el control de *Colletotrichum asianum*

RESUMEN

El nance (*Byrsonima crassifolia*) es conocido por sus diversas aplicaciones en la alimentación, la medicina y la farmacología. Sin embargo, debido a su limitada industrialización y distribución, alrededor del 60% de la producción total se pierde. Este estudio tiene como objetivo utilizar el fruto de nance como una fuente valiosa de extractos acuosos para controlar al hongo *Colletotrichum asianum*. La investigación implica cuantificar e identificar a los compuestos fenólicos (CF) en los extractos del fruto y realizar pruebas *in vitro* para evaluar el crecimiento micelial, la esporulación y la germinación. Los CF clave identificados fueron el ácido gálico, la catequina y la galocatequina. Se emplearon cuatro tratamientos con diferentes concentraciones de extracto acuoso (0%, 1%, 0.5% y 0.1%). La inhibición más notable del crecimiento micelial ocurrió al 0.1%, con un $34.7\% \pm 1.60$. En la prueba de esporulación, la concentración del 1% no solo mostró el menor recuento de esporas a 2.5×10^5 sino también en la germinación con un 89%. La evaluación del potencial antifúngico en los compuestos bioactivos del nance (*B. crassifolia*) mostró una significativa eficacia antifúngica *in vitro* contra *C. asianum*, un patógeno crítico en el cultivo de mango que causa la antracnosis. Los extractos acuosos de nance muestran su eficacia para ser utilizados como una alternativa ecológica en el tratamiento de la antracnosis en el cultivo de mango.

Palabras clave: compuestos bioactivos, inhibición, patógeno de frutas, bioactivos de frutas.

INTRODUCTION

The nance (*Byrsonima crassifolia*), a species indigenous to Mesoamerica, exhibits a natural distribution spanning from Mexico to Panama. Revered for its delectable, bittersweet, and mildly acidic fruits, the nance finds applications in various consumables such as jellies, soft drinks, and ice creams (Alves & Franco, 2003). Flourishing in warm, semi-warm, and temperate climates, the fertile habitat of México fosters its prolific growth. The Agrifood and Fisheries Information Service (SIAP) reported a substantial production of 7.1 thousand tons in 2016 alone (SIAP, 2021), creating a promising avenue for diverse nance-based products. Like many fruits, nance serves as a rich source of potentially functional compounds, particularly phenolic compounds. While previous studies have explored the antifungal properties of phenolic compounds in fruits for plant disease control (Ansari, M. A., Anurag, A., Fatima, Z. & Hameed, S., 2013; Li & Jennings, 2017; Mohammadi, A., Nazari, H., Imani, S. & Amrollahi, H., 2014), there is a notable absence of research on nance as an antifungal agent. Despite its abundance in phenolic compounds with antimicrobial potential, there remains an unexplored potential. This research aims to assess the viability of nance fruit as a source for extracting biologically relevant compounds with antifungal properties for controlling *Colletotrichum asianum* an important pathogen of mango fruits.

MATERIALS AND METHODS

Collection and preparation of the sample

The samples were sourced from local distributors at the Tepic, Nayarit market. Stringent criteria were applied to ensure they reached the necessary physiological maturity for handling. Initially, selected samples underwent a thorough wash with running water to eliminate impurities and solid particles. Subsequently, they were disinfected, washed with a commercial detergent (1.5% w/v), and finally rinsed with distilled water. The samples were then homogenized using an ultraturrax (T25, IKAworks, Wilmintong, NC) and subjected to lyophilization using a Labconco model 77522020 (Kansas, USA). Following lyophilization, the samples were further processed through grinding, sieving, and stored hermetically for subsequent use.

Aqueous Extraction of phenolic compounds from nance (*Byrsonima crassifolia*)

The aqueous extracts of nance were obtained using the method proposed by Cortés-Rivera, H. J., Blancas-Benitez, F. J., Romero-Islas, L.C., Gutiérrez-Martinez, P. & González-Estrada, R. R. (2019), by mixing 0.5 g of sample with 25 mL of SDW, later shaken for 1 h, and then centrifuged, to be later filtered with acrodisk (MilliporeTM, 0.45 µm, Darmstadt, Germany). The extract solutions were stored in amber flasks prior to the experiments.

Phenolic compounds determination and antioxidant capacity of Bioactive Compounds in nance aqueous extracts

Phenolic compounds (PC) contents were determined in the extracts, previously obtained, this determination was carried out following the Montreau method (1972), with modifications by Alvarez-Parrilla, E., Rosa, L. A. D. La, Amarowicz, R. & Shahidi, F. (2012). This method is based on the formation of phosphomolybdic-phosphotungstic complexes, as the molybdate and sodium tungstate salts contained in the reagent react with any type of phenol. Subsequently, with a pH change, the phosphomolybdic-phosphotungstic complexes are reduced to intensely blue chromogenic oxides of tungsten and molybdenum. The intensity of this blue color is proportional to the number of hydroxyl groups in the molecules, enabling their spectrophotometric quantification against a gallic acid standard curve.

A total of 250 µL of the sample extract was added to 1250 µL of 10% Folin Ciocalteu and the pH was changed with 1 mL of 7.5 % sodium carbonate. The mixture was incubated in a water bath at 50 °C for 15 minutes. After the incubation time, it was cooled to room temperature. A volume of 270 µL was transferred to the microplate wells and spectrophotometrically read at 750 nm using a microplate reader (Biotek Synergy HT). Gallic acid was used as standard (0.0125–0.2 mg/mL, $R_2 \geq 0.9997$) and results are expressed as mg of gallic acid equivalents per gram of sample on a dry weight basis (mg GAE/g DW).

Free Radical Scavenging Activity (DPPH)

The assessment of free radical scavenging activity was conducted using the DPPH assay, following the methodology outlined by Usia, T., Banskota, A. H., Tezuka, Y., Midorikawa, K., Matsushige, K. & Kadota, S. (2002) with slight adjustments. Samples of PC were dissolved in methanol (100 µL) and combined with 300 µM DPPH solution (100 µL) in a flat 96-well plate (Costar, Corning, Corning, NY, USA). The mixture was then incubated in darkness for 30 minutes. Subsequently, the absorbance was measured at 517 nm using an ELISA plate reader (Multiskan GO, Thermo Scientific, Waltham, MA, USA). Results were expressed as millimol of trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) equivalents per gram DW (mmol TE/g DW).

Ferric reducing antioxidant power (FRAP)

The assay was performed as described by Benzie & Strain (1996), with some modifications. FRAP solution 10:1:1 (v/v/v) dissolved in a sodium acetate buffer (0.3 M; pH 3.6), TPTZ-HCl (10 mM, 40 mM), and ferric chloride hexahydrated (20 mM) was warmed to 37 °C before mixing it with the samples. Briefly, 24 µL of sample from the aqueous extraction was mixed with 180 µL of FRAP solution and the absorbance was measured at 595 nm after 30 min using a microplate reader

(Multiskan GO, Thermo Scientific, Waltham, MA, USA). Results were expressed as millimol of trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic) equivalents per gram DW (mmol TE/g DW).

Identification of Bioactive Compounds in nance aqueous Extracts Using HPLC-DAD

Phenolic acids were identified using an HPLC-DAD system (Agilent 1200, Santa Clara, CA) equipped with a Zorbax Eclipse Plus C18 reversed-phase column (4.6 mm x 100 mm, 3.5 μ m), following the methodology outlined by Ayala-Soto, F. E., Serna-Saldivar, S. O., García-Lara, S. & Pérez-Carrillo, E. (2014) with some modifications. Operating conditions included an injection volume of 5.0 μ L and a column temperature of 30 °C. The elution gradient consisted of water acidified with trifluoroacetic acid at pH 2 (A) and acetonitrile (B) as follows: 15% B at 0 min, 35% B after 10 min, 55% B at 11 min, all at a flow rate of 0.6 mL/min; 75% B at 12 min with a flow rate of 0.8 mL/min; and 100% B at 13 min with a flow rate of 1.0 mL/min, maintaining a temperature of 25 °C.

Phenolic compound characterization was performed by comparing the retention time (RT) observed in the diode array detector (DAD) with the RT of analytical standards and previously reported data (Abu-Reidah, I. M., Ali-Shtayeh, M. S., Jamous, R. M., Arráez-Román, D. & Segura-Carretero, A., 2015; Arce-Reynoso, A., Mateos, R., Mendivil, E. J., Zamora-Gasga, V. M. & Sáyago-Ayerdi, S., 2023; Durán-Castañeda *et al.*, 2023). External calibration curves were used to quantify the identified compounds, including the calibration equations ($y = a + bx$), R^2 values, and the limits of detection (LOD) and quantification (LOQ) for each compound: Quercetin ($y = 1324209x + 78664$; R^2 : 0.9979; LOD 0.85 μ M/mL; LOQ 2.59 μ M/mL), Catechin ($y = 10910x + 115$; R^2 : 0.9909; LOD 1.77 μ M/mL; LOQ 5.37 μ M/mL), 3,4-Hydroxybenzoic acid ($y = 6945.7x + 11924$; R^2 : 0.9883; LOD 2.68 μ M/mL; LOQ 8.94 μ M/mL), Sinapic acid ($y = 17539x - 8742.1$; R^2 : 0.9981; LOD 1.29 μ M/mL; LOQ 4.30 μ M/mL), Ellagic acid ($y = 39246x + 31226$; R^2 : 0.995; LOD 1.31 μ M/mL; LOQ 3.97 μ M/mL), Gallic acid ($y = 36198x - 25330$; R^2 : 0.9966; LOD 1.08 μ M/mL; LOQ 3.27 μ M/mL).

In vitro Evaluation of Antifungal Activity

To assess the antifungal activity, the pathogenic fungus, previously isolated and identified from infected mango fruits (Moreno-Hernández, C. L., Zambrano-Zaragoza, M. L., Velázquez-Estrada, R. M., Sánchez-Burgos, J. A. & Gutierrez-Martinez, P., 2022), was cultivated on potato dextrose agar (PDA) at 27 °C for 6 days before the experiments.

Preparation of Treatments

The extracts obtained from nance were filtered using sterile acrodisks (Millex-HN filtration unit; 0.45 μ m, nylon, 33

mm) and subsequently incorporated into PDA medium at concentrations of 0.1%, 0.5%, and 1% (v/v) in a biosafety hood.

Mycelial Growth Test

For the evaluation, the poisoned agar technique was applied (Balouiri, M., Sadiki, M. & Ibsouda, S. K., 2016), with the extract addition following the protocol proposed by Cortés-Rivera *et al.* (2019). The agar plates were then incubated at 27 °C for four days. The control consisted of PDA medium without the extract. Antifungal activity was expressed as the percentage inhibition of mycelial growth (%IMD) and calculated using the following equation:

All tests were done in triplicate, and each experiment was repeated twice.

Sporulation

To assess the impact of different treatments on sporulation, we followed the procedure outlined by Gutierrez-Martinez, P., Ramos-Guerrero, A., Cabanillas-Beltran, H., Romero-Islas, J. & Cruz-Hernandez, A. (2015). Following the completion of the mycelial growth inhibition test, the Petri dishes were repurposed for the sporulation test. In brief, 8 mL of sterile distilled water was added to the fungal lawn and then scraped with a sterile glass rod to disrupt the mycelial structure, releasing the formed spores. The resulting spores were filtered using sterile gauze, and the spore concentration per mL was determined by counting in a Neubauer chamber with an optical microscope (Motic BA300, USA).

Germination Evaluation

The spore germination test followed the methodology described by Gutiérrez-Martínez *et al.* (2015) with some modifications. For the test, a spore suspension of 6-day-old of *C. asianum* was prepared using the protocol proposed by Cortés-Rivera *et al.* (2019). PDA plates were prepared to contain the extracts at 0.1%, 0.5%, and 1%. Discs of PDA (10 mm in diameter) were cut, inoculated with 10 μ L of the spore suspension, and incubated at 25 °C for 8 h. Germinated spores were counted using a microscope (Motic BA300, USA) at 40X magnification.

Statistical Analysis

Phenolic compounds determination and antioxidant capacity of bioactive compounds in nance aqueous extracts were conducted in triplicate, with two repetitions and mean and standard deviation were calculated for each determination. *In vitro* analyses of mycelial development, sporulation, and germination were also performed in triplicate with two repetitions and mean and standard deviation were calculated for each determination. To determine significant differences between concentrations (1%, 0.5% and 0.1% (v/v)), Statistica 10.0 Software for Windows (STATISTICA®, STATSOFT Inc., USA) was used with an analysis of variance (ANOVA) in a multifactorial design and an LSD test with a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Phenolic compounds (PC) and antioxidant capacity of Bioactive Compounds in Aqueous nance (*Byrsonima crassifolia*) Extracts

In general, PC is an important parameter in the study of fruits and their by-products due to its high content and contribution to antioxidant activity. Table I shows the content of PC in the nance extracts, obtaining values of 29.70 ± 1.58 g GAE/100 g DW. Whole fruits are generally a good source of PC, such as apples that contain close to 1.81 g GAE/100 g DW (Sun, J., Chu, Y.-F., Wu, X. & Liu, R. H., 2002); pink guava, 0.61 g GAE/100 g DW (Luximon-Ramma, A., Bahorun, T. & Crozier, A., 2003); mango, 0.34 g GAE/100 g DW (Lu *et al.*, 2021). However, these values are lower than those obtained in this study, indicating that nance could be an important source of PC, with potential antifungal effects (Simonetti, G., Brasili, E. & Pasqua, G., 2020). Antioxidant capacity has been evaluated in food products using various methodologies with different mechanisms. The antioxidant activities of PC in nance extracts are shown in Table I, the reported values are 184.87 ± 1.02 mMol TE/g y 34.37 ± 8.89 mMol TE/g obtained by the DPPH and FRAP methods, respectively. The major PC in nance are phenolic acids, which are small molecules, and therefore can react with the radical and be more reactive, resulting in higher DPPH values. The antioxidant capacity could be directly related to the antimicrobial potential of the

extracts, making nance a candidate to be used as a source of compounds with antifungal potential (Ahmadi, S., Ahmadi, G. & Ahmadi, H., 2022).

Identification of the main bioactive compounds present in aqueous extracts of nance by HPLC-DAD

The analysis of nance aqueous extracts using HPLC-DAD shows in Table II, gallic acid, a phenolic acid identified as the major PC in nance aqueous extracts, has previously demonstrated antimicrobial effects against certain fungi (Oulahal & Degraeve, 2022). For its part, catechin, a flavonoid identified in nance aqueous extracts, has demonstrated inhibitory effects against various microorganisms (Mora, A., Parra, J., Chaverri, J. M. & Arias, M. L., 2013). The presence of these bioactive compounds as sinapic acid and quercetin, suggests the potential antimicrobial properties of nance aqueous extracts, highlighting their significance in natural product-based research for microbial control.

Inhibition of mycelial growth, sporulation, and germination *in vitro* of *Colletotrichum asianum*

Table III illustrates the impact of different treatments on the mycelial growth, sporulation, and germination of *C. asianum*. Mycelial growth inhibition was relatively consistent across the three treatments, with 34.7%, 34.4%, and 32.9% inhibition observed at concentrations of 0.1%, 0.5%, and 1%, respectively.

Table I. Total Soluble Phenols (TSP) and Antioxidant Capacity in Aqueous nance (*Byrsonima crassifolia*) Extracts.^a

Sample	TSP (mg GAE/g)	Antioxidant Capacity	
		DPPH (mMol TE/g)	FRAP (mMol TE/g)
Aqueous Nance Extracts	29.70 ± 1.58	184.87 ± 1.02	34.37 ± 8.89

^aThe values are expressed as the mean \pm standard deviation of n=3 observations. GAE: Gallic Acid Equivalents. TE: Trolox equivalents.

Table II. Phenolic compounds identified and quantified by HPLC-DAD, in Aqueous Nance (*Byrsonima crassifolia*) Extracts.^a

Compound	RT (min)	Concentration (μ g/ mL)
Gallic acid	5.5	114.60 ± 5.06
Ellagic acid	17.7	6.63 ± 0.040
Sinapic acid	19.3	56.023 ± 2.10
3,4-Dihydroxybenzoic acid	9.9	17.9 ± 0.84
Quercetin	20.7	43.39 ± 2.63
Catechin	14.1	38.98 ± 0.05

^aThe data represent the mean \pm standard deviation (n=3).

Table III. Percentage of inhibition of mycelial growth, sporulation and germination of the different treatments during 4 days at 27 °C.^a

Treatments	1%	0.5%	0.1%
Mycelial growth (% of inhibition)	32.9±2.13 ^B	34.43±3.40 ^A	34.77±1.60 ^A
Sporulation (% of reduction)	98.29%	96.59%	96.59%
Germination (% of inhibition)	89%±1.5 ^A	72%±2.0 ^B	41%±3.0 ^C

^aThe values in a row followed by the same letter are not significantly different ($p < 0.05$).

The inhibitory effect on mycelial development is attributed to gallic acid and catechin present in the soluble fraction of nance. Gallic acid acts on polyphenoloxidase, leading to the accumulation of oxidation products that affect protein synthesis in saprophytic and phytopathogenic fungi (Gautam, A. K., Singh, P. K. & Aravind, M., 2020). Catechin induces rapid leakage of small molecules, causing liposome aggregation and potential damage to the cell membrane, resulting in conidia and hyphae lysis (Ncama, K., Mditshwa, A., Tesfay, S. Z., Mbili, N. C. & Magwaza, L. S., 2019). This aligns with findings reported by Toyoshima, Y., Okubo, S., Toda, M., Hara, Y. & Shimamura, T. (1994) regarding the impact of catechin on *T. mentagrophytes*. The compromised function of the cell wall due to gallic acid and catechin may result in structural damage and reduced hyphal length.

In the sporulation test, all treatments demonstrated significant effectiveness against *C. asianum*, with up to 98% reduction in sporulation. Notably, treatments did not exhibit the characteristic salmon coloration associated with spore formation, while the control showed spores in the PDA medium (Figure 1). This outcome may be linked to phenolic compound-induced damage to the mycelium, crucial for spore formation consistent with findings by Cortés-Rivera *et al.* (2019) on *Penicillium italicum*.

The germination test revealed that the 1% extract concentration maintained the highest inhibition of spore germination. Gallic acid, causing sterol damage vital for fungal cell membrane integrity, likely contributed to this inhibition (Li & Jennings, 2017). Results are in line with studies by Li & Jennings (2017) on the effects of gallic acid against *Candida albicans*, demonstrating a reduction comparable to the drug fluconazole.

In a recent study, Morelia-Jiménez *et al.* (2023) reported significant effects on the fungus *Fusarium musae* isolated from bananas exposed to natural extracts from coconut mesocarp. The authors showed promising results in the *in vitro* control of the fungus by affecting its mycelial growth, sporulation process, and germination. Additionally, micrographs in SEM analysis revealed that spores were unable to germinate in banana wounds treated with the aqueous extracts.

The growth cycle of *C. asianum* exhibited similar patterns across treatments, with extract concentration being a limiting factor. Influencing the growth cycle of pathogenic fungi is crucial for effective disease management. Affecting mycelial development, sporulation, and the germination process can help control or prevent fungal infection (De Silva, D. D., Crous, P. W., Ades, P. K., Hyde, K. D. & Taylor, P. W. J., 2017).

Although the results of the *in vitro* tests are promising, it is crucial to evaluate these treatments not only *in vivo* but also for their feasibility of application at the pre-harvest stage. Furthermore, it would be interesting to assess the economic feasibility of producing these natural treatments and their chemical stability in the future. These additional analyses would contribute significantly to the implementation of sustainable and economically viable solutions for crop protection in agriculture.

CONCLUSIONS

The assessment of antifungal potential in bioactive compounds from nance (*Byrsonima crassifolia*) exhibited significant *in vitro* antifungal efficacy against *Colletotrichum asianum*, a critical pathogen in mango cultivation causing anthracnose. Nance aqueous extracts show promise as an eco-friendly alternative for anthracnose treatment in mango cultivation.

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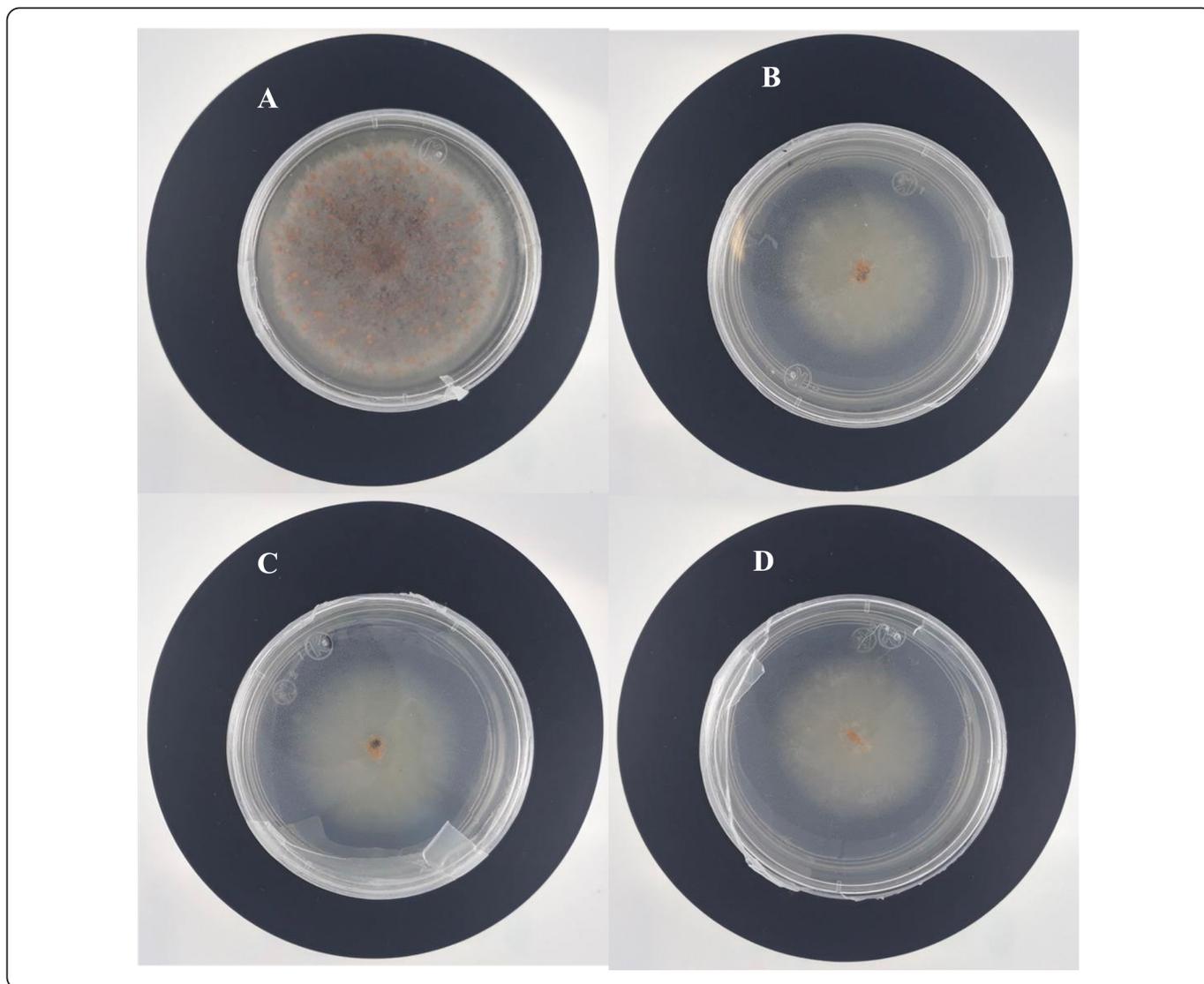


Figure 1. Final growth of *Colletotrichum asianum* after 4 days of incubation at 27 °C. A) Control treatment, B) 1% treatment, C) 0.5% treatment, D) 0.1% treatment.

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