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Identification of biosurfactant-producing fungi isolated from the Costa Rican mangrove

Identificación de hongo productor de biosurfactante, aislado de manglar costarricense

Identificação de fungo produtor de biossurfactante, isolado de mangue costarriquenho

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Abstract

In Costa Rica, mangrove potential has not been studied from a biotechnological perspective, despite reports from other latitudes of promising fungi associated with these ecosystems, including biosurfactant potential. These molecules are primarily used in industry as emulsifiers; however, studies indicate their potential in healthcare, pharmaceutical, and agricultural applications. **[Objective]** This study aimed to screen and preliminarily identify fungal strains capable of producing biosurfactants by implementing a set of effective, low-cost, straightforward assays for future exploitation. **[Methodology]** Five fungal strains from the Manuel Antonio mangrove in Puntarenas, Costa Rica, were isolated, molecularly identified, and assessed for biosurfactant production utilizing the crude biosurfactant broth. The drop collapse test, oil displacement test, emulsification index, and hemolysis tests were assessed. **[Results]** The use of crude biosurfactant broth minimized the cost and time required for purifications, which enabled the reduction of screening time for all fermentations. Isolate MP4 displayed, overall and consistently, better performance than the other strains evaluated for growth and biosurfactant production assays, especially for the emulsification index and blood hemolysis test. ITS and LSU molecular markers were analyzed, and MP4 was taxonomically assigned as *Trichoderma* sp.; however, it was shown to be closely related to *T. melanomagnum*. **[Conclusions]** Results emphasize the unexplored potential of mangrove resources in Costa Rica for biosurfactant production, and although preliminary, they demonstrate the value of conducting future comprehensive studies to evaluate this fungal potential.

Keywords: hemolysis; drop-collapse; *Trichoderma* sp; molecular identification; secondary metabolites; emulsification index, ITS.

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Resumen

En Costa Rica, el potencial de los manglares no ha sido estudiado desde una perspectiva biotecnológica, a pesar de múltiples reportes de hongos prometedores asociados a estos ecosistemas en otras latitudes, incluyendo potencial biosurfactante. Tales moléculas se utilizan principalmente en la industria como emulsificadores; sin embargo, también se reportan aplicaciones en áreas como la salud, la farmacia y la agricultura. **[Objetivo]** El objetivo de este estudio fue tamizar e identificar preliminarmente cepas fúngicas capaces de producir biosurfactantes, mediante la implementación de ensayos efectivos, de bajo costo y fáciles de efectuar, para su futura explotación. **[Metodología]** Cinco cepas fúngicas del manglar de Manuel Antonio en Puntarenas, Costa Rica, fueron aisladas, identificadas molecularmente y evaluadas para producción biosurfactante, utilizando el caldo crudo del biosurfactante. Se evaluaron la prueba de colapso de gota, el desplazamiento de aceite, el índice de emulsificación y las pruebas de hemólisis. **[Resultados]** El uso del caldo crudo de biosurfactante minimizó el costo y el tiempo requeridos para las purificaciones, lo que nos permitió reducir el periodo de evaluación en todas las fermentaciones. El aislamiento MP4 mostró, entre todas y de forma consistente, un mejor desempeño en el índice de emulsificación y en la prueba de hemólisis. Se analizaron los marcadores ITS y LSU; MP4 fue taxonómicamente asignado como *Trichoderma* sp. y mostró una cercana relación a *T. melanomagnum*. **[Conclusiones]** Nuestros resultados enfatizan el potencial no explorado de los recursos de manglar en Costa Rica para la producción de biosurfactante y, a pesar de ser preliminares, demuestran el valor de realizar futuros estudios exhaustivos que evalúen este potencial fúngico.

Palabras clave: hemólisis; colapso de gota; *Trichoderma* sp.; identificación molecular; metabolitos secundarios; índice de emulsificación; ITS.

Resumo

Na Costa Rica, o potencial dos manguezais não foi estudado do ponto de vista biotecnológico, apesar dos inúmeros relatos sobre fungos promissores associados a esses ecossistemas em outras latitudes, incluindo o potencial biossurfactante. Essas moléculas são utilizadas principalmente na indústria como emulsificantes; no entanto, também há relatos de aplicações em setores como saúde, farmácia e agricultura, entre outros. **[Objetivo]** O objetivo deste estudo foi selecionar e identificar preliminarmente cepas fúngicas capazes de produzir biossurfactantes, por meio da implementação de testes eficazes, de baixo custo e fáceis de implementar, para sua futura exploração. **[Metodologia]** Cinco cepas fúngicas do manguezal de Manuel Antonio em Puntarenas, Costa Rica, foram isoladas, identificadas molecularmente e avaliadas para a produção de biossurfactantes, utilizando o caldo bruto do biossurfactante. Foram avaliados o teste de colapso de gota, o deslocamento de óleo, o índice de emulsificação e os testes de hemólise. **[Resultados]** O uso do caldo bruto de biossurfactante minimizou o custo e o tempo necessários para as purificações, o que nos permitiu reduzir o tempo de avaliação em todas as fermentações. O isolamento MP4 apresentou, entre todos e de forma consistente, um melhor desempenho no índice de emulsificação e no teste de hemólise. Os marcadores ITS e LSU foram analisados; o MP4 foi taxonomicamente classificado como *Trichoderma* sp. e apresentou uma relação próxima com *o T. melanomagnum*. **[Conclusões]** Nossos resultados enfatizam o potencial inexplorado dos recursos de manguezais na Costa Rica para a produção de biossurfactantes e, apesar de serem preliminares, demonstram o valor de realizar estudos futuros exaustivos que avaliem esse potencial fúngico.

Palavras-chave: hemólise; colapso da gota; *Trichoderma* sp.; identificação molecular; metabólitos secundários; índice de emulsificação; ITS.

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Introduction

Surfactants produced by microorganisms are referred to as biosurfactants (BS). These compounds typically perform better than their chemical counterparts in terms of high biodegradability, low toxicity, high specificity, and biocompatibility, in addition to a wide range of chemical structures (Gayathiri *et al*. 2022). In general terms, surfactants are amphiphilic molecules that can interact with hydrophobic and hydrophilic compounds simultaneously, giving them their characteristics, such as reducing surface and interfacial tension at oil/air and water/oil interfaces, respectively, and acting as emulsifiers (Raddadi *et al*. 2018; Solano-González and Solano-Campos 2022).

Currently, the primary usage of surfactants at an industrial scale is predominantly synthetic. However, the cost of these molecules has increased due to the COVID-19 pandemic and subsequent political disagreements (Allam *et al*. 2022). Furthermore, synthetic surfactants tend to be more environmentally toxic (Johnson *et al*. 2021). Therefore, increasing efforts have been aimed towards searching for both economically and environmentally friendly surfactants. An excellent spot to look for metabolites with these characteristics is mangroves, as they shelter interesting microorganisms that exhibit a plethora of biochemical versatility by encoding genes for proteins, enzymes, and metabolites with important biotechnological applications (de Souza Sebastianes *et al*. 2013; Alberti *et al*. 2017), such as ligninases, cellulases, xylanases, and biosurfactants (Martinho *et al*. 2019). Fungal biosurfactants (BS) have been reported mainly from yeast (Fernandes *et al*. 2023) and filamentous fungi (Geiser *et al*. 2014; Konishi *et al*. 2013; Laurie *et al*. 2012; Lorenz *et al*. 2014; Morita *et al*. 2006; Saika 2014; Schirawski *et al*. 2010; Solano-González *et al*. 2019; Taniguti *et al*. 2015; Wada *et al*. 2021; Wege *et al*. 2021) with higher production yields in comparison to bacteria (Bhardwaj *et al*. 2013). In addition, fungal BS has been categorized as Generally Regarded as Safe (GRAS) by the FDA (El-Enshasy 2007; Sewalt *et al*. 2016), making them an attractive target for production.

Biosurfactant capability can be studied from different perspectives; nonetheless, cost-effective assays for screening are important. For these purposes, drop-collapse, hemolysis, oil displacement, and emulsifying index tests are considered adequate and informative options (da Silva *et al*. 2021). Few studies report environmental fungal isolates for BS production in Latin America, with most studies conducted in Brazil (Silva *et al*. 2018; Martinho *et al*. 2019) and Mexico (Villagrán *et al*. 2023), the former from mangrove isolates. In Costa Rica, mangroves account for 0.8% of the country's total surface area (Zamora-Trejos and Cortés 2009). However, comprehensive research on these ecosystems remains missing (Jia *et al*. 2020). Moreover, research into fungal BS remains underexplored. Only two studies have reported bacterial production of BS (Rodríguez-Rodríguez *et al*. 2012), both of marine origin, specifically from the Gulf of Nicoya. Given Costa Rica's diverse ecosystems, studying fungal isolates from mangroves is imperative to identify their potential for BS production (Martinho *et al*. 2019). Based on the background mentioned above, the present study aimed to determine the BS potential of fungal strains isolated from Costa Rican mangroves, providing insights into their biotechnological manipulation and subsequent exploitation by implementing easy, effective, and low-cost screening methods.

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Methodology

Sampling of fungal strains

All strains in the study were isolated from mangrove sediments from Puntarenas, Costa Rica, by taking 10 g of sediment and dissolving it in 90 mL of sterile saline solution (0.85% NaCl). A 10-fold serial dilution from 10-1 to 10-6 was prepared, and 100 µL of each dilution was plated onto Petri dishes containing 20 mL of potato dextrose agar (PDA), Sabouraud dextrose agar (SDA), and yeast extract peptone glycerol (YPG) medium. The incubation period was seven days at 25°C. Fungal isolates coded as A2.1, A3.1, B5.3, and A4.1 were deposited at Laboratorio de Biología Marina (R-024-2020-OT-CONAGEBIO), while organism MP4, isolated from the Costa Rican National Park Manuel Antonio (R-CM-UNA-0010-2022), was deposited at Laboratorio de Bioinformática Aplicada. Both laboratories are affiliated with Escuela de Ciencias Biológicas at Universidad Nacional (UNA) in Costa Rica.

Media composition

YPG media (Yeast extract 10 g/L, Peptone 20 g/L, Glucose 20 g/L, pH 6.0) and Vogel's minimal media, supplemented with 20 g/L of glucose as a carbon source, were used. The media to induce BS production (hereinafter referred to as induction media) was YPG without glucose supplemented with 1% (v/v) commercial soy oil (Clover ®).

Media evaluation for fungal growth

YPG and Vogel's media were tested to determine optimal fungal growth; both were prepared using micro-filtered (5 µm) marine-rested water as the solvent and autoclaved at 121°C and 15 PSI for 15 minutes. To obtain solid media, 15 g/L of Agar-Agar was supplemented. All strains were incubated at 25 °C. After seven days, growth was evaluated in solid media by diameter measurements, following Sharma and Pandey' (2010) methodology, whereas dry weight, as proposed by Singh *et al*. (2012), was used for liquid media.

Inoculum for biosurfactant production

A 10-day mycelial culture (growth on PDA) of 6 mm was established for each strain in 75 mL of YPG in 250 mL Erlenmeyer flasks, with six replicates per strain. The cultures were maintained at 120 rpm and 30 ºC for five days to obtain sufficient biomass.

According to the literature, fungal BS production occurs when two factors are met, typically in the presence of a fatty acid source and nitrogen starvation (Solano-González and Solano-Campos 2022). After optimal growth was observed, all biomass was inoculated into 50 mL of inducing medium supplemented with 1% (v/v) commercial soy oil in 250 mL flasks, maintained at 120 rpm and 30 °C for seven days; six replicates were prepared for each isolate. After the seventh day, samples were centrifuged for 15 min at 10,000 g to separate the biomass from the suspension. Each ferment was stored at 4 ºC for further processing (hereinafter referred to as crude ferment (CF)).

Assessment of biosurfactant production

Efficient screening for BS production in fungal strains requires rapid, simple, and cost-effective tests. Consequently, this study has incorporated a range of assays to detect

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and validate the presence of BS in fungal fermentations. These assays include the drop collapse test (DCT), oil displacement assay (ODA), emulsification index (E24 %), and blood hemolysis test (BHT). For the analysis, crude-BSB was used as the sample, with cells removed by centrifugation.

- **Drop collapse test (DCT)**

This semi-quantitative test was modified from the method by Bodour and Miller-Maier (1998). To ensure the validity of the results, each well in sterile 96-well plates was rinsed as follows: hot water, 70% alcohol, and Milli-Q water. After complete drying, each well was filled with 2 µL of autoclaved commercial soy oil, and the plates were allowed to stand for 2 hours. Subsequently, a micropipette was used to dispense 5 µL of CF onto the oil layer; after 1 minute, the drop diameter was measured under a stereoscope using a calibrated micrometer. The analysis was performed on six biological replicates, with two technical replicates for MP4 and A3.1, and four technical replicates for A2.1, A4.1, and B5.3. A 3% Tween 80 (v/v) solution served as the positive control, and Milli-Q water and media without inoculum as the negative controls.

- **Oil displacement assay (ODA)**

This semi-quantitative, sensitive, reliable, fast, and easy-to-perform methodology (da Silva *et al*. 2021) was implemented following the Morikawa *et al*. (2000) protocol with slight modifications. One hundred and fifty (150) mm-diameter Petri dishes rinsed with 70% alcohol were used, and 40 mL of distilled water was applied as the first coating layer. On this layer, 10 µL of commercial soy oil was carefully dispensed as a thin layer using a micropipette. Consecutively, 10 µL of CF was dispensed at the center of the oil layer, and photographs were taken using a measuring rule. To determine the potential effect of Milli-Q water or media on the ODA 3% Tween 80 (v/v) solution, Milli-Q water and uncultured media were used as controls. Two technical replicates were evaluated for MP4 and A3.1, whereas four technical replicates were used for A2.1, A4.1, and B5.3. Diameter displacement images were processed using ImageJ v.1.5.

- **Emulsification index E24 (E24%)**

The Cooper and Goldberg (1987) protocol was used by rinsing conventional 10 x 100 mm test tubes with 70% ethanol and Milli-Q water and using 2 mL of commercial soy oil and 2 mL of CF. Each test tube was vigorously vortexed for two minutes and incubated at room temperature for 24 hours undisturbed. Subsequently, the emulsification index (E24%) was calculated by dividing the height of the emulsified layer by the total height, multiplied by 100. For MP4 and A3.1, two technical replicates were performed, while A2.1, A4.1, and B5.3 were subjected to four technical replicates. Positive and negative controls corresponded to a 1% soap preparation and media with and without commercial oil, respectively.

- **Blood hemolysis Test (BHT)**

To evaluate hemolytic activity, YPG plates supplemented with 5% (v/v) horse blood were used, donated by the School of Veterinary Medicine at Universidad Nacional. Each plate was inoculated at the center with a 6 mm piece of mycelial growth on YPG and incubated at 37 °C for 5 days. Afterwards, the hemolysis diameter hale was measured according to Thavasi *et al*. (2011). At least six repetitions were conducted for each isolate, with a non-inoculated plate serving as the negative control.

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- **DNA extraction and molecular identification**

Fungal strains that consistently produced BS were subjected to molecular identification. DNA extraction was performed from YPG plates after a seven-day incubation period. A total of 50 mg of biomass was transferred to 2 mL tubes for further processing. Organic extraction was performed following the methodology of Syedd-León *et all*. (2022) with slight modifications: a 10% SDS solution, proteinase K (10 mg/mL), and 0.6% v/v β-mercaptoethanol were used, and the samples were incubated for 1.5 hours at 65 °C. Subsequently, a chloroform mixture was prepared: octanol (24:1) was added to the solution, following the steps described in the article mentioned above. 0.8% agarose gels were used to determine DNA integrity and quality. DNA samples were amplified to obtain the corresponding genetic regions of the internal transcribed spacer (ITS) of the ribosomal DNA using the universal primers ITS1/ITS4 (White *et all*. 1990), and the region corresponding to 28S rRNA gene using the primers LROR/LR6 (Schoch *et all*. 2012). These amplifications were performed through a contracted service with Macrogen Inc. Geneious software v.R9.1.8 was used to manually curate sequences and generate the consensus sequences for each primer set; taxonomy determination was carried out using BLASTn (http://www.ncbi.nlm.nih.gov) and UNITE (https://unite.ut.ee). For the former, only type material was selected, and environmental isolates were excluded; for the latter, solely the fungal dataset was used (excluding HTS sequences). Taxonomic identification was based on hits with identity values greater than 99.6% for ITS and 99.8% for LSU (Vu *et all*. 2019). The best results were used to determine the optimal substitution model in MEGA X v. 10.2 (Stecher *et all*. 2020) using the Akaike Information Criterion (AIC). A maximum-likelihood tree was then constructed in MEGA X v. 10.2 using 1000 bootstraps to assess the clustering pattern. Finally, TrichOKEY (https://trichokey.com) was used to validate the genus.

- **Statistical analysis**

All collected data were subjected to a variance analysis to determine statistically significant differences between assays and controls using the R package v.4.1.1. Specific considerations for each test are described in the corresponding section.

Analysis and results

Media growth evaluation

Among the tested media, significantly better growth was observed in YPG (Fig. 1A and B). Regardless of the medium, isolate A3.1 exhibited the most significant growth fluctuation, whereas MP4 showed the least (Fig. 1). Based on these results, the YPG medium was used for downstream analysis.

Assessment of biosurfactant production: Drop Collapse Test (DCT)

By exclusively using the CF, it was observed that A2.1, A4.1, and MP4 strains are not significantly different in the DCT test and, therefore, display a similar behavior to the 3% Tween 80 solution (Fig. 2). Interestingly, isolates B5.3 and A3.1 had comparable DCT values to the media without inoculum (Fig. 2). Nonetheless, the most interesting results correspond to MP4, A4.1 and A2.1 isolates, which shared no

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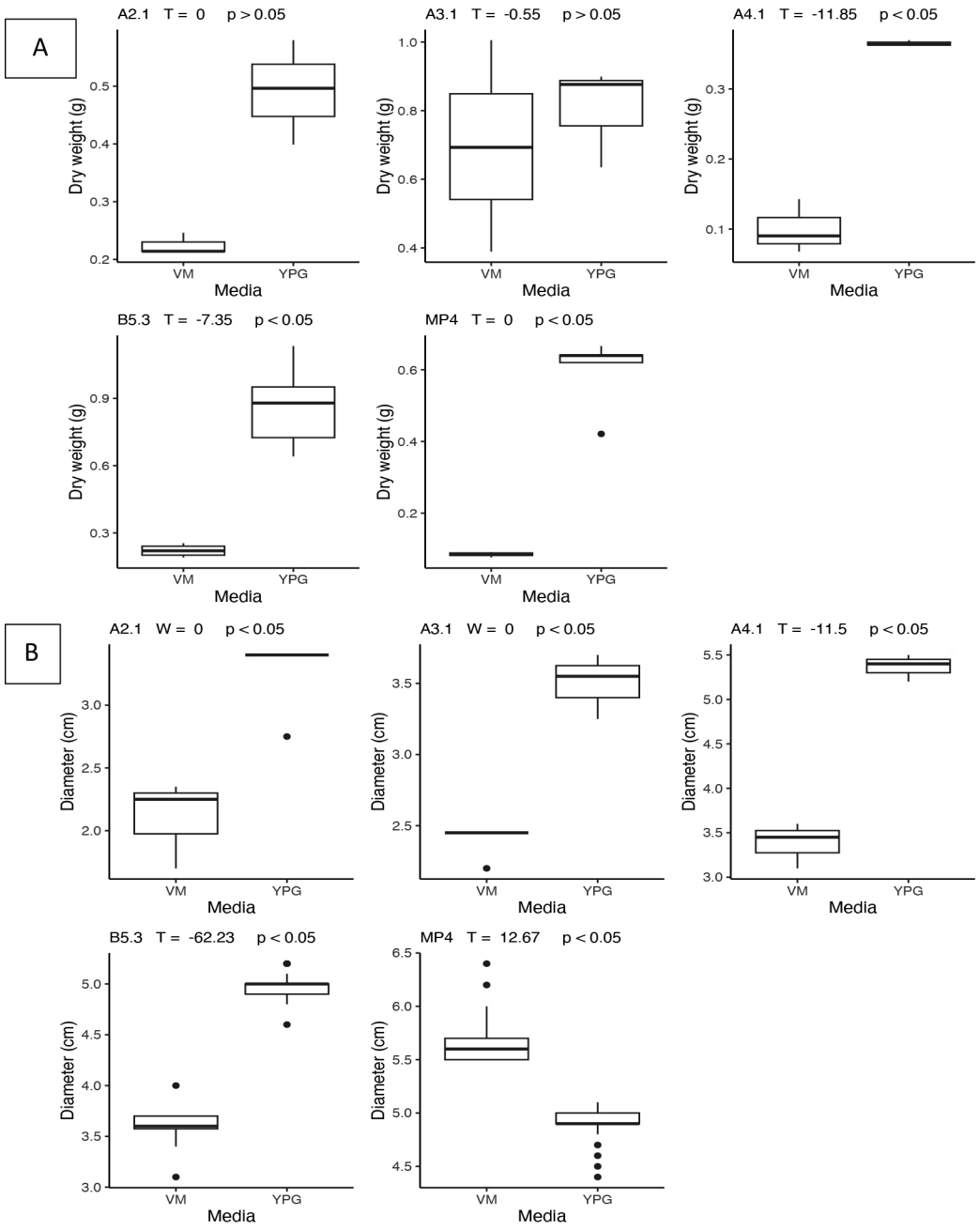

Figure 1. *Fungal growth comparison for YPG and 20 g/L glucose-supplemented Vogel's minimal media for A2.1, A3.1, A4.1, B5.3, and MP4 isolates.* ***A*** *Average boxplots for dry weight.* ***B*** *Average boxplots for diameter growth on agar plates.*
Note: derived from research.

significant differences with Tween 80. Finally, despite the M-i sample exhibiting a higher effect than the negative control (Milli-Q water), its overall behavior was closer to that observed for Milli-Q water. This suggested that the media would not create a false positive in the DCT test.

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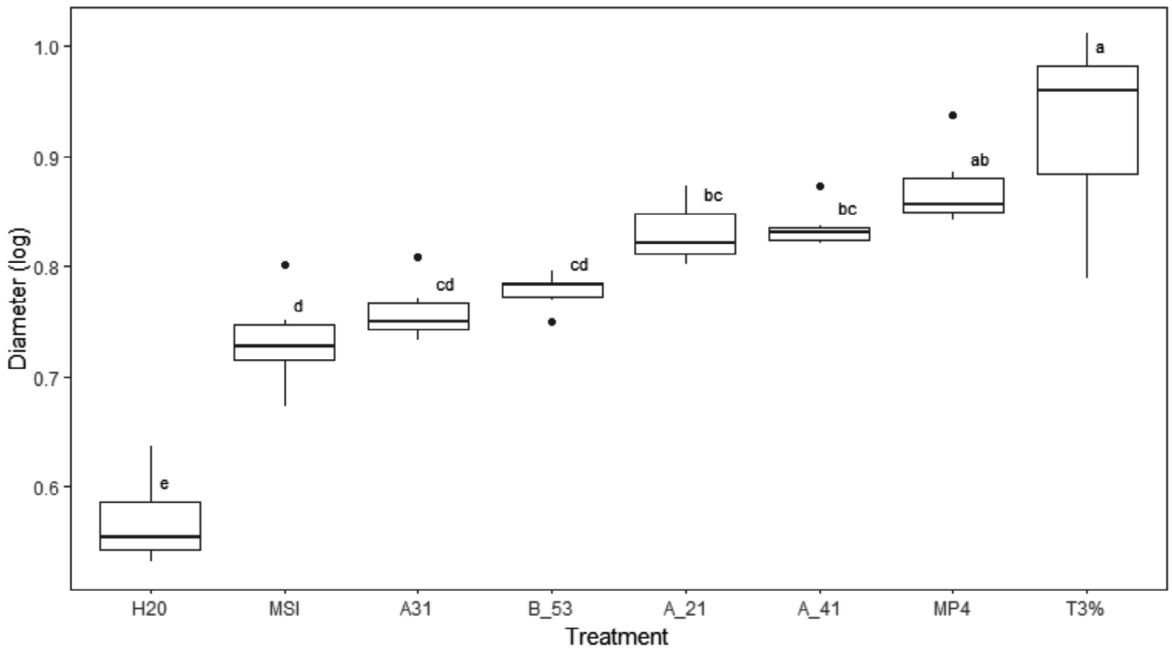

Figure 2. *Drop collapse test (DCT) for fungal strains in this study and controls. Water and media without inoculum (M-i) correspond to negative controls, whereas the 3% Tween 80 solution (T3%) corresponds to a positive control. The same letters in the plot indicate the absence of significant differences.*
Note: derived from research.

Assessment of biosurfactant production: Oil displacement assay (ODA)

To ensure the statistical robustness of this test, the results were subjected to a Shapiro test ($p < 0.05$). Subsequently, data transformation was performed to proceed with a one-way analysis of means (not assuming equal variances, $F = 219.16$, $p < 0.05$). This ODA test validates the surfactant activity for the positive controls (3% soap and 1% Tris) and demonstrates that the MP4 and A2.1 isolates exhibited a similar performance. On the other hand, the medium exhibited activity similar to that of B5.3, A4.1, and A3.1; the latter two consistently indicated a potential lack of surfactant capacity (Fig. 3).

Assessment of biosurfactant production: Emulsification index E24 (E24%)

A complete statistical analysis was conducted for this test, beginning with a Shapiro test ($W = 0.94679$, $p < 0.05$) and a log transformation of the data. Once the data was normal ($W = 0.9817$, $p > 0.05$), variances were reviewed to determine if they were homogeneous ($\chi^2 = 25.439$, $p < 0.05$), and a Welch one-way analysis of means (not assuming equal variances) ($F = 183.64$, $p < 0.05$) was calculated. Subsequently, a nonparametric Dunnett post hoc test (Table 1) was performed to identify variables with statistical significance. The E24% index showed that isolate MP4 and 1% soap exhibited similar emulsification potential (Fig. 4). This was supported by the absence of statistically significant differences in

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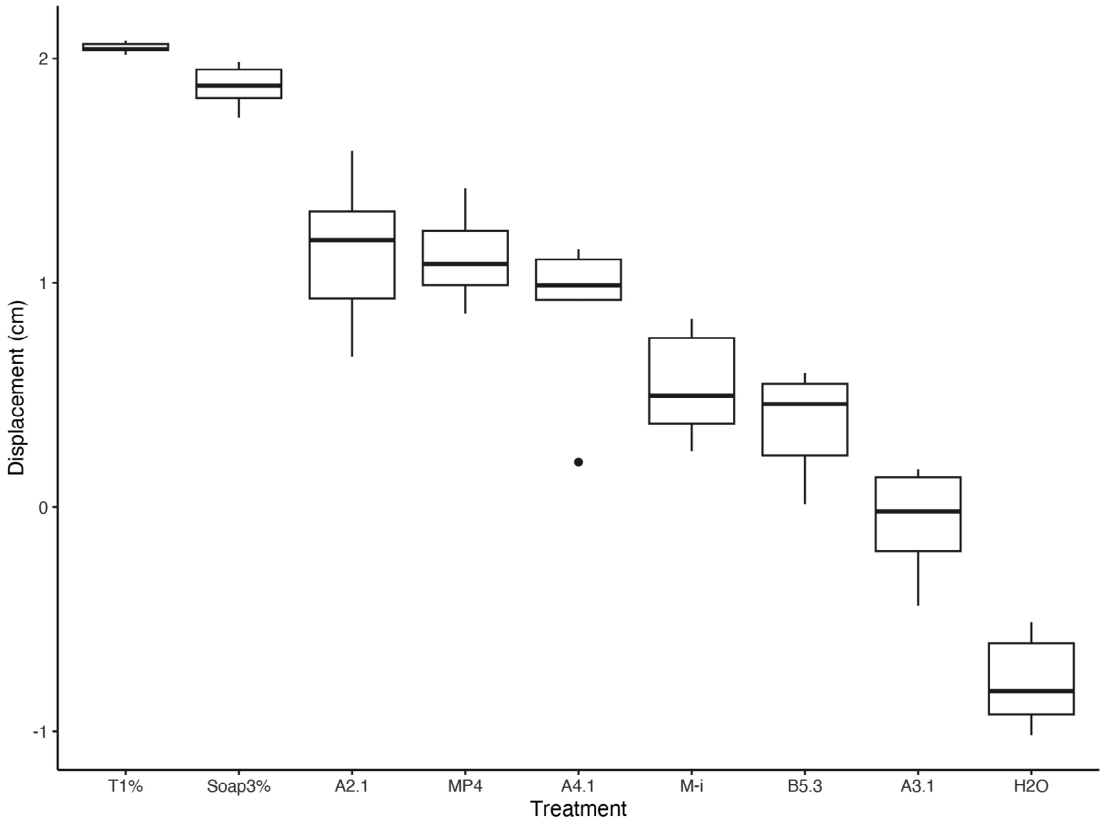

Figure 3. *Oil displacement test (ODT) for fungal strains in this study and controls (n=6). Water and media without inoculum (Medium) serve as negative controls, whereas Tween 80 (T1) and 3% soap serve as positive controls.*
Note: derived from research.

Table 1. *Dunnett's pairwise comparison between the E24% index among replicates, using 1% soap as the control. The asterisk indicates no statistically significant differences. MSI stands for media without inoculum.*

Condition	A3.1	A2.1	A4.1	B5.3	H20	MP4	M-i
A2.1	0.00374	-	-	-	-	-	-
A4.1	0.00533	1.00*	-	-	-	-	-
B5.3	0.02277	0.97165*	0.79648*	-	-	-	-
H_20	0.6559*	0.00035	0.00086	0.00128	-	-	-
MP4	0.00038	0.02958	0.12539*	0.00742	6.90E-06	-	-
MSI	0.99484*	0.00675	0.00416	0.02515	1.00*	0.00174	-
Soap 1%	0.00051	0.05111	0.22198*	0.01122	1.80E-05	0.12458*	0.00214

Note: derived from research.

pairwise Dunnett comparisons (Table 1). Another interesting finding was the similarity observed between the negative control (Milli-Q water) and the MSI (media without inoculum); this particular result indicates that the media most likely did not attribute any emulsification activity to the assayed inoculum. Nonetheless, isolates A3.1, A2.1, A4.1, and B5.3 overall showed a very similar behavior during the E24% test.

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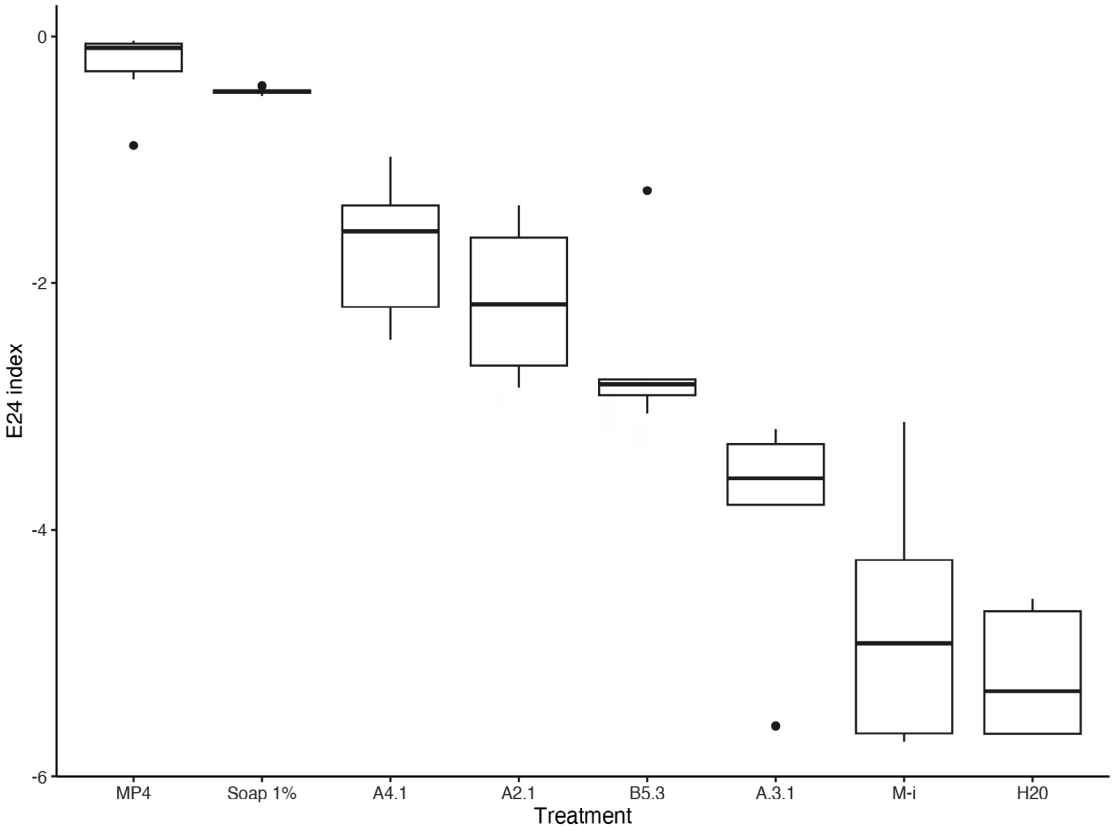

Figure 4. *The E24% index was applied to transformed data for fungal isolates in this study and controls (n=6). Water and media without inoculum (M-i) served as negative controls, whereas 1% soap served as the positive control.*
Note: derived from research.

The Dunnett test is used to identify significant differences between samples. A clear contrast was observed between the positive and negative controls (1% soap and Milli-Q water, respectively), as their reported p-values were among the lowest (Table 1). In addition, Milli-Q water and MSI showed no significant differences, whereas MP4 and A4.1 E24% index p-values were not significantly different from 1% soap.

Assessment of biosurfactant production: Blood hemolysis Test (BHT)

The evaluated strains exhibited behavior distinct from that of the A4.1 and A3.1 isolates, which lack hemolytic activity. However, while isolate A4.1 grew on the plate, isolate A3.1 had difficulty growing, as only two of six plates showed poor growth. For isolate B5.3, a fragmented growth pattern was observed in four of six plates (Fig. 5, A1), precluding a proper evaluation of hemolytic activity. Furthermore, when the strain grew uniformly on certain plates, its hemolytic activity was inconsistent (Fig. 5, A2 and A3). On the other hand, isolate MP4 consistently demonstrated efficient lysis of red blood cells among all six evaluated replicates (Fig. 5. D1-D3).

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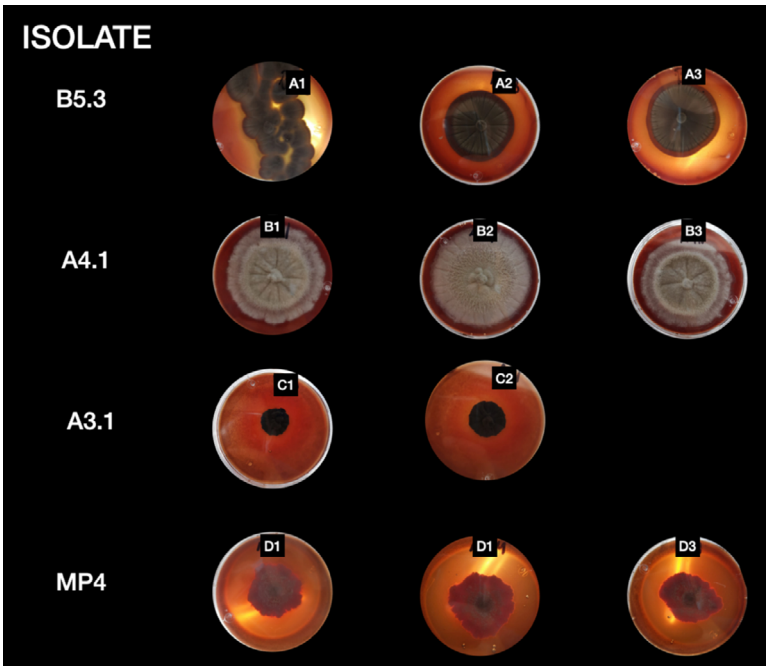

Figure 5. *Blood hemolysis test (BHT) for four fungal strains isolated from the Costa Rican mangrove. The test was performed in six replicates; however, only sets of three are displayed, corresponding to each line and its respective replicates per column. For isolate A3.1, only two of six plates showed growth. Positive hemolytic activity was observed for isolate B5.3 replicate a.3; however, this was inconsistent across plates. On the other hand, isolate MP4 exhibited both optimal growth and persistent hemolytic activity.*
Note: derived from research.

DNA extraction and molecular identification

BLASTn and UNITE searches for LSU and ITS molecular markers revealed a clear lack of sequence availability for LSU rRNA. An attempt was made to retrieve sequences with an identity percentage higher than 99.8% for LSU and 99.6% for ITS [33]. However, the highest reported value across both databases was 97.8%. Hence, a threshold of 97% or higher was used. Nonetheless, because many LSU sequences were unavailable for comparison with ITS, only ITS results are presented in this investigation. The corresponding nucleotide sequences were recovered, and the best nucleotide substitution models were calculated using MEGA: T92+G for the ITS molecular marker and TN93 for the LSU molecular marker.

Subsequently, a tree implementing ML and 1000 bootstraps was implemented. For the ITS alignment, it was observed that the MP4 isolate did not share a clade with any other species; it derives from *Trichoderma melanomagnum* (Fig. 6). On the other hand, for LSU, the isolate clustered with *T. reesei* (Figure S1), both with bootstrap values higher than 90%. *A. niger* was included as an outgroup.

Discussion

Despite Costa Rica's significant contribution to the global marine biodiversity (3.5%) (Wehrtmann *et al*. 2009), the study of marine mangrove fungi, particularly from an industrial standpoint, had remained unexplored until now (Cortés and Wehrtmann 2009). These environments play an important ecological role because their convulsion of distinctive nutrients promotes fungal colonization, which can produce highly valuable enzymes and compounds, thereby creating rich sources for bioindustries (Jia *et al*. 2020). However, to fully unlock their potential, further exploration and identification of mangrove-associated fungi is necessary (Martinho *et al*. 2019). For

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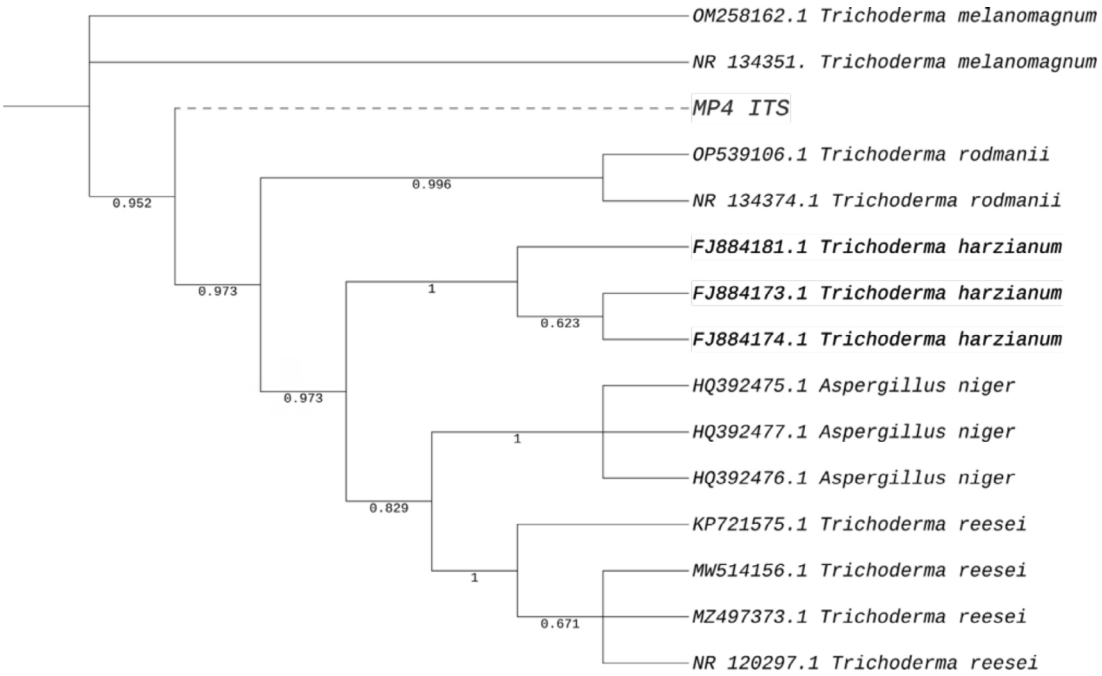

Figure 6. *Phylogenetic tree of the ITS sequences of rDNA from 5 fungi taxa belonging to the Trichoderma species and A. niger as an outgroup. Tree built by the Neighbor-joining method and distance calculation using the Kimura 2-parameter model. Bootstrap values based on 1000 replicates are displayed for each branch.*
Note: derived from research.

such purposes, affordable BS screening techniques are required. This research employed approaches grounded in well-established scientific literature and validated reasoning to examine five fungal isolates from multiple perspectives and dimensions (Gayathiri *et al*. 2022; Kosaric and Sukan 2014). In terms of growth, we observed that, regardless of the media used, isolate A3.1 showed the highest fluctuation in growth rate, whereas MP4 exhibited the lowest.

Regarding media preference, irrespective of strain, a clear difference was observed between YPG and Vogel-supplemented media, with the former supporting better fungal growth. This difference in media growth is supported by McIntyre *et al*. (2002), who found that comparing YPG and Vogel media under aerobic and anaerobic conditions influenced fungal growth because these media contained different nutrient levels. Additionally, Azmi and Seppelt (1997) reported that fungal radial growth can be affected by factors such as pH, temperature, and culture media composition, where the use of undefined media, such as yeast extract and peptone, can improve fungal growth since these compounds supplement the media with micronutrients and growth factors (Basu *et al*. 2015).

With respect to the screening tests used to determine emulsification potential, Trichoderma sp. (MP4 isolate) exhibited overall and consistent performance comparable to the positive controls (1% soap, 3% soap, and 1% Tween), providing strong evidence of biosurfactant validation. The screening methods were selected based on

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the emulsifying properties of the BS (da Silva *et al*. 2021; Ferreira *et al*. 2020; Marcelino *et al*. 2019; Mulligan and Cooper 1984; Youssef *et al*. 2004). The inclusion of media without inoculum in the results helped determine that the emulsifying activity of the MP4 isolate is not due to media effects and validated its BS production.

Despite using two different molecular markers, taxonomy assignment was difficult, primarily due to the lack of LSU sequence availability in the GenBank and UNITE databases. Therefore, it was not possible to compare the same dataset with both molecular markers. In addition, among the available datasets, those type materials accounted for a smaller portion. Our molecular identification test indicates the MP4 isolate corresponds to a Trichoderma sp. because, in addition to BLASTn and UNITE, BarCode analysis using TrichoMARK (https://trichokey.com/index.php/trichomark) successfully identified all four unique ITS molecular markers associated with the Trichoderma genus, confirming the inclusion of this strain within the genus. Even though we were unable to determine its exact species, the phylogeny test demonstrates its divergence from T. melanomagnum. Nonetheless, the results might imply that MP4 corresponds to an unrecognized species. This supports the shortage of available molecular markers for potentially new Hypocrea/Trichoderma species, reported by Zhang *et al*. (2007).

Fungal BS production has been previously reported (da Silva *et al*. 2014), even implementing the DCT and E24% index (Martinho *et al*. 2019). In general, Trichoderma species have been reported as BS or bioemulsifier (BE) producers (Silva *et al*. 2018), although the chemical nature of these compounds remains unknown. In addition, species such as T. viridae and T. reesei have been suggested as BS producers (Maheswari and Parveen 2012); however, exploration of this area remains limited.

Conclusions

To our knowledge, this is the first report of a fungal species that produces biosurfactants (BSs) isolated from the Costa Rican mangrove, namely a Trichoderma species. This demonstrates the potential of native biodiversity and its applicability to different industrial scales. It also suggests the need to broaden the scope of marine fungal studies to understand their metabolic capabilities better. This result paves the way for our ongoing research with Trichoderma sp. (MP4 isolate) as a BS producer. In addition, we are conducting supplementary studies to determine whether MP4 corresponds to a new Costa Rican marine fungal species.

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Conflict of Interest

The authors declare no competing interests.

Author contribution statement

All the authors declare that the final version of this paper was read and approved.

Authors and Credit Roles: S.S.G., J.A.R.R.: Conceptualization, Methodology, Supervision. K.V.A., S.S.G., J.A.R.R., A.F.M., S.S.T.: Data Curation, Formal Analysis; Software, Writing - Original Draft, Writing - Review & Editing, Visualization. K.V.A., S.S.G., J.A.R.R., A.F.M., S.S.T.: Validation, Investigation, Resources, Writing - Original Draft.

The total contribution percentages for this paper were as follows: K.V.A., 35%; A.F.M., 20%; J.A.R.R., 10%; S.S.T., 10%; S.S.G., 25%.

Data availability statement

Genomic data is available at the NCBI GenBank under accession number PP853654.

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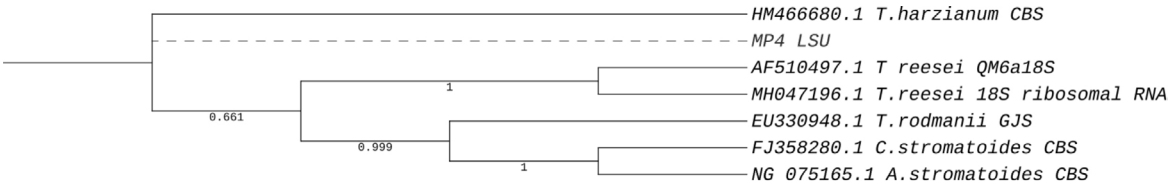

Figure S1. *Phylogenetic tree of the LSU sequences of rDNA from 6 fungi taxa belonging to the Trichoderma species and A. stromatoides as outgroups. Tree built by the Neighbor-joining method and distance calculation using the Tamura-Nei model. Bootstrap values based on 1000 replicates are displayed for each branch.*
Note: derived from research.

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