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# Control of *Colletotrichum plurivorum* on Papaya Using a Nanoemulsion of *Schinus terebinthifolia* (Pink Pepper) Essential Oil

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## ABSTRACT

*Schinus terebinthifolia*, a plant which is widely distributed throughout South America, is commonly known as pink pepper or Brazilian peppertree. Its essential oil has demonstrated antioxidant, wound-healing, antibacterial and antifungal properties. To enhance its antifungal potential, nanoemulsions of *S. terebinthifolia* essential oil were prepared to assess their antifungal effect on *Colletotrichum plurivorum*, the causal agent of anthracnose in papaya. To achieve this, in vitro assays were evaluated to determine the minimum inhibitory concentration (MIC) of the nanoemulsion using 96-well microplates and petri dishes. Subsequently, the most effective concentrations were tested in vivo on papaya fruits under both preventive and curative conditions. The in vitro results indicated that the *S. terebinthifolia* essential oil nanoemulsion inhibited the growth of *C. plurivorum*. In vivo evaluations on papaya fruits inoculated with *C. plurivorum* revealed that the nanoemulsion reduced both lesion size and disease incidence, demonstrating preventive and curative effects. The use of *S. terebinthifolia* essential oil nanoemulsions proved to be an innovative and effective post-harvest strategy for the control of papaya anthracnose at concentrations of 0.13% and 0.26%, showing promising results for the management of *C. plurivorum* infections.

## 1 | Introduction

Fruit cultivation represents a significant sector within the broader context of global agriculture. According to data from the Food and Agriculture Organization of the United Nations—FAO (Food and Agriculture Organization of the United Nations) (2024), global fruit production reached 933 million tons in 2022. Brazil is the third-largest producer globally, and papaya is a significant crop within the country. It is therefore vital to enhance the post-harvest quality of papaya, given the substantial losses incurred due to diseases caused by fungi belonging to the *Colletotrichum* genus. These diseases pose a grave threat, jeopardizing fruit quality for commercial sale (FAO (Food and Agriculture Organization of the United Nations) 2024).

The most prevalent post-harvest disease that affects papaya is anthracnose, a condition caused by fungi belonging to the *Colletotrichum* species complex. This disease manifests most frequently during the post-harvest phase of fruit (Ventura and Rezende 2016). A comprehensive post-harvest disease control strategy should be initiated within the field itself; it is widely recognized that diseases manifest during storage often originate in the orchard. The treatment of these diseases typically involves the application of synthetic fungicides during fruit management

to minimize infection effects. To ensure efficacious disease management, it is imperative to identify treatments that are not only efficient and safe, but also ideally, natural, with minimal impact on consumer health and the environment (Dantas et al. 2003).

In this context, the application of essential oils to prevent the spread of plant diseases has gained attention as a natural alternative to synthetic fungicides and represents a promising approach for pathogen control and an emerging strategy in the agricultural sector (Donsì and Ferrari 2016). However, the direct application of essential oils on fruit surfaces is limited due to their high volatility. Consequently, essential oils are often incorporated into polymer-based coatings or emulsions to extend shelf life (Aider 2010). Nevertheless, their high degree of hydrophobicity hinders uniform dispersion across the fruit surface (Vargas et al. 2008).

Among essential oils, *Schinus terebinthifolia*, a plant native to South America, is commonly known as the Brazilian peppertree or pink pepper. The essential oil extracted from its fruits predominantly contains monoterpenes, such as $\alpha$-pinene, $\beta$-myrcene, $\alpha$-phellandrene, $\delta$-3-carene, limonene and $\beta$-phellandrene (Barraqui et al. 2023; Schimitberger et al. 2018). The essential oil from the fruits of *S. terebinthifolia* has been shown to exhibit several biological activities, including antioxidant activity (Oliveira et al. 2020), antifungal (Mohamed et al. 2020), insecticidal (Belhoussaine et al. 2022), anti-inflammatory (Marangoni et al. 2023) and antibacterial effects (Uliana et al. 2016; Costa da Silva et al. 2023; Barraqui et al. 2025). Specifically, regarding fungi, studies have shown that the essential oil of *S. terebinthifolia* exhibits fungistatic and fungicidal properties (Bernardi et al. 2024). These results provide a solid foundation for the development of novel fungal control methodologies and agrochemical applications.

A strategy to improve the activity of essential oils is the use of nanoemulsions—stable formulations in which extremely small droplets of an active liquid (such as essential oils) are dispersed in water or another aqueous solvent. The diffusion of oil droplets across microbial cell membranes is facilitated by nanoemulsions, resulting in intracellular disruption and cell death, and thereby significantly enhances antimicrobial effectiveness (Pandey et al. 2022). The use of nanotechnology in the development of nanoformulations and nanomaterials has been demonstrated to enhance the activity of bioactive compounds (Sheth et al. 2020). This approach has improved the bioavailability of active ingredients, increased the permeability of poorly soluble compounds, and reduced turbidity compared to conventional emulsion systems (Sheth et al. 2020). The potential applications of nanoemulsions are diverse, including cosmetics, topical drugs and polymeric films (Fernandes et al. 2023).

Furthermore, nanoemulsions have become a promising strategy for the development of new bioproducts by improving both bioavailability and efficacy (Gharsan et al. 2022). This technology facilitates a more uniform distribution and greater efficacy of active components while concomitantly reducing the amount of product required for application (Brusare 2021).

Despite these advantages, little is known about the effectiveness of *S. terebinthifolia* essential oil nanoemulsions against phytopathogenic fungi in tropical fruits. No study has evaluated their potential for the control of anthracnose in papaya caused by species of the *Colletotrichum orchidearum* complex, including *C. plurivorum*, which represents a significant gap in the literature. Therefore, the present study aims to evaluate the antifungal potential of nanoemulsions prepared from the essential oil of *Schinus terebinthifolia* fruits in controlling the *Colletotrichum plurivorum* in post-harvest papaya fruits.

## 2 | Materials and Methods

### 2.1 | Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Conde SA (Spain, SP). Streptomycin sulphate, sodium hypochlorite and tebuconazole fungicide (commercial formulation) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Emulsifiers Polysorbate 20 (Tween 20) and Sorbitan trioleate (Span 85) were supplied by Sigma-Aldrich. Distilled water was used in all experiments.

### 2.2 | Essential Oil and Chemical Analysis

The essential oil of *Schinus terebinthifolia* fruits was supplied by Nativa da Foz, a company representing family farmers in São Mateus, Espírito Santo, Brazil. The chemical composition of essential oil was analysed using an Agilent GCMS-5977C system equipped with an HP-5MS UI capillary column and a temperature program increasing from 50°C to 250°C at 3°C/min. Helium served as the carrier gas at a flow rate of 1 mL/min for a 2 μL injection in 1:10 split mode, while the mass spectrometer operated at 70 eV with a scan range of 35–550 amu. Compound identification was finalized by comparing mass spectra and calculated retention indices—derived from a series of n-alkanes against the NIST library database and established literature (Adams 2009).

### 2.3 | Nanoemulsions Production

The nanoemulsion was prepared according to the methodology outlined by Barraqui et al. (2025) and employed the low-energy phase inversion method. It was previously established that the most stable and effective formulation was achieved with 15% emulsifier (which consisted of Polysorbate 20 and Sorbitan Trioleate, with a hydrophilic–lipophilic balance (HLB) of 15). The oil phase incorporated 5% *Schinus terebinthifolia* essential oil, while the aqueous phase comprised distilled water added to reach the desired solution volume. For in vitro assays, the preparation process involved the controlled addition of the aqueous phase to the oil phase under constant agitation using a vortex mixer (Model VX-38) to produce 1 g of nanoemulsion (NE). For larger-scale preparation (1 L), the same process was employed but using a mechanical stirrer (Fisatom 713DS) at 500 rpm (Barraqui et al. 2025).

### 2.4 | Nanoemulsion Characterisation

The large-scale nanoemulsion (1 L) was prepared using the concentration determined as effective in the in vitro assays, ensuring

**TABLE 1** | Primers and cycles were used in this study.

| | | PCR amplification protocols | | | | |
|---|---|---|---|---|---|---|
| **Gene** | **Primer** | **Initial denaturation (95°C)** | **Denaturation (95°C)** | **Annealing** | **Extension (72°C)** | **Final extension (72°C)** |
| APN2/MAT-IGS | AMR | 3 min | 30 s | 50°C | 1 min | 10 min |
| | AMF | | | 1 min | | |
| GAPDH | GAP-95 | 3 min | 30 s | 60°C | 1 min 30 s | 10 min |
| | GAP-1174 | | | 45 s | | |
| GS | GS-64F | 3 min | 30 s | 55°C | 1 min | 10 min |
| | GS-967R | | | 45 s | | |
| TUB2 | BT1 | 3 min | 30 s | 53°C | 1 min 30 s | 10 min |
| | BT22 | | | 1 min | | |

consistency between laboratory-scale and applied-scale formulations. The droplet size distribution, polydispersity index (PDI) and zeta potential were measured using Dynamic Light Scattering (DLS) with a Litesizer 500 (Anton Paar, Austria). To evaluate stability over time, DLS analyses were conducted one day after preparation (Day 1) and subsequently after 8, 21 and 30 days of storage at room temperature (25°C). This monitoring allowed the assessment of droplet size variation, polydispersity and colloidal stability throughout the storage period.

## 2.5 | Fungal Isolation

Isolates of the *Colletotrichum* species complex were obtained from naturally infected papaya fruits (*Carica papaya*). The samples were collected from the Experimental Farm of the Capixaba Institute for Research, Technical Assistance and Rural Extension (Incaper), located in Sooretama, Espírito Santo, Brazil.

The isolation process involved the scraping of fungal structures from the fruits using a sterile scalpel and transferring to petri dishes containing PDA medium (Conde SA, Spain, SP), supplemented with 0.05% streptomycin sulphate. The plates were then placed in a Biological Oxygen Demand (BOD) growth chamber and incubated at 28°C for a period of seven days in the dark. Following this incubation, inoculum was prepared for pathogenicity tests on commercial papayas to evaluate the pathogenic ability of the fungus on papaya fruits.

## 2.6 | Molecular Identification of Fungal Isolates

### 2.6.1 | DNA Extraction, PCR and Sequencing

*Colletotrichum* isolates were grown on PDA media at 25°C ± 2°C for 7 days and 12 h light. Genomic DNA was extracted using the CTAB (cetyl trimethyl ammonium bromide) protocol described by Doyle and Doyle (1990). The following loci were amplified for multilocus analyses: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the intergenic spacer between DNA lyase and the mating-type locus MAT1-2-1 (APN2/MAT-IGS), glutamine synthetase (GS) and β-tubulin (TUB2). These genomic regions are reported to be the most informative for identifying species from several *Colletotrichum* species complexes (Vieira et al. 2020). Sequences obtained were compared with those from GenBank using the BLAST tool (Table 1).

The PCR amplifications were performed in a 12.5 μL volume reaction containing 4 μL PCR-grade water, 1 μL DNA template, 0.625 μL each primer (10 μM), and 6.25 μL 2× PCR master mix (Promega GoTaq Master Mix; Madison, Wisconsin, USA). PCR products were visualized in a 1.5% agarose/TAE gel by electrophoresis. The primers and cycles used in this study are listed in Table 1.

The PCR products were purified by ethanol and ammonium acetate precipitation and sequenced on an ABI 3730xl DNA analyser (Applied Biosystems, Foster City, California, USA) on a DNA sequencing platform located at Laboratório de Bioinformática e Biologia Evolutiva—LABBE from Universidade Federal de Pernambuco (Pernambuco, Recife, Brazil). Sequence reads were assembled into contigs and edited using the Staden package (Staden et al. 1998).

### 2.6.2 | Phylogenetic Analyses

Sequences from *Colletotrichum* ex-type and reference isolates from previous studies were retrieved from GenBank and included in phylogenetic analyses. Multiple sequence alignments (MSA) were generated with the online version of MAFFT 7 with the Q-INS-i iterative refinement method (Katoh et al. 2002). For the multilocus analysis, the loci were concatenated using Sequence Matrix v. 1.8 (Vaidya et al. 2011).

A phylogeny for each locus and concatenated alignments was inferred using maximum likelihood (ML). ML analyses were performed using IQ-TREE v. 2.1.2 (Nguyen et al. 2015), keeping identical sequences in the alignment. The ML tree search was estimated to use a specific substitution model for each locus.

Model parameters were separately estimated for each partition using ModelFinder, allowing each partition to have its evolution rate (−m MFP −p). The best ML tree was found after 1000 iterations with a perturbation strength of 0.2. ML analyses were carried out with 1000 bootstrap pseudoreplicates under the GTR-GAMMA model (−m GTRGAMMA −p 12345 −k −f a –N 1000 −x 12,345).

Species were recognized utilizing the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) criteria, as described by Taylor et al. (2000) and Dettman et al. (2003).

## 2.7 | Inoculum Preparation

The inoculum was prepared from fungal cultures grown on Petri dishes with PDA medium and incubated for a period of 7 days at 28°C in the dark. After this period, the fungal mycelium was scraped using a microbiological needle to obtain a suspension. The conidial concentration was determined by direct microscopic counting with the use of a Neubauer chamber. For this, the suspension was subjected to serial dilutions to achieve a final concentration of $1.0 \times 10^6$ conidia $mL^{-1}$. Fungal viability was assessed by adding 100 μL of a diluted fungal suspension ($1 \times 10^3$ spores $mL^{-1}$) to Petri dishes containing PDA and spreading it using a Drigalski loop. Plates were incubated at 28°C for 7 days, and colony formation was observed.

## 2.8 | In Vitro Antifungal Activity

The antifungal activity was evaluated in sterile, flat-bottom 96-well microplates. A total of 100 μL of Potato Dextrose Broth (PDB—Conde SA, Spain, SP), 100 μL of nanoemulsion (at varying concentrations), and 100 μL of fungal inoculum ($1.0 \times 10^6$ conidia $mL^{-1}$ *C. plurivorum*) were added to each well. The *S. terebinthifolia* essential oil nanoemulsion was tested at concentrations of 2.5%, 1.25%, 0.62%, 0.31%, 0.15%, 0.07%, 0.03%, 0.015%, 0.007% and 0.003%. The highest concentration of nanoemulsion was added to the first well, followed by serial dilution across the plate. The plates were then incubated at 28°C for 7 days in the dark. The negative control consisted of only PDB and the fungus only. The positive control contained the commercial fungicide tebuconazole at a final concentration of 0.02%. A solvent control containing only the emulsifiers Polysorbate 20 and sorbitan trioleate at the highest concentration used in the NE tests—(2.5% *w*/*v*) was also included. The experiment was conducted in triplicate, with three repetitions. Results were evaluated visually by observing the presence or absence of fungal growth inhibition in each well to determine the minimum inhibitory concentration (MIC).

Mycelial growth inhibition assays were also conducted in Petri dishes (60 × 15 mm) containing PDA medium amended with nanoemulsions at concentrations of 0.5%, 0.26%, 0.13%, 0.05% and 0.005%. Controls included: a positive control (PDA plus tebuconazole, with 10 μL of the fungicide at a concentration of 0.02%), solvent control (PDA plus emulsifier at concentrations 0.5%), and a negative growth control (PDA without any added substances). Following solidification of the medium, a 5 mm diameter wells were created in the centre of each plate and filled with 3 μL of a *C. plurivorum* spore suspension at an initial concentration of $1.0 \times 10^6$ conidia per millilitre. The plates were then incubated in a BOD chamber at 28°C in the dark. Mycelial growth diameters were measured along two perpendicular axes using a digital calliper on Days 2, 4, 6 and 8 after inoculation.

The experiment was conducted with three replicates for each treatment. The collected data were subjected to statistical analysis, as Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test ($p < 0.05$). All analyses were performed using RStudio (version 2.14).

## 2.9 | In Vivo Antifungal Activity

In vitro results guided the design of in vivo tests aimed at the evaluation of the preventive and curative effects of the nanoemulsion. For this stage, papaya fruits at ripening stage 1, as described by Martins et al. (2024), were selected. All fruits were harvested at stage 1 in the field and then allowed to ripen naturally during storage, progressing through stages 2–4 and reaching stage 5 (fully ripe) by the end of the 8-day evaluation period.

Papayas at ripening stage 1 were obtained from an organic farm located in the municipality of Aracruz, Espírito Santo, Brazil. The fruits were selected based on uniformity in size, colour and absence of visible defects or injuries.

Preventive treatment: Fruits were surface-sterilised, dipped in 1% sodium hypochlorite for 1 min, rinsed with sterile water and air-dried. They were then immersed for 5 min in 1 L of the nanoemulsion at concentrations that demonstrated high activity in vitro. Following immersion, the fruits were air-dried at room temperature for 2 h prior to inoculation with *Colletotrichum plurivorum*.

Curative treatment: Fruits were prepared and inoculated 24 h before the nanoemulsion treatment (immersion as described for the preventive treatment).

Inoculation: For both preventive and curative assays, inoculation was performed by pipetting 100 μL of a spore suspension ($1.0 \times 10^6$ conidia $mL^{-1}$) onto a superficial wound (5 mm diameter, 1 mm in depth) created on the fruit centre using a sterile cork borer.

Controls: For both assays, positive control fruits were immersed in the commercial fungicide Tebuconazole at a concentration of 0.02%, either before (preventive) or after (curative) inoculation. Negative control fruits were wounded and non-inoculated in sterile distilled water.

Incubation and Evaluation: After treatment and/or inoculation, the fruits were placed in plastic boxes, with five fruits per box and one box per treatment. The boxes were not hermetically sealed and were kept on benches in a controlled-temperature room at 25°C. The experiment was conducted under the ambient relative humidity of the room, which was approximately 80%. Under these conditions, the boxes allowed for sufficient humidity to favour disease development while avoiding water condensation on the fruit surface.

## 2.10 | Fruit Quality Evaluation

Fruit quality parameters were assessed at the end of the storage period (Day 8). Fresh mass loss (FML) was calculated as the percentage difference between the initial and final fresh weight of each fruit. Total soluble solids (TSS) were measured in juice extracted near the inoculation site using a digital refractometer, and the results were expressed in °Brix. These variables were used to evaluate whether the nanoemulsion treatments affected the postharvest physicochemical quality of papaya fruits and are not direct measures of antifungal activity.

## 2.11 | Experimental Design and Statistical Analysis

The in vivo experiment used a completely randomized design. Lesion diameter data were analysed considering treatment and evaluation time in a factorial design (NE 0.13% preventive, NE 0.26% preventive, Fungicide preventive, Water preventive, NE 0.13% curative, NE 0.26% curative, Fungicide curative, Water curative) and evaluation time (Days 2, 4, 6, 8) in a factorial arrangement. Four replicates (individual fruits) were used per treatment combination. FML and TSS were analysed at the end of the experiment (Day 8). Statistical analysis was performed with RStudio version 2.14. Data were subjected to ANOVA, and treatment means were compared with Tukey's test ($p < 0.05$).

# 3 | Results

## 3.1 | Essential Oil Composition

The GC–MS analysis of *S. terebinthifolia* essential oil revealed a chemical profile predominantly composed of monoterpenes, representing 71.67% of the total identified compound (Table 2). A total of ten compounds were identified, with *p*-cymene (30.6%), *α*-pinene (16.9%), limonene (15.3%) and myrcene (2.4%) being the major constituents. Other compounds detected in lower concentrations included *α*-phellandrene, *δ*-3-carene, *α*-thujene, camphene, *β*-pinene and terpinolene. These results are consistent with the findings of Barraqui et al. (2023), who identified *δ*-3-carene, *α*-pinene, and limonene as primary components in mature fruits. Variations in the relative abundance of specific compounds reported in the literature are commonly attributed to factors such as fruit maturity, geographical origin and analytical conditions (Cavalcanti et al. 2015; Schimitberger et al. 2018). Overall, the predominance of monoterpenes in the essential oil provides a chemical basis for the antifungal activity evaluated in this study.

## 3.2 | Dynamic Light Scattering (DLS) Characterisation

The dynamic light scattering (DLS) analysis was performed to evaluate the droplet size distribution, polydispersity index (PDI) and zeta potential of the *Schinus terebinthifolia* nanoemulsion prepared at large scale (1 L) and stored for 30 days (Table 3 and Figure 1).

**TABLE 2** | Chemical composition of the essential oil from fruits of *Schinus terebinthifolia*.

| | RT (min) | Compound | Area % | $AI_{calc}$ | $AI_{lit}$ |
|---|---|---|---|---|---|
| 1 | 6.934 | *α*-Thujene | 0.993 | 926 | 924 |
| 2 | 7.201 | *α*-Pinene | 16.937 | 934 | 932 |
| 3 | 7.631 | Camphene | 0.364 | 947 | 946 |
| 4 | 8.603 | *β*-Pinene | 0.431 | 976 | 974 |
| 5 | 9.158 | Myrcene | 2.386 | 992 | 988 |
| 6 | 9.639 | *α*-Phellandrene | 1.429 | 1005 | 1002 |
| 7 | 9.868 | *δ*-3 Carene | 2.776 | 1011 | 1008 |
| 8 | 10.560 | *p*-Cymene | 30.630 | 1028 | 1020 |
| 9 | 10.709 | Limonene | 15.327 | 1031 | 1024 |
| 10 | 13.072 | Terpinolene | 0.398 | 1088 | 1086 |

*Note:* Total identified: 71.67%. Monoterpenes: 71.67%.
Abbreviations: $AI_{calc}$, arithmetic index calculated; $AI_{lit}$, arithmetic index from literature; RT, retention time.

**TABLE 3** | Hydrodynamic diameter (Z-average), peak size, polydispersity index (PDI) and zeta potential of *Schinus terebinthifolia* nanoemulsions at different concentrations and storage times.

| Concentration (%) | Time (days) | Z-average (nm) | PDI | Zeta potential (mV) |
|---|---|---|---|---|
| 0.13 | 1 | 20.9 | 0.237 | −31.6 |
| | 8 | 19.7 | 0.228 | −32.7 |
| | 21 | 18.5 | 0.221 | −34.1 |
| | 30 | 21.0 | 0.235 | −33.0 |
| 0.26 | 1 | 28.5 | 0.254 | −36.8 |
| | 8 | 19.7 | 0.229 | −37.4 |
| | 21 | 21.4 | 0.240 | −35.9 |
| | 30 | 20.9 | 0.231 | −34.6 |
| 0.50 | 1 | 36.2 | 0.287 | −32.5 |
| | 8 | 28.9 | 0.263 | −31.8 |
| | 21 | 28.6 | 0.271 | −33.7 |
| | 30 | 33.3 | 0.280 | −32.9 |

The nanoemulsions maintained particle sizes within the nanometric range (20–40 nm) throughout the storage period, confirming the stability of the formulation. At Day 1, the hydrodynamic diameters ranged from 20.86 (0.13%) to 36.21 nm (0.5%). By Day 8, a slight reduction in size was observed, with values between 19.74 (0.26%) and 28.92 nm (0.5%). On Day 21, the diameters remained relatively stable, varying from 18.46 (0.13%) to 28.57 nm (0.5%). At Day 30, small variations occurred, with droplet sizes ranging from 20.90 (0.26%) to 33.30 nm (0.5%). These results indicate that the nanoemulsion exhibited good physical stability, with no evidence of aggregation or phase separation during storage.

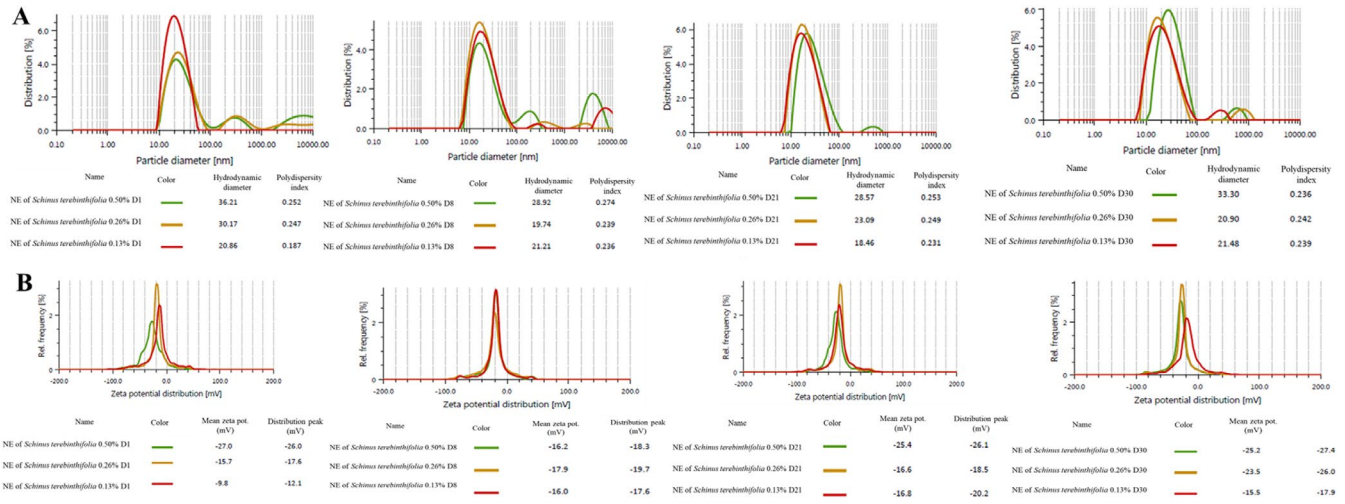


**FIGURE 1** | (A) Hydrodynamic diameter (nm) and (B) Zeta potential (mV) of *Schinus terebinthifolia* nanoemulsions at different concentrations (0.5%; 0.26% and 0.13%) during 30 days of storage. Measurements were performed using dynamic light scattering (DLS).

The polydispersity index (PDI) values were consistently below 0.3 for all formulations and time points, confirming the monodisperse nature of the droplets and uniform distribution. Zeta potential values ranged between −31.6 and −37.4 mV, indicating high electrostatic repulsion between droplets, which further supported the stability of the colloidal system. Overall, the DLS characterization confirmed that the large-scale nanoemulsion of *S. terebinthifolia* retained its nanometric characteristics and physicochemical stability for at least 30 days under storage at room temperature (25°C).

### 3.3 | Molecular Identification and Phylogenetic Delimitation of Colletotrichum Species

BLAST searches of the GAPDH, APN2/MAT-IGS, GS and TUB2 sequences generated from the papaya isolate (strain 898) indicated high similarity to species within the *Colletotrichum orchidearum* species complex, with the closest matches to *C. plurivorum*. To confirm its taxonomic placement, a multilocus phylogenetic analysis was performed using a concatenated alignment of GAPDH and TUB2 sequences from our isolate together with ex-type and reference strains of recognised species in the *C. orchidearum* complex.

In the maximum-likelihood tree, rooted with *C. piperis*, strain 898 grouped within a well-supported clade corresponding to *C. plurivorum*, together with the ex-type strain CBS 125474 and other reference isolates (Figure 2). No other species of the complex clustered with this lineage, and branch support values for the *C. plurivorum* clade were high, confirming the affiliation of our isolate to this species. Based on these multilocus phylogenetic data and the GCPSR criteria applied, the pathogen used in this study was identified as *Colletotrichum plurivorum*.

### 3.4 | In Vitro Antifungal Activity

The minimum inhibitory concentration (MIC) of the *Schinus terebinthifolia* essential oil nanoemulsion against *Colletotrichum plurivorum* was determined with a 96-well microplate assay. Visual assessment after 7 days of incubation revealed that nanoemulsion concentrations of 0.15% and higher (0.31%, 0.62%, 1.25% and 2.5%) completely inhibited fungal growth (Figure 3). Lower concentrations allowed fungal development. Therefore, the MIC was determined to be 0.15%.

In the Petri dish assays, nanoemulsion inhibited the mycelial growth of *C. plurivorum* in a concentrations-dependent manner (Table 4 and Figure 4). Complete inhibition of mycelial growth (0 mm diameter) was observed throughout the 8-day incubation period at nanoemulsion concentrations of 0.50%, 0.26% and 0.13%. This level of inhibition was statistically similar ($p > 0.05$) to that observed for the positive control (Tebuconazole fungicide). A moderate inhibitory effect was observed at the 0.05% concentration, where mycelial growth was significantly reduced compared to the negative control (PDA medium only) but significantly higher than the 0.13% concentration ($p < 0.05$). The lowest concentration tested (0.005%) exhibited significantly less inhibition compared to the 0.05% concentration, although it still showed some reduction compared to the negative control at earlier time points ($p < 0.05$). The solvent control (emulsifiers only) did not significantly inhibit fungal growth compared to the negative control (PDA medium) ($p > 0.05$), which indicates that the observed antifungal activity was due to the essential oil within the nanoemulsion.

In the 96-well microplate assay, a wide concentration range of the nanoemulsion (0.003%–2.5%) was tested to take advantage of the high-throughput format of this method and to accurately determine the minimum inhibitory concentration (MIC) in a single experiment. For the mycelial growth assay in petri dishes, we subsequently selected a narrower range of concentrations (0.005%–0.50%), centred around and above the MIC obtained in the microplate assay. This strategy allowed us to confirm the inhibitory effect under solid-medium conditions while avoiding the use of unnecessarily high concentrations and excessive amounts of emulsifier in the agar medium.

### 3.5 | In Vivo Antifungal Activity

Evaluation of the nanoemulsion's efficacy in the control of anthracnose on inoculated papaya fruits revealed significant

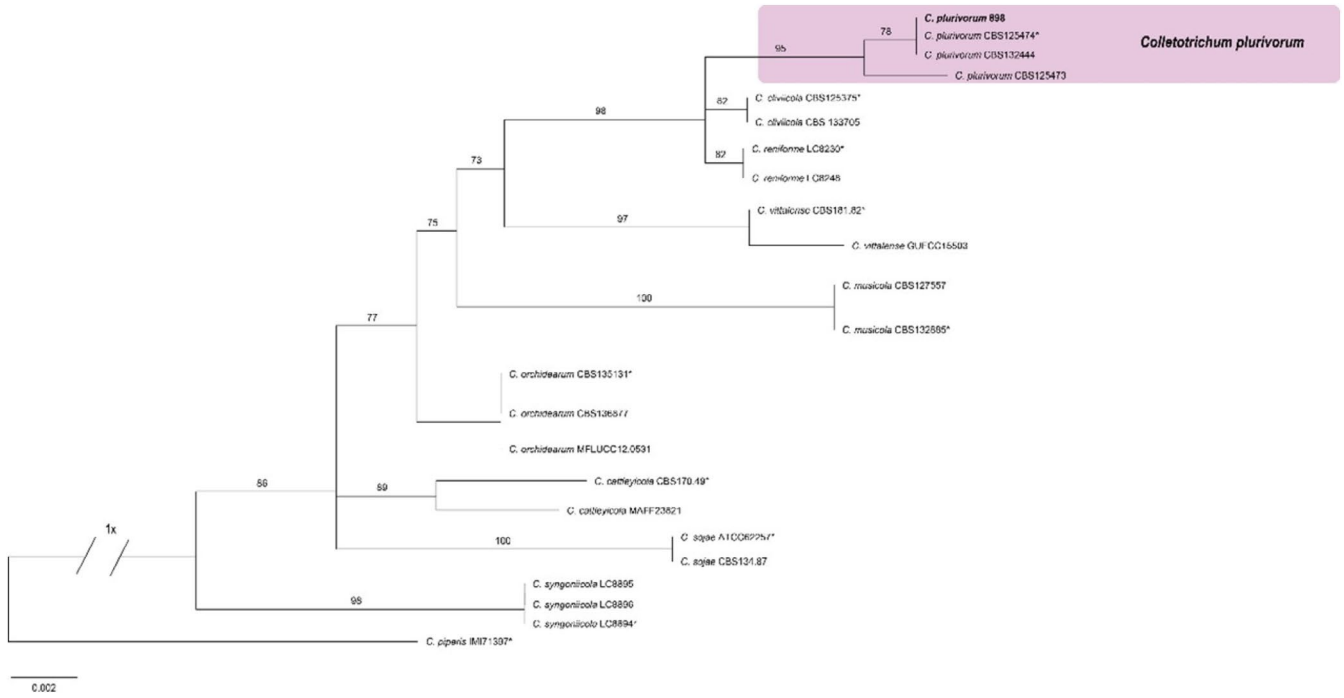


**FIGURE 2** | Maximum likelihood tree of the *Colletotrichum orchidearum* species complex inferred on IQ-TREE from a concatenated alignment of GAPDH and TUB2. Significant supports for ML (SH-alrt bootstrap ≥ 70) are shown above the nodes. The tree was rooted with *Colletotrichum piperis*. Ex-type isolates are indicated with '*' at the end of the taxa labels. Isolates from the study are highlighted in bold. The scale bar indicates the average number of substitutions per site.

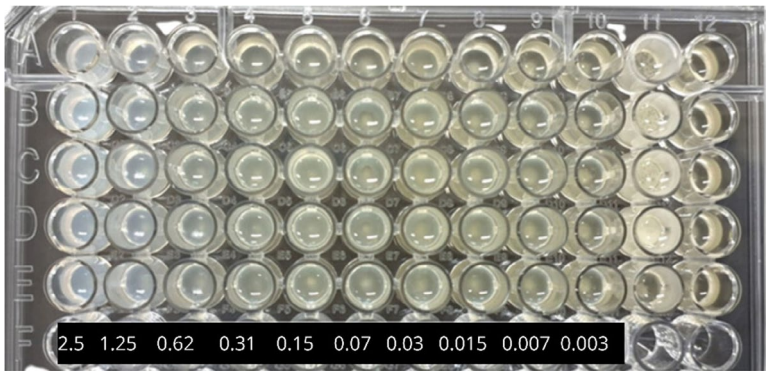


**FIGURE 3** | Biological activity of the *Schinus terebinthifolia* essential oil nanoemulsion against the fungus *Colletotrichum plurivorum* in 96-well microplate assays.

preventive and curative effects. After eight days of storage at 25°C, anthracnose symptoms (lesions around the inoculation site) were observed only in the negative control fruits (treated with sterile distilled water) (Figure 5). Fruits treated with the *S. terebinthifolia* nanoemulsion at concentrations of 0.26% and 0.13%, applied either preventively (before inoculation) or curatively (after inoculation), showed no visible lesion development, similar to the positive control fruits treated with tebuconazole. Disease incidence was 0% for all nanoemulsion and fungicide treatments, compared to 100% incidence in the negative controls for both preventive and curative assays. Consequently, lesion diameter was 0 mm for all nanoemulsion and fungicide treatments throughout the 8-day evaluation period, and differed significantly ($p < 0.05$) from the negative controls where lesions progressively enlarged.

During the 8-day storage period, an increase in fresh mass loss (FML) was observed in all treatments (Table 5). However, fruits treated with *S. terebinthifolia* essential oil nanoemulsion (0.13% and 0.26%), under both preventive and curative applications, exhibited significantly lower FML ($p < 0.05$) compared to the negative control. The lowest FML was observed in the preventive treatments (1.47% for 0.13% NE and 1.79% for 0.26% NE). The positive control (fungicide) also showed significantly lower FML than the negative control but generally higher than the nanoemulsion treatments. The highest FML occurred in the negative controls (6.18% curative, 8.26% preventive).

Regarding total soluble solids (TSS), no significant differences ($p > 0.05$) were observed among the treatments at the

**TABLE 4** | Colony diameter (mm) of *C. plurivorum* grown on PDA medium with different concentrations of *S. terebinthifolia* nanoemulsion.

| Treatment (concentration) | Day 02 | Day 04 | Day 06 | Day 08 |
|---|---|---|---|---|
| 0.005% | 12.04 ± 0.96 bc | 31.67 ± 0.49 ab | 51.4 ± 3.41 ab | 67.2 ± 5.14 ab |
| 0.05% | 12.04 ± 0.96 bc | 32.33 ± 5.56 ab | 50.21 ± 8.45 b | 67.0 ± 0.98 ab |
| 0.13% | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c |
| 0.26% | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c |
| 0.50% | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c |
| PC—Fungicide | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c | 0.00 ± 0.00 c |
| NC—Polysorbate 20%–0.05% | 14.11 ± 0.52 ab | 30.34 ± 1.72 b | 43.23 ± 6.18 ab | 73.55 ± 4.39 ab |
| NC—Medium | 25.05 ± 1.19 a | 56.47 ± 0.49 a | 68.85 ± 3.41 a | 81.26 ± 3.45 a |

*Note:* Mean colony diameter of *C. plurivorum*. Values in the same column followed by the same letter do not differ significantly according to Tukey's test ($p < 0.05$).
Abbreviations: NC = negative control; PC = positive control (Tebuconazole).

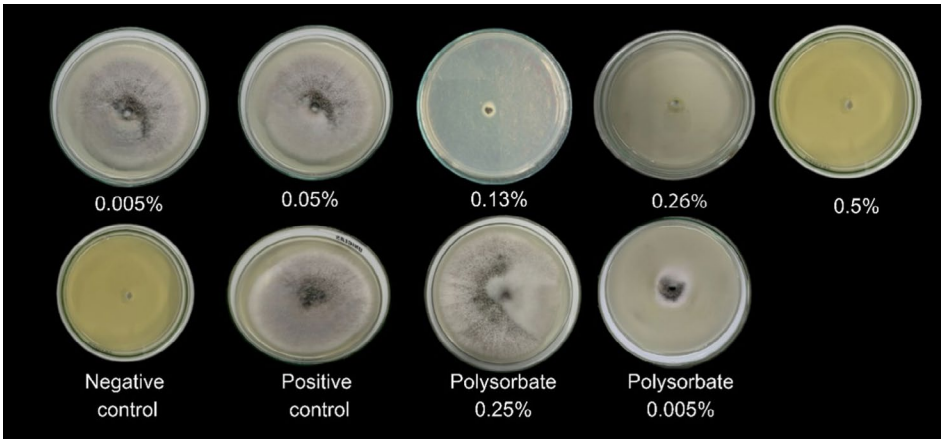


**FIGURE 4** | —Biological activity of the *Schinus terebinthifolia* essential oil nanoemulsion against the fungus *Colletotrichum plurivorum* in petri dish assay.

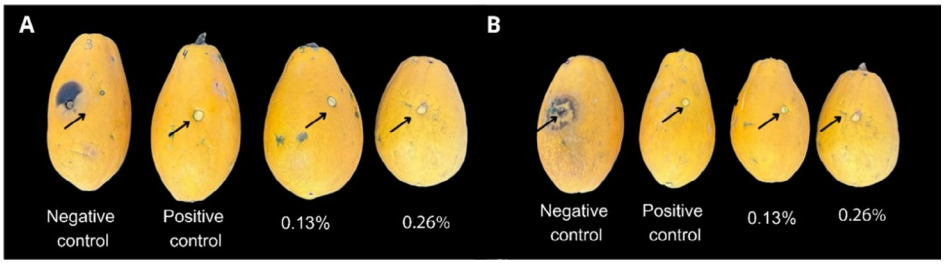


**FIGURE 5** | Curative treatment (A) and preventive treatment (B) with the essential oil nanoemulsion from *S. terebinthifolia* fruits against the *Colletotrichum plurivorum* species in papaya fruits.

end of the storage period, with values that ranged from 12.22° to 12.98°Brix. The colour progression of the fruits followed the natural ripening process and transitioned from green (stage 1) towards yellow-orange (stage 4) uniformly across all treatments.

## 4 | Discussion

This study demonstrated the significant antifungal potential of a nanoemulsion formulated with essential oil from *Schinus terebinthifolia* fruits against *Colletotrichum plurivorum*, the causal agent of papaya anthracnose. The in vitro assays clearly showed that the nanoemulsion inhibited fungal growth, with an MIC of 0.15% in microplate assays and complete inhibition of mycelial growth at 0.13% in petri dish assays over 8 days. This inhibitory effect was concentration-dependent.

Crucially, the efficacy observed in vitro translated successfully to the in vivo experiments on papaya fruits. Both preventive and curative applications of the nanoemulsion at 0.13% and 0.26% completely prevented the development of anthracnose lesions over an 8-day storage period at 25°C. This level of control was comparable to that achieved with the commercial fungicide

**TABLE 5** | Fresh mass loss (FML) in papaya fruits subjected to curative and preventive treatments with *S. terebinthifolia* nanoemulsion.

| **Curative** | | |
|---|---|---|
| **Treatment** | **FML (%)** | **SST (°Brix)** |
| 0.13% | 2.09% | 12.73 ± 0.88 a |
| 0.26% | 1.82% | 12.59 ± 0.71 a |
| Positive control antifungal | 4.35% | 12.98 ± 0.75 a |
| Negative control | 6.18% | 12.78 ± 0.75 |
| **Preventive** | | |
| **Treatment** | **FML (%)** | **SST (°Brix)** |
| 0.13% | 1.47% | 12.54 ± 0.73 a |
| 0.26% | 1.79% | 12.22 ± 1.06 a |
| Positive control antifungal | 5.12% | 12.79 ± 0.74 a |
| Negative control | 8.26% | 12.59 ± 0.75 a |

Abbreviation: FML = fresh mass loss.

Tebuconazole, which highlights the potential of the *S. terebinthifolia* nanoemulsion as a viable alternative for post-harvest disease management.

The antifungal capacity of *S. terebinthifolia* essential oil is likely attributable to its chemical composition, particularly the presence of monoterpenes like $\alpha$-pinene, $\beta$-myrcene, $\alpha$-phellandrene, $\delta$-3-carene and limonene, as reported previously (Barraqui et al. 2023). These compounds are highly lipophilic and are known to interact with fungal cell membranes, leading to increased membrane fluidity and permeability, disruption of membrane-embedded proteins and leakage of intracellular contents. In addition, these effects may interfere with key metabolic processes such as respiration and ion transport, ultimately compromising cell viability (Rao et al. 2010).

Beyond the action of individual constituents, the antifungal effect of essential oils is strongly influenced by the phytocomplex, which involves synergistic interactions among major and minor terpenes. These interactions can enhance membrane destabilisation and promote a multitarget mode of action, resulting in a cumulative antifungal effect that is more pronounced than that of isolated compounds. Due to their lipophilic nature, these molecules can simultaneously affect multiple cellular targets, including membrane structure, enzymatic activity and intracellular homeostasis (Radice et al. 2025).

An important advantage of the nanoemulsion formulation observed in this study was the lack of phytotoxicity on the papaya fruits. Previous research that used *S. terebinthifolia* essential oil directly, even when diluted, reported phytotoxic effects at concentrations required for fungal control, which limited its practical application (Oliveira Júnior et al. 2013). In contrast, our nanoemulsion formulation, even at 0.5% (tested in vitro), did not cause observable damage when applied to fruits at 0.13% and 0.26% in vivo. This suggests that the nanoencapsulation reduces direct contact of phytotoxic compounds with fruit tissues, while ensuring controlled release of active compounds.

The characterization by DLS confirmed that the nanoemulsion produced at large scale maintained nanometric droplet sizes (20–40 nm), low polydispersity (PDI $< 0.3$), and stable zeta potential (−31 to −37 mV) for at least 30 days of storage. These findings demonstrate that the formulation was stable and reproducible between small- and large-scale preparations, addressing a key requirement for its potential application (Barraqui et al. 2025).

The use of nanoemulsions offers several advantages over direct application of essential oils. The nanometric droplet size enhances stability, improves surface coverage on the fruit, and potentially allows for controlled release of the active compounds (Pandey et al. 2022; Sheth et al. 2020). This likely explains the high efficacy observed in vivo in our study, in contrast with some reports with *Lippia sidoides* oil, where the in vivo activity of essential oils was lower than expected from in vitro results, possibly due to poor incorporation or high volatility (Zillo et al. 2018). Through the improvement of the delivery and persistence of the active components, nanoemulsification can enhance antifungal efficacy and potentially reduce the amount of essential oil required, which offers both biological and economic benefits.

In addition to disease control, the nanoemulsion treatment reduced fresh mass loss (FML), likely due to the formation of a thin coating that limited water vapour diffusion and transpiration (Oliveira et al. 2020). Importantly, no significant differences in total soluble solids (TSS) were observed among treatments, indicating that the natural ripening process was not affected. However, this study did not evaluate fruit colour and firmness, parameters that are highly relevant for consumer acceptance. The absence of these measurements should be acknowledged as a limitation (Ali et al. 2011).

The combination of effective anthracnose control and maintenance of fruit quality suggests that the *S. terebinthifolia* essential oil nanoemulsion is a promising, natural alternative for post-harvest management of papaya. The ability to achieve control comparable to a synthetic fungicide at a relatively low concentration (0.13%) is particularly encouraging. The use of these nano-structured systems, by enhancing the efficacy of bioactive compounds and ensuring better stability and controlled release, constitutes a promising strategy for integrated postharvest disease management (Palhano et al. 2004).

Future research should focus on the optimisation of the nanoemulsion formulation for long-term stability under commercial storage and transport conditions. Further investigation into the precise mechanisms of antifungal action and potential synergistic effects within the essential oil is warranted. Additionally, the evaluation of the impact on fruit sensory attributes and the conduction of thorough cost–benefit analyses are necessary steps towards potential commercial application.

## 5 | Conclusion

The application of nanoemulsions that contain *Schinus terebinthifolia* essential oil represents an innovative and effective strategy for the control of anthracnose, caused by *Colletotrichum*

*plurivorum*, in post-harvest papaya fruits. This study demonstrated that nanoemulsion concentrations of 0.13% and 0.26% effectively inhibited the fungus in vitro and provided complete control of the disease in vivo, and exhibited both curative and preventive activity comparable to a commercial fungicide. Importantly, the nanoemulsion formulation enhanced the essential oil's efficacy without causing phytotoxicity or negatively impacting fruit ripening. The effectiveness of the lower concentration (0.13%) is particularly noteworthy, which suggests a potentially sustainable and cost-effective alternative for papaya producers. This approach offers significant potential for the extension of fruit shelf life and maintenance of post-harvest quality and makes a valuable contribution to integrated disease management strategies in papaya production.


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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