ARTICLE | Article in Press

Essential oil nanoemulsions as a sustainable postharvest treatment to control anthracnose caused by *Colletotrichum* spp. in papaya and banana

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Received: 30 May 2026 / Accepted: 18 August 2026

Abstract

Brazilian fruit production faces substantial postharvest losses due to anthracnose, creating demand for control strategies less dependent on synthetic fungicides. This study developed, characterized, and evaluated essential oil nanoemulsions of *Cymbopogon flexuosus*, *Cymbopogon winterianus*, and *Zingiber officinale* against *Colletotrichum plurivorum* and *C. musae* in papaya and banana. Formulations were prepared by low-energy emulsification and showed nanometric droplet sizes (95.9 to 171.3 nm) and low polydispersity indices (<0.28); zeta potentials ranged from −4.3 to -24.5 mV, consistent with steric stabilization typical of nonionic (polysorbate) systems. GC/MS identified geranial and neral in *C. flexuosus*, citronellal, geraniol and citronellol in *C. winterianus*, and ar-curcumene and α-zingiberene in *Z. officinale* as main constituents. In vitro, the *C. winterianus* and *C. flexuosus* nanoemulsions inhibited mycelial growth of both pathogens at low concentrations (0.03–0.07% essential oil). In fruit assays, the *C. winterianus* nanoemulsion reduced anthracnose severity in papaya in a concentration-dependent manner, with no visible lesion at 0.16% and no deterioration of the physicochemical parameters measured. Under the same conditions, the formulations did not reduce disease severity in banana, and the *Z. officinale* nanoemulsion showed low activity throughout. The *C. winterianus* nanoemulsion is therefore a potential candidate for postharvest control of papaya rather than a confirmed alternative to conventional fungicides, warranting further evaluation before broader application.

Keywords Postharvest preservation · Nanoemulsions · Essential oils · Anthracnose control · *Colletotrichum* spp. · Tropical fruits

https://doi.org/10.1038/s41598-026-67933-9

Published online: 03 September 2026

Introduction

Bananas and papayas are among the most economically and socially relevant tropical fruits cultivated in Brazil. In 2021, Brazil was the world's largest consumer and the fourth-largest producer of bananas, harvesting approximately 6.6 million tons over 455,000 hectares[1]. Papaya is also a relevant export commodity: approximately 44,000 tons were exported in 2024, generating around US$58.1 million in revenue[1]. Both production chains are affected by postharvest losses caused mainly by fungal diseases, which reduce fruit quality and commercial value.

Anthracnose, caused by species of the genus *Colletotrichum*, is among the most frequent postharvest diseases of tropical fruits, affecting papaya, mango, banana and passion fruit[2]. In banana, the disease is mainly associated with *Colletotrichum musae*, whereas papaya anthracnose is caused by species belonging to different *Colletotrichum* complexes, including *C. plurivorum*. Because morphological characters are conserved within these complexes, reliable species assignment requires multilocus phylogenetic analysis, a point of practical relevance when antifungal treatments are evaluated against defined pathogen targets[3]. At a global scale, the Food and Agriculture Organization of the United Nations (FAO) estimates that up to 40% of crop production is lost annually to plant pests, with plant diseases costing the world economy over US$ 220 billion and invasive insects at least US$ 70 billion per year[4].

Although various agricultural practices help reduce the occurrence of diseases, modern agriculture still relies predominantly on synthetic fungicides to sustain productivity and fruit quality. This reliance faces two converging constraints. First, regulatory restrictions have progressively removed active ingredients from the market: carbendazim, a benzimidazole fungicide widely used for fungal disease control, was banned in Brazil because of its environmental persistence and potential risks to human health[5]. Azole fungicides, in turn, have been detected at elevated concentrations in wastewater, raising environmental concerns and creating potential barriers to international trade through maximum residue limits[6]. Second, resistance to the main fungicide classes used against anthracnose has been documented in *Colletotrichum*. The Fungicide Resistance Action Committee (FRAC) reports resistance in *Colletotrichum* spp. to methyl benzimidazole carbamates (MBC), quinone-outside inhibitors (QoI) and demethylation inhibitors (DMI), associated with mutations in the *TUB2*, *CYTB* and *CYP51* genes[7]. In Brazil, *C. musae* isolates from banana carrying thiophanate-methyl resistance and displaying reduced sensitivity to postharvest fungicides have already been characterized, indicating that the useful life of the currently registered chemical options may be limited[8,9].

In this context, there is a growing demand for disease control strategies that combine efficacy, environmental safety, and regulatory compliance. Essential oils (EOs) have been investigated as one such alternative. EOs are complex mixtures of volatile compounds obtained mainly from aromatic plants by steam distillation or cold pressing. They concentrate plant secondary metabolites, such as monoterpenes, sesquiterpenes, aldehydes, alcohols, and esters, which account for their biological and aromatic properties. EOs have documented antimicrobial, antiparasitic, antifungal, and allelopathic activities[10–12].

Their direct application in agriculture faces is nevertheless limited by high volatility, low water solubility, and instability under field and storage conditions. These factors reduce practical efficacy and require frequent reapplication, which increases costs. Delivery systems capable of stabilizing and protecting the active compounds are therefore needed, and nanoemulsions are one such system. Nanoemulsions are colloidal dispersions of oil droplets in an aqueous phase, typically with diameters between 20 and 200 nm, and stabilized by surfactants. This structure can promote the encapsulation and protection of active ingredients and has been associated with controlled release, greater dispersibility in water, improved stability and reduced volatilization of essential oils[13]. These properties may facilitate the use of EOs in postharvest disease management, although efficacy remains dependent on the specific oil, pathogen and host involved.

Among the species with reported antifungal activity, *Cymbopogon winterianus* essential oil reduced mycelial growth and conidial production of *Fusarium oxysporum* f. sp. *Lycopersici*[14]. The essential oil of *Cymbopogon flexuosus* was active against *Fusarium avenaceum* with reported effects on the plasma membrane and reduced

pathogenicity associated with inhibition of enzymatic activity[12]. *Zingiber officinale* essential oil inhibited the mycelial growth of *Colletotrichum theobromicola*, the causal agent of anthracnose, by 52% to 68.6% at 150 and 200 µL mL^{-1}, respectively, with a concurrent reduction in sporulation[15].

Despite this body of evidence, most reports on EO nanoemulsions address in vitro activity or pathogens of field crops, and comparatively few evaluate postharvest anthracnose in tropical fruits under conditions resembling commercial handling. In addition, studies in this area frequently rely on isolates identified by morphology alone, which is unreliable within *Colletotrichum* species complexes. The present study addresses this gap by combining chemical and colloidal characterization of three EO nanoemulsions with antifungal assays against host-associated *C. plurivorum* and *C. musae* isolates identified by multilocus phylogeny, followed by fruit immersion assays that reproduce a treatment format used in packinghouses. The contribution therefore lies not in the nanoemulsification technique itself, which is well established, but in the evaluation of defined formulation–pathogen–host combinations under postharvest conditions.

This study aimed to develop and characterize nanoemulsions based on the essential oils of *Cymbopogon flexuosus*, *Cymbopogon winterianus*, and *Zingiber officinale* and to evaluate their antifungal activity against phylogenetically identified *Colletotrichum plurivorum* and *Colletotrichum musae* isolates, both in vitro and on papaya and banana fruits under postharvest storage conditions.

Materials and methods

Essential oils and reagents

The essential oils (EOs) of *Cymbopogon flexuosus* (lemongrass, Lot 220), *Cymbopogon winterianus* (citronella, Lot 161), and *Zingiber officinale* (ginger, Lot 294) were commercially acquired from FERQUIMA Indústria e Comércio Ltda (Brazil) and stored at 4 °C in amber glass bottles until use. The surfactants used for nanoemulsion preparation were sorbitan trioleate (Sigma-Aldrich), sorbitan monooleate (Sigma-Aldrich), polysorbate 20 (Neon, Brazil), and polysorbate 80 (Synth, Brazil). Distilled water was used as the aqueous phase in all formulations. All reagents were of analytical grade and used without further purification.

Chemical characterization

The chemical characterization of the EOs was performed using an Agilent 7890B gas chromatograph coupled to a 5977 A mass selective detector (MSD) operating under electron impact ionization at 70 eV and scanning from m/z 50 to m/z 550. An HP-5 column (30 m × 250 µm × 0.25 µm) was used for compound separation, with Helio as carrier gas at 1.0 mL min^{-1}. Samples were diluted in dichloromethane at 0.1% and 2 µL was injected in split (1:10) mode. The injector temperature was set at 290 °C, and the MSD transfer line, ion source and quadrupole were maintained at 310 °C, 230 °C and 150 °C, respectively. The oven temperature program started at 40 °C, followed by a heating rate of 5 °C min^{-1} up to 280 °C, and subsequently 15 °C min^{-1} up to 310 °C.

A homologous series of n-alkanes (C10–C40) was analysed under identical chromatographic conditions, and linear retention indices (LRI) were calculated according to van den Dool and Kratz[16], which is the appropriate formulation for temperature-programmed runs. Compounds were identified by comparison of their mass spectra with those of the NIST (2.4) spectral library and by comparison of retention indices with literature values[17].

Development and characterization of nanoformulations

Nanoemulsions (NEs) containing EOs were prepared using a low-energy emulsification method. Formulations consisted of 5% (w/w) essential oil (EO), a surfactant or surfactant mixture at 15% −20% (w/w), and distilled water to 100% (w/w).

Four target hydrophilic–lipophilic balance (HLB) values — 11, 13, 15 and 16.7 — were screened for each essential oil. These values were obtained by combining four surfactants of different HLB: sorbitan trioleate (Span 85, HLB 1.8), sorbitan monooleate (Span 80, HLB 4.3), polysorbate 80 (Tween 80, HLB 15) and polysorbate 20 (Tween 20, HLB 16.7). For each surfactant pair, the required mass ratio was calculated from the weighted-average equation[18]:

$$HLB = (mA \times HLBA + mB \times HLBB) / (mA + mB)$$

where HLB represents the hydrophilic-lipophilic balance of the surfactant system, and mA and mB correspond to the mass (g) of surfactants A and B, respectively.

HLB 11 and 13 were reached using a sorbitan ester combined with a polysorbate; HLB 15 was obtained either with Tween 80 alone or with a sorbitan ester plus Tween 20; and HLB 16.7 was obtained with Tween 20 alone. Each target HLB was tested at two total surfactant concentrations, 15% and 20% (w/w), giving 24 candidate formulations per essential oil (Supplementary Table S1). Candidate formulations were first screened by visual inspection for macroscopic homogeneity, absence of phase separation and the presence of the Tyndall effect, over the first 24 h of storage. For each essential oil, the formulation that remained macroscopically stable and showed the smallest droplet size and lowest polydispersity index by dynamic light scattering was selected for the subsequent assays.

For in vivo assays, 1 L of nanoemulsion was produced, maintaining the proportional composition described above. Throughout this study, all nanoemulsion concentrations are expressed as the essential oil (EO) content, not as the proportion of nanoemulsion. The stock nanoemulsion contained 5% EO, and working concentrations were obtained by volumetric dilution of this stock in distilled water; each dilution is designated by its resulting EO concentration.

Low-energy emulsification procedure

In the preparation procedure, the oil phase (EO and surfactants pre-mixed according to the target HLB) was added to a glass vial and homogenized using a vortex mixer (model VX-38-BI) at 500 rpm for 2 min. Next, the aqueous phase (distilled water) was slowly added under continuous agitation until the final formulation mass was reached. After the complete addition of the aqueous phase, the system was homogenized for an additional 2 min. All preparations were carried out at room temperature (25 °C). NE formation and stability were initially evaluated qualitatively by visual inspection, considering homogeneity, absence of phase separation, and the presence of the Tyndall effect, characterized by a bluish light scattering typical of nanometric colloidal dispersions.

The selected stock nanoemulsions (5% EO) were diluted in distilled water to EO concentrations of 2.5%, 1%, 0.5% and 0.2% for characterization. These concentrations correspond, respectively, to 50%, 20%, 10% and 4% (v/v) of the stock nanoemulsion.

Hydrodynamic diameter and zeta potential

The hydrodynamic diameter, polydispersity index (PdI), and zeta potential were determined by dynamic light scattering (DLS) using a Litesizer 500® instrument (Anton Paar) at 25 °C. Only formulations that remained macroscopically stable on visual inspection were analyzed. Measurements were performed directly on the working dilutions (2.5%, 1%, 0.5% and 0.2% EO); no additional dilution was applied prior to analysis. Refractive index and viscosity of the dispersant were set at 1.330 and 0.8931 mPa·s, respectively, and zeta potential was calculated using the Smoluchowski approximation. One batch was prepared for each formulation, and each sample was analysed in a single measurement on each evaluation day; the number of runs per measurement (up to 1000) refers to the internal acquisition cycles averaged by the instrument to produce that single measurement and does not constitute experimental replication. Values are therefore reported as single determinations, without an

associated dispersion measure, and neither replicate-measurement nor batch-to-batch variability was assessed. Measurements were conducted on days 1, 17, and 34 after preparation to evaluate the temporal stability of the nanoemulsions.

Isolation and identification of fungal strains

The fungal isolates of *Colletotrichum musae* (E-892) and *Colletotrichum plurivorum* (E-898) were obtained from Plant Pathology Laboratory of the Capixaba Institute for Research, Technical Assistance, and Rural Extension (Incaper), and isolated from naturally infected fruits of banana cv. Pome (*Musa* AAB) and papaya (*Carica papaya*) collected in October, 2024 at the Experimental Farms in Alfredo Chaves (−20.616944, −41.782778), and Sooretama (−19.118363, −40.0802547), respectively, Espírito Santo, Brazil.

Fragments (approximately 1 mm) were excised from the margin between symptomatic and healthy tissue, surface-disinfested in sodium hypochlorite (1%) for 1 min, rinsed twice in sterile distilled water, blotted dry and plated onto potato dextrose agar (PDA). Plates were incubated at 25 ± 2 °C under a 12 h photoperiod, and emerging colonies were purified by by monosporic culture. Identification based on morphological characteristics was performed by one of the authors (J.A.V.); the isolates were designated E-892 and E-898 and maintained at the Incaper Plant Pathology Laboratory. Pathogenicity of both isolates was confirmed on the respective hosts in the in vivo assays described below, in which inoculated non-treated fruits developed typical anthracnose symptoms.

DNA extraction, PCR amplification, and sequencing

Colletotrichum isolates were cultured on potato dextrose agar (PDA) at 25 ± 2 °C under a 12 h photoperiod for 7 days. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method[19]. The loci *GAPDH*, *APN2/MAT-IGS*, *GS*, *and TUB2* were amplified for multilocus phylogenetic analysis, as these markers are informative for species delimitation in *Colletotrichum* complexes[3]. Primer pairs and thermal cycling conditions for each locus are given in Supplementary Table S2.

Polymerase chain reaction (PCR) was performed in 12.5 µL reaction mixtures containing PCR-grade water, genomic DNA, primers (10 µM each), and 2× PCR Master Mix (GoTaq® Green Master Mix, Promega). Amplicons were separated on 1.5% agarose gel and visualized under UV light. PCR products were purified by ethanol and ammonium acetate precipitation and sequenced in both directions using an ABI 3730xl DNA Analyzer at LABBE, Universidade Federal de Pernambuco. Sequence assembly and editing were performed with the Staden Package[21]. Consensus sequences were compared with GenBank entries using BLASTn and deposited under the accession numbers listed in the Data Availability statement. No custom code or bespoke computational pipeline was developed in this study; all analyses were performed with publicly available software using the parameters described.

Phylogenetic analyses

Reference sequences of *Colletotrichum* were retrieved from the GenBank database and aligned using MAFFT v.7 with the Q-INS-i algorithm[21]. Loci were concatenated using SequenceMatrix v.1.8[22]. Phylogenetic trees were inferred using the maximum likelihood (ML) method in IQ-TREE v.2.1.2[23], with the best-fit substitution model selected independently for each partition using ModelFinder[24]. Branch support was estimated with 1000 bootstrap replicates under the GTR+GAMMA model. Species delimitation followed the GCPSR concept[25,26].

Microdilution assay in 96-well plates

Minimum inhibitory concentration (MIC) was determined using a broth microdilution method in 96-well plates, based on visual assessment of fungal growth inhibition[27]. Each well received potato dextrose broth (PDB),

serially diluted NE, and fungal inoculum. Essential oil concentrations in the wells ranged from 2.5% to 0.003% (v/v), obtained by two-fold serial dilution. Microplates were incubated at 27 °C for 7 days without agitation under dark conditions. MIC was defined as the lowest concentration at which no fungal growth was visible.

Conidial suspensions were prepared from 7-day-old PDA cultures, filtered, adjusted in a Neubauer chamber and added to each well to give a final density of 1.5×10^6 conidia mL^{-1}. This inoculum density is higher than the $0.4–5 \times 10^4$ CFU mL^{-1} recommended by CLSI document M38 for filamentous fungi[28]; it was adopted to approximate the high inoculum pressure typical of postharvest anthracnose infection, and MIC values reported here should therefore not be directly compared with values obtained under CLSI conditions.

The negative control consisted of PDB containing fungal inoculum without treatment, and the positive control consisted of PDB containing fungal inoculum and 0.02% tebuconazole (dissolved in water). Surfactant controls were prepared at the surfactant concentration corresponding to the highest essential oil concentration tested (0.16%), reproducing the surfactant-to-oil ratio of each stock nanoemulsion: 0.64% polysorbate 20 for the *C. flexuosus* and *C. winterianus* nanoemulsions, and 0.48% polysorbate 80 for the *Z. officinale* nanoemulsion. This design allowed verification of a possible effect of the surfactant alone, at the highest surfactant load used in the bioassays, on fungal growth. Each treatment was tested in five wells per plate (technical replicates), and the whole assay was performed twice on independent occasions using independently prepared nanoemulsion batches (biological replicates). The minimum fungicidal concentration (MFC) was not determined. Accordingly, complete absence of visible growth is reported as growth inhibition and is not interpreted as fungicidal activity.

In vitro antifungal activity in Petri dishes

In vitro antifungal assay was conducted in Petri dishes (90 × 15 mm) containing PDA medium supplemented with NE concentrations derived from the microplate assay. Treatments consisted of five replicates with two repetitions. Nanoemulsions were added to molten PDA cooled to approximately 45 °C, homogenized, and poured immediately, in order to limit thermal loss of volatile constituents. Control treatments included PDA without additives, PDA with 0.02% tebuconazole, and PDA containing the corresponding surfactant at the equivalent concentration (0.5% v/v).

Each plate was inoculated centrally with 50 µL of a conidial suspension (1.5×10^6 conidia mL^{-1}) of *C. plurivorum* and *C. musae*, sealed and incubated at 27 °C in the dark. Plates of different treatments were incubated in separate containers to prevent vapour-phase transfer of volatile compounds between treatments. Colony diameter was measured in two perpendicular directions on days 2, 4, 6, and 8 after inoculation using a digital caliper, and the mean of the two readings was used[29]. Percentage inhibition of mycelial growth was calculated as $[(Dc - Dt)/Dc] \times 100$, where Dc and Dt are the mean colony diameters of the control and treated plates, respectively. Treatments consisted of five plates each (technical replicates), and the assay was performed twice on independent occasions (biological replicates).

In vivo antifungal activity

Papaya and banana fruits were obtained from organic cultivation and selected for uniformity of size and absence of visible injury. All fruits were harvested at stage 1 in the field and then allowed to ripen naturally during storage, passing through stages 2 to 4 and reaching stage 5 (fully ripe) at the end of the evaluation period[30]. Fruits were disinfected with 1% sodium hypochlorite solution for 1 min, rinsed in sterile distilled water and air-dried at room temperature.

Fruits were immersed for 4 min in nanoemulsions at EO concentrations of 0.04%, 0.08%, 0.12% and 0.16% (v/v). Control fruits were immersed in distilled water under the same conditions. No commercial fungicide treatment was included in the fruit assays. After 2 h of drying, two wounds of approximately 1 × 5 mm were made on the equatorial region of each fruit with a sterile needle, and 50 µL of a conidial suspension (1.0×10^6 conidia

mL^{-1}) of *C. plurivorum* (papaya) or *C. musae* (banana) was deposited into each wound. Fruits were incubated for 8 days at 25 °C and 72% relative humidity.

Four fruits per treatment were used, arranged in a completely randomized design. The fruit was taken as the experimental unit; the two wounds per fruit were averaged before analysis and were not treated as independent replicates. Disease severity was assessed at day 8. For papaya and banana, lesion area (mm²) was determined from digital images using ImageJ (National Institutes of Health, Bethesda, MD, USA), calibrated against a scale reference included in each photograph.

Fresh mass loss was calculated as [(Wi − Wf)/Wi] × 100, where Wi and Wf are the initial and final fruit weights. Total soluble solids (°Brix) and refractive index were determined in homogenized pulp using a Abbe-type bench-top refractometer, model RTA-100 at 25 °C, and pH was measured with a Digital pHmeter PHS-3 C. Determinations were performed in three replicates per fruit.

Statistical analysis

Collected data were subjected to analysis of variance (ANOVA), and treatment means were compared using Tukey's test ($p < 0.05$). All analyses were performed using InfoStat software, version 2011[31].

Results and discussion

Chemical composition of essential oils

The chemical composition of the essential oils (EOs) was determined by gas chromatography coupled with mass spectrometry (GC-MS). The identified compounds and their relative abundances are summarized in Table 1.

In *C. flexuosus* essential oil, the major constituents were geranial (38.3%) and neral (30.8%), which together accounted for 69.1% of the total chromatographic area. These compounds belong predominantly to the class of oxygenated monoterpenes, which have been reported as major contributors to the antifungal activity of *Cymbopogon* oils in other pathosystems[11]. In those studies, oxygenated monoterpenes were associated with disruption of fungal membrane integrity and interference with enzymatic systems; because these mechanisms were not investigated in the present work, they are referred to here only as previously proposed modes of action and not as processes demonstrated for the isolates studied. These findings are consistent with, who reported the presence of 22 compounds in the essential oil of *C. flexuosus*, with geranial and neral as the predominant constituents. In addition to these major constituents, other oxygenated compounds (13.4%) and hydrocarbon terpenes (7.8%) were also detected. Although present in lower concentrations, minor constituents may contribute significantly to the overall biological activity of essential oils[32].

For *C. winterianus* (citronella), 18 compounds were identified, comprising 77.1% monoterpenes and 15.6% sesquiterpenes. The major compounds – citronellal (28.6%), geraniol (18.3%), and citronellol (14.5%) - represented 61.4% of the total identified area. Rathore et al.[32] reported citronellal (41.24%), citronellol (9.91%), geraniol (16.8%) and other constituents in *C. winterianus* oil. Differences between profiles are commonly attributed to chemotype, geographic origin and agronomic conditions, and such variability should be considered when standardizing EO-based formulations.

In the *Z. officinale* (ginger) essential oil, 28 compounds were identified. The principal constituents were *ar*-curcumene (19.45%), *α*-zingiberene (11.53%), *β*-sesquiphellandrene (10.22%), *β*-bisabolene (9.59%), and camphene (7.79%). The chemical profile was dominated by sesquiterpenes (59% of the total area) with smaller monoterpenes fraction. Sulieman et al.[34] reported 22 compounds in Sudanese samples, with a higher proportion of gingerol-related compounds, illustrating the compositional variability of this species.

Table 1 Chemical constituents of essential oils identified by GC-MS.

Retention time (min)	RI_{cal}	RI_{lit}	Compound name	Relative area (%)			
				C. flexuosus	C. winterianus	Z. officinale	Chemical class
6.766	921	921	Tricyclene	–	–	0.54	M
7.144	933	932	*α*-Pinene	–	–	1.95	M
7.631	948	946	Camphene	1.36	–	7.79	M
8.603	976	974	*β*-Pinene	–	–	0.26	M
9.004	988	981	6-Methyl-5-hepten-2-one	1.14	–	0.51	M
9.152	992	988	Myrcene	–	–	0.60	M
10.440	1025	1022	*o*-Cymene	–	–	0.20	M
10.583	1028	1024	Limonene	1.18	–	–	M
10.611	1029	1025	Sylvestrene	–	3.81	–	M
10.726	1032	1026	1,8-Cineole	–	–	2.91	M
13.610	1101	1095	Linalool	1.46	0.81	–	M
15.590	1146	1145	Isopulegol	–	1.48	–	M
16.225	1161	1148	Citronellal	–	28.61	–	M
16.436	1166	1165	Borneol	0.59	–	1.57	M
17.249	1184	1177	(E)-Isocitral	0.57	–	–	M
17.581	1191	1186	*α*-Terpineol	–	–	0.87	M
19.521	1235	1223	Citronellol	–	14.47	–	M
20.150	1250	1235	Neral	30.77	–	–	M
20.774	1256	1249	Geraniol	5.18	18.30	0.28	M
21.592	1282	1264	Geranial	38.32	0.52	–	M
22.147	1295	1293	2-Undecanone	–	–	0.31	M
23.475	1326	1312	Citronellic acid	–	0.43	–	M
23.984	1338	1335	*δ*-Elemene	–	–	0.26	S
24.802	1357	1350	Citronellyl acetate	–	3.60	–	M
24.922	1360	1356	Eugenol	–	1.07	–	M
25.123	1364	1369	Cyclosativene	–	–	0.45	S
25.609	1376	1374	*α*-Copaene	–	–	1.20	S
26.021	1385	1379	Geranyl acetate	4.42	3.68	0.42	M
26.336	1393	1389	*β*-Elemene	–	2.75	1.60	S
26.908	1406	1405	Sesquithujene	–	–	0.49	S
27.418	1420	1417	(E)-Caryophyllene	2.16	–	–	S
28.115	1436	1432	*α*-trans - Bergamotene	–	–	0.20	S
29.002	1458	1454	(E)-*β*-Farnesene	–	–	0.88	S
29.963	1481	1480	Germacrene D	–	1.69	0.99	S
30.284	1489	1479	ar-Curcumene	–	–	19.45	S
30.490	1494	1489	β-Selinene	–	–	1.55	S
30.730	1500	1493	*α*-Zingiberene	–	–	11.53	S
30.759	1501	1500	*α*-Muurolene	–	1.03	–	S
30.925	1505	1509	*α*-Bulnesene	–	0.92	–	S
31.234	1513	1505	*β*-Bisabolene	–	–	9.59	S
31.291	1515	1513	*γ*-Cadinene	3.05	1.25	–	S
31.703	1525	1522	*δ*-Cadinene	–	3.70	–	S
31.880	1530	1521	*β*-Sesquiphellandrene	–	–	10.22	S
32.699	1551	1548	Elemol	–	–	0.58	S
32.733	1552	1546	Edcaryol (epi-*α*-cadinol)	–	4.30	–	S
TOTAL AREA %				90.19	91.99	82.27	

RI_{cal}: Calculated Retention Index; RI_{lit}: Literature Retention Index; M: Monoterpenoid; S: Sesquiterpenoid.

Development of nanoformulations: macroscopic stability and HLB value

The nanoemulsions (NEs) of lemongrass, citronella, and ginger were evaluated on days 1, 17, and 34, with stability monitored by visual inspection. Different formulations and surfactant blends were prepared for each EO: *Cymbopogon flexuosus* – 5% (w/w) EO, 20% (w/w) polysorbate 20, 75% (w/w) water (HLB 16.7); *Zingiber officinale* – 5% (w/w) EO, 15% (w/w) polysorbate 80, 80% (w/w) water (HLB 15); and *Cymbopogon winterianus* – 5% (w/w) EO, 20% (w/w) polysorbate 20, 75% (w/w) water (HLB 16.7). All formulations were nonionic-surfactant systems. On the day of preparation (day 1), the formulations appeared transparent or translucent with a bluish characteristic of the Tyndall effect (Fig. 1), a phenomenon where light is scattered by colloidal particles[35]. Increased dilution of the NE resulted in a more translucent appearance.

By day 17, NEs of 2.5% *C. flexuosus* and of 2.5% and 1% *C. winterianus* showed phase separation by creaming[36]. The remaining concentrations remained macroscopically stable until day 34. *Zingiber officinale* remained macroscopically stable throughout the evaluation.

Colloidal stability of nanoemulsions: hydrodynamic size, polydispersity index (PdI), and zeta potential

Droplet size, size distribution and surface charge were determined for the macroscopically stable formulations (Fig. 2). Throughout this section, macroscopic stability refers to the absence of visible phase separation, whereas colloidal stability refers to the maintenance of droplet size and size distribution over time. The two properties were not equivalent in the present system: among the formulations selected as macroscopically homogeneous, some nevertheless showed droplet growth over storage, indicating that the absence of visible separation did not

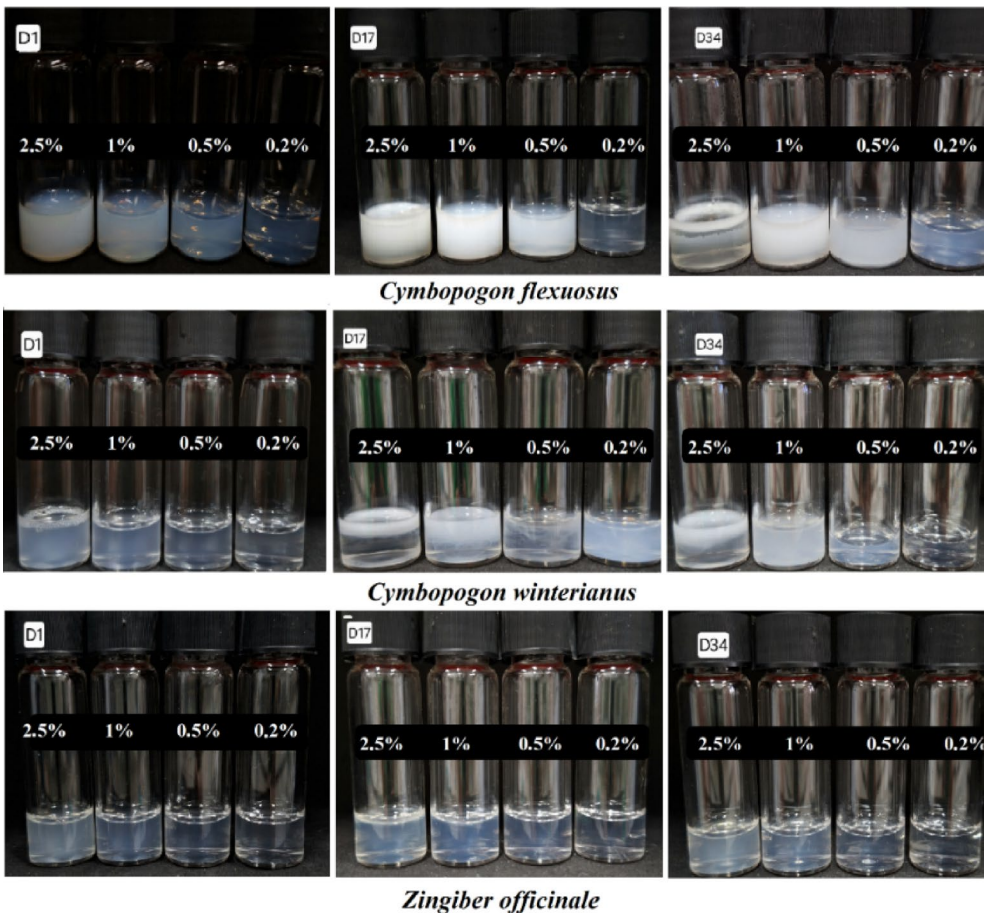

Fig. 1 Macroscopic appearance of *Cymbopogon flexuosus*, *Cymbopogon winterianus*, and *Zingiber officinale* nanoemulsions at different essential oil concentrations during the storage period. D1 = day 1; D17 = day 17 and D34 = day 34.

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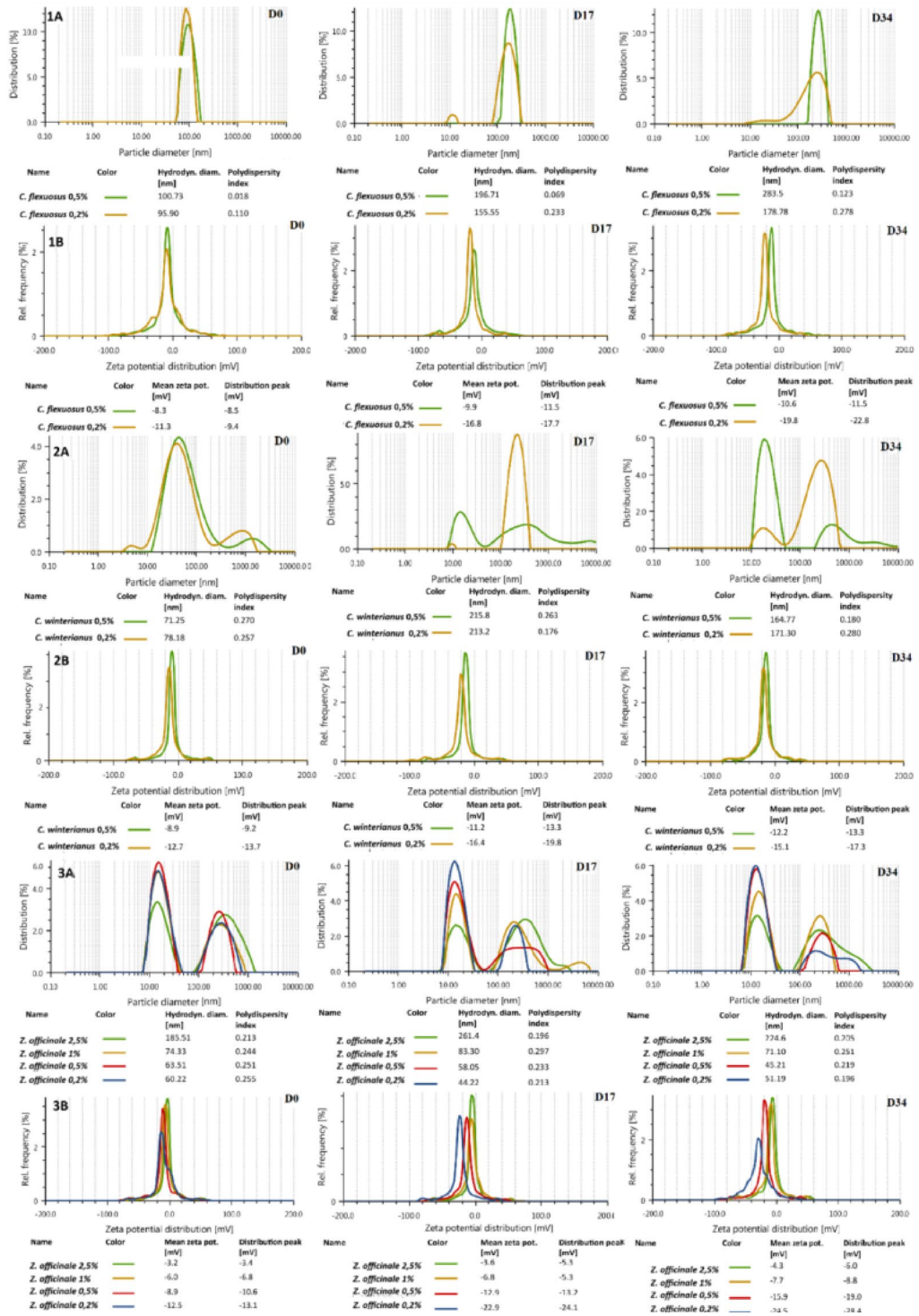

Fig. 2 Hydrodynamic diameter, polydispersity index (PdI), and zeta potential of nanoemulsions formulated with *Cymbopogon flexuosus*, *Cymbopogon winterianus*, and *Zingiber officinale* essential oils evaluated on days 1, 17, and 34.

ensure colloidal stability. Because the formulations were stabilized with nonionic surfactants (polysorbate 20 or 80), stabilization is expected to be governed primarily by steric repulsion from the polyoxyethylene chains rather than by electrostatic repulsion; the small negative zeta potentials measured here are consistent with this mechanism and are typical of Tween-stabilized emulsions, in which zeta potential is not the main predictor of physical

stability[37,38]. For this reason, the zeta potential values are reported below as descriptive parameters and not as evidence of high electrostatic stabilization.

For *C. flexuosus*, initial mean droplet diameters ranged from 95.90 to 117.68 nm, with a positive correlation between oil concentration and mean particle size was observed. DLS analyses showed a single peak for all measured samples, indicating a uniform droplet-size distribution. PdI values were consistently < 0.25, indicative of a narrow size distribution. Zeta potential values became more negative at lower concentrations, reaching − 11.3 mV at 0.2%. This variation is consistent with the reduced ionic strength of the more diluted systems, which decreases screening of the electrical double layer, and is therefore not interpreted as an increase in colloidal stability. By day 17, an increase in mean diameter was observed relative to day 0, consistent with partial droplet growth, indicating a loss of colloidal stability over storage even in the absence of visible phase separation; formulations at 0.2% and 0.5% retained low PdI values and comparable zeta potentials through day 34. These observations are within the range reported by Gündel et al.[39] for lemongrass nanoemulsions.

All *C. winterianus* concentrations were nanometric on day 1 (Fig. 2). By day 17, mean diameters increased for the most diluted samples (215.8 nm at 0.5% and 213.2 nm at 0.2%), a change compatible with Ostwald ripening and indicating reduced colloidal stability in these samples, which nevertheless remained macroscopically homogeneous. Zeta potential measured − 11.2 mV (0.5%) and − 16.4 mV (0.2%). On day 34, the 0.5% and 0.2% formulations were 164.8 nm and 171.3 nm, with PdI of 0.280 and 0.180, respectively. The smaller droplet sizes obtained here with polysorbate 20, relative to some reports using polysorbate 80, are consistent with a surfactant-dependent effect on droplet size[40].

The *Zingiber officinale* formulations exhibited multimodal size distributions at day 1 with zeta potentials around − 12.5 mV. By day 17 hydrodynamic diameters ranged from 44 to 261 nm, and by day 34 the reduction of the secondary size population suggested the dispersion of aggregates rather than the complete breakdown of the emulsion, yielding diameters of 51–225 nm and zeta potentials from − 4.3 to −24.5 mV. Overall, the values obtained (− 4.3 to − 24.5 mV) are below the ± 30 mV threshold often associated with predominantly electrostatic stabilization, which is expected for nonionic systems and does not by itself indicate colloidal instability.

Noori et al.[41] reported smaller droplet sizes for ginger nanoemulsions prepared with higher surfactant content. Although macroscopically stable throughout the evaluation period, the ginger nanoemulsion showed low antifungal activity in the assays below, which indicates that, in this system, chemical composition rather than droplet size was the main factor associated with antifungal efficacy.

Isolates and phylogenetic identification

Multilocus phylogenetic analyses revealed that one isolate clustered within the *C. musae* clade with strong maximum likelihood (ML) support (Fig. 3), whereas the second isolate was assigned to *C. plurivorum* with 95% ML support (Fig. 4).

Pronounced morphological variability within the genus *Colletotrichum* makes species identification based solely on phenotypic characteristics unreliable. Therefore, accurate species delimitation requires a polyphasic approach combining morphological examination with multilocus phylogenetic analysis[42]. The identification of the isolates as *C. musae* and *C. plurivorum* is particularly relevant in the context of postharvest disease management, since these species are important etiological agents of anthracnose in tropical fruits. Consequently, the use of host-associated isolates strengthens the bological relevance of the antifungal assays conducted in this study, allowing the evaluation of nanoemulsion formulations under pathogen–host combinations representative of postharvest infection scenarios. The corresponding sequence data were deposited in GenBank under the following accession numbers: isolate 892, PZ531034 (*GAPDH*), PZ531035 (*TUB2*), PZ531037 (*GS*), and PZ531039 (*APMAT*); isolate 898, PZ531036 (*TUB2*) and PZ531038 (*GAPDH*).

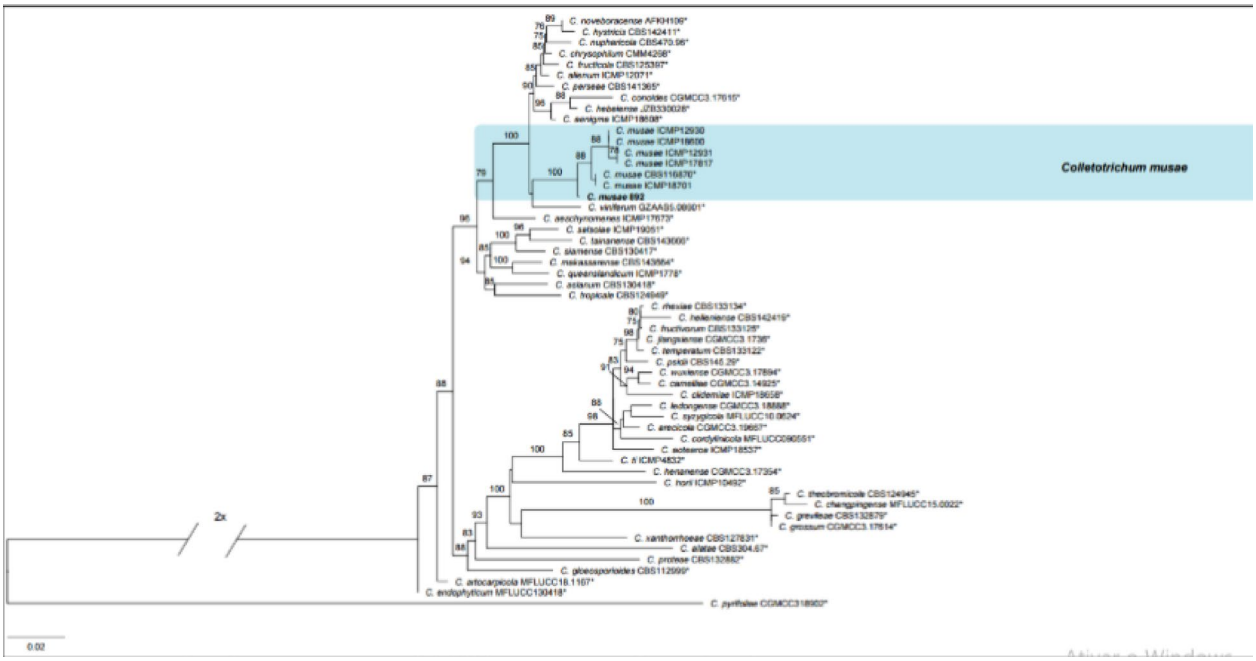

Fig. 3 Maximum likelihood phylogenetic tree inferred from concatenated multilocus sequences (*GAPDH*, *APN2/MAT-IGS*, *GS*, *and TUB2*) showing the placement of the isolate from banana fruit within the *Colletotrichum musae* clade. Significant ML support values (SH-aLRT bootstrap ≥ 70) are shown above the corresponding nodes. The phylogenetic tree was rooted with *Colletotrichum pyrifoliae*. Ex-type isolates are indicated by an asterisk (*) at the end of the taxon labels, and isolates obtained in the present study are highlighted in bold. The scale bar represents the average number of nucleotide substitutions per site.

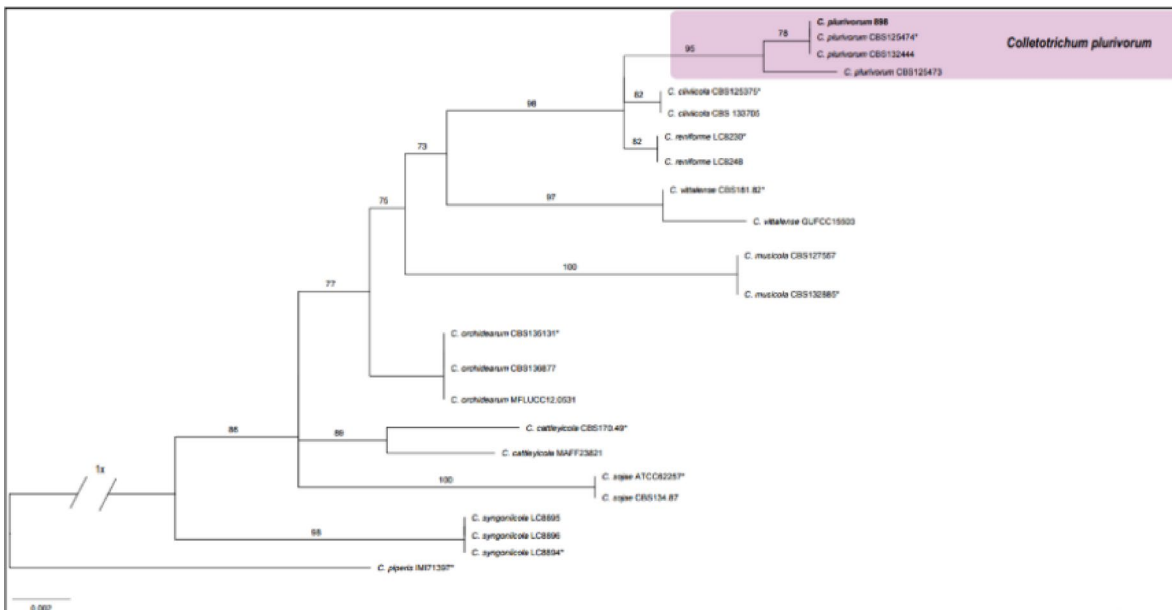

Fig. 4 Maximum likelihood phylogenetic tree inferred from concatenated multilocus sequences (*GAPDH*, *APN2/MAT-IGS*, *GS*, *and TUB2*), showing the placement of the isolate from papaya fruit within the *Colletotrichum plurivorum* clade. In the second phylogenetic reconstruction, significant ML support values (SH-aLRT bootstrap ≥ 70) are also shown above the nodes, with the tree rooted using *Colletotrichum piperis*. Ex-type isolates are marked with an asterisk (*), and isolates from this study are highlighted in bold. The scale bar indicates the mean number of substitutions per site.

Antifungal activity

In the microdilution assays, the *Cymbopogon flexuosus* and *Cymbopogon winterianus* NEs inhibited *C. plurivorum* at 0.07% (EO basis), whereas the *Zingiber officinale* nanoemulsion did not inhibit growth at the concentrations tested (Fig. 5). For *C. musae*, the *C. flexuosus* nanoemulsion showed the lowest MIC (0.03%), followed by *C. winterianus* (0.07%); the *Z. officinale* formulation again showed no detectable inhibition.

In Petri-dish assays, *C. flexuosus* and *C. winterianus* nanoemulsions at 0.03% fully prevented visible mycelial growth of *C. plurivorum* over 8 days (0 mm colony diameter) (Fig. 6). The *Z. officinale* nanoemulsion reduced growth without preventing it.

For *C. musae* (Table 2), the greatest inhibition was observed for *C. flexuosus* (0.03%) and *C. winterianus* (0.04%); the *Z. officinale* nanoemulsion produced partial growth reduction only at markedly higher concentrations (0.6–1.4%). The surfactant control did not differ from the negative control for the tested formulations indicating that, under these conditions, the growth inhibition observed was associated with the encapsulated oils rather than with the surfactant. Ali et al.[43] reported that ginger oil (2%) combined with gum arabic (10%) reduced *C. gloeosporioides* conidial germination by 93% in postharvest papaya, corroborating the relevance of EO-based systems for anthracnose control.

The MIC values obtained (0.03–0.07% v/v) guided the selection of working dilutions for fruit immersion (0.04–0.16% v/v), chosen to combine the antifungal effect observed in vitro with an immersion format compatible with packinghouse handling.

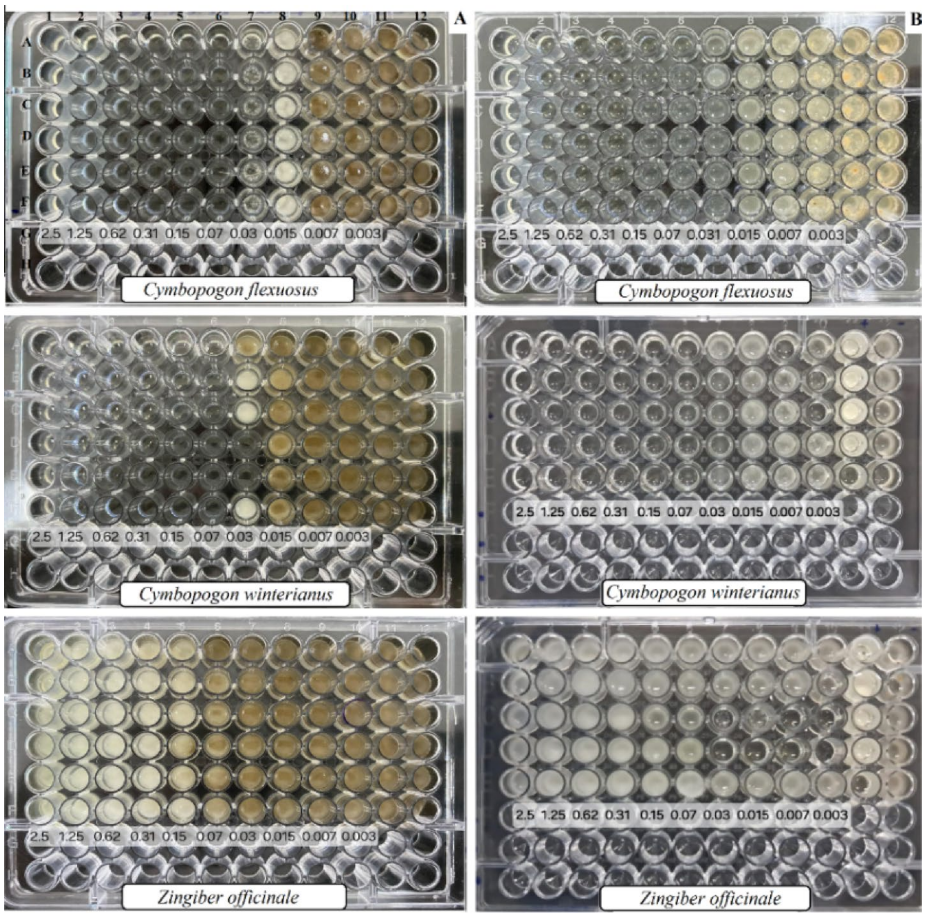

Fig. 5 Minimum inhibitory concentration (MIC) of nanoemulsions against *Colletotrichum plurivorum***(A)** and *Colletotrichum musae***(B)**. *Columns 1–10: oils diluted in series. *Columns 11 and 12: Negative control. A: *Colletotrichum plurivorum*. B: *Colletotrichum musae*.

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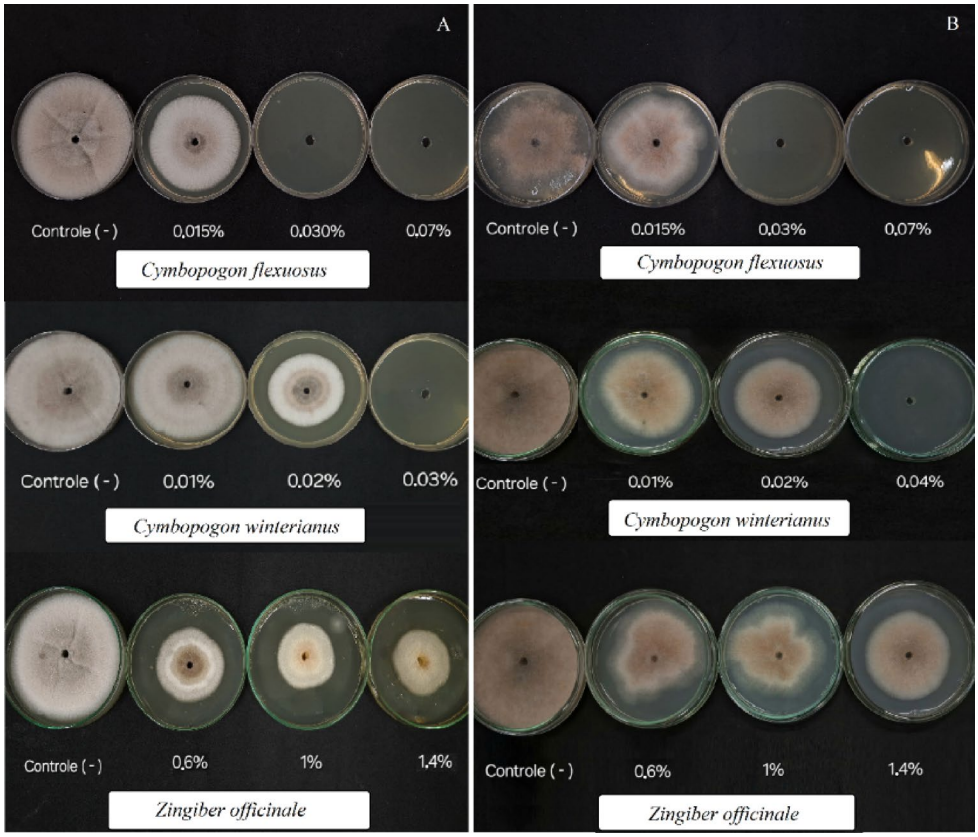

Fig. 6 Effect of essential oil nanoemulsions on the mycelial growth of *Colletotrichum plurivorum* and *Colletotrichum musae* in PDA medium at different concentrations. **A**: *Colletotrichum plurivorum***B**: *Colletotrichum musae*. Colony diameter measurements were performed in two perpendicular directions and averaged for each replicate.

Table 2 Average colony diameter (mm) of *Colletotrichum plurivorum* and *Colletotrichum musae* treated with essential oil nanoemulsions *in vitro*.

Essential Oil (NE)	Treatment (%)	C. plurivorum Diameter (mm)	C. musae Diameter (mm)
C. flexuosus	0.07	0.00 ± 0.00a	0.00 ± 0.00a
	0.03	0.00 ± 0.00a	0.00 ± 0.00a
	0.015	64.87 ± 5.68b	69.24 ± 2.72b
	Negative Control (NC)	91.26 ± 1.02c	80.26 ± 1.69c
	Positive Control (PC)	0.00 ± 0.00a	0.00 ± 0.00a
C. winterianus	0.04	0.00 ± 0.00a	0.00 ± 0.00a
	0.02	57.24 ± 5.39a	61.24 ± 9.82b
	0.01	79.68 ± 3.00b	78.38 ± 4.70c
	Negative Control (NC)	83.96 ± 1.71c	89.43 ± 2.96d
	Positive Control (PC)	0.00 ± 0.00a	0.00 ± 0.00a
Z. officinale	1.4	36.96 ± 2.49a	40.09 ± 5.80a
	1.0	37.93 ± 2.09a	43.03 ± 2.10a
	0.6	40.41 ± 3.94a	43.9 ± 2.70a
	Negative Control (NC)	77.69 ± 0.82b	80.3 ± 1.69b
	Positive Control (PC)	0.00 ± 0.00a	0.00 ± 0.00a

*Average colony diameter in mm. Values in the same column followed by the same letter do not differ significantly according to Tukey's test ($p < 0.05$). PC = Positive control (Tebuconazole); NC = Negative control.

In vivo assays (fruit tests)

In papaya assays, inoculated with *C. plurivorum*, the *C. winterianus* nanoemulsion reduced lesion area in a concentration-dependent manner (Table 3). with a significant reduction relative to the control observed from 0.08% and reaching complete control at 0.16%. *C. flexuosus* showed a less pronounced dose-response: only the

Table 3 Effect of *Cymbopogon* essential oils nanoemulsions on the physicochemical parameters and mycelial growth of *Colletotrichum plurivorum* in papaya fruit.

Colletotrichum plurivorum						
Treatment (%)	**Initial weight (g)**	**Final weight**	**°Brix**	**Refractive index (nD)**	**pH**	**Mycelial growth (mm^2)**
Cymbopogon winterianus						
0.04	296±38a	316±39a	6.7±0.45a	1.34a	4.57a	1664.60±100a
0.08	341±17a	323±11a	7.14±2.58a	1.34a	4.63ab	868.67±352b
0.12	334±30a	309±29a	10.76±1.14b	1.35b	4.52a	327.60±196c
0.16	380±34a	361±6a	11.00±0.24b	1.35b	5.08b	0.00±0.00c
control	359±53a	347±53a	5.65±1.72a	1.34a	4.67ab	1375.50±397a
Cymbopogon flexuosus						
0.04	334±37a	328±38a	6.50±1.92a	1.34a	4.33a	1344±317a
0.08	361±20a	354±20a	7.58±1.84a	1.34a	4.64a	1456±415a
0.12	353±34a	343±31a	8.55±0.23a	1.35a	4.34a	1461±284a
0.16	368±39a	360±40a	9.00±3.25a	1.34a	5.45b	317±90b
control	359±53a	347±53a	5.65±1.72a	1.34a	4.67a	1375±397a

Values in the same column followed by the same letter do not differ significantly according to Tukey's test ($p<0.05$).

highest concentration (0.16%) significantly reduced the lesion area (72% reduction relative to the control). The *Z. officinale* formulation did not control the disease under the tested conditions (Fig. 7).

Soluble solids (°Brix) increased at 0.12% and 0.16% (10.76–11.00 °Brix) relative to the control (5.65 °Brix). This difference is most plausibly explained by disease status rather than by a direct effect of the treatment: control fruits were extensively colonized and at an advanced senescence stage, whereas fruits protected at 0.16% ripened normally. According to MAPA Normative Instruction No. 4/2010[44], the recommended soluble solids content for papaya is at least 11 °Brix. Carnelossi et al.[45] reported 8.8 to 11.60 °Brix and pH between 6.15 and 5.60 in minimally processed papaya. The refractive index followed the increase in soluble solids (1.35), and the pH remained within the range for papaya, indicating that disease control at 0.16% was not accompanied by deterioration of the physicochemical parameters measured.

The superior in vivo performance of the *C. winterianus* nanoemulsion, relative to *C. flexuosus*, coincides with its higher content and diversity of oxygenated monoterpenes (citronellal, geraniol, citronellol). These constituents have been associated with fungal membrane disruption and ROS accumulation in other systems[46,47]; as these mechanisms were not evaluated here, they are cited as possible explanations rather than as demonstrated causes, and warrant direct investigation (e.g., membrane permeability, ROS quantification, ergosterol content) in future work.

Regarding the assays with *Colletotrichum musae*, in general, neither the *Cymbopogon winterianus* nor the *Cymbopogon flexuosus* nanoemulsions significantly reduced disease severity: lesion values did not differ from the control at any concentration (Table 4). °Brix, refractive index, pH and mycelial growth did not differ among treatments. Under the conditions tested, therefore, the formulations did not control *C. musae* on banana and did not alter the physicochemical parameters measured (Fig. 8).

The figures show representative images of fruits inoculated with *Colletotrichum musae* and subjected to different concentrations of nanoemulsions. The white areas in the image correspond to the intact fruit tissue, originally yellowish in color, which did not show development of fungal infection. The damaged areas are delimited on the surface of the fruit and appear in dark shade, contrasting with the healthy tissue.

The differential antifungal performance among formulations can be rationalized by (i) chemical composition of the oils and (ii) colloidal properties of the nanoemulsions at working dilutions. *C. winterianus* oil is rich in oxygenated monoterpenes (citronellal, geraniol, citronellol), a class repeatedly associated with antifungal activity in the literature[11,45]. The complete inhibition of *C. plurivorum in vitro* by the *C. winterianus* and *C. flexuosus* nanoemulsions and the concentration-dependent control in papaya are consistent with these reports.

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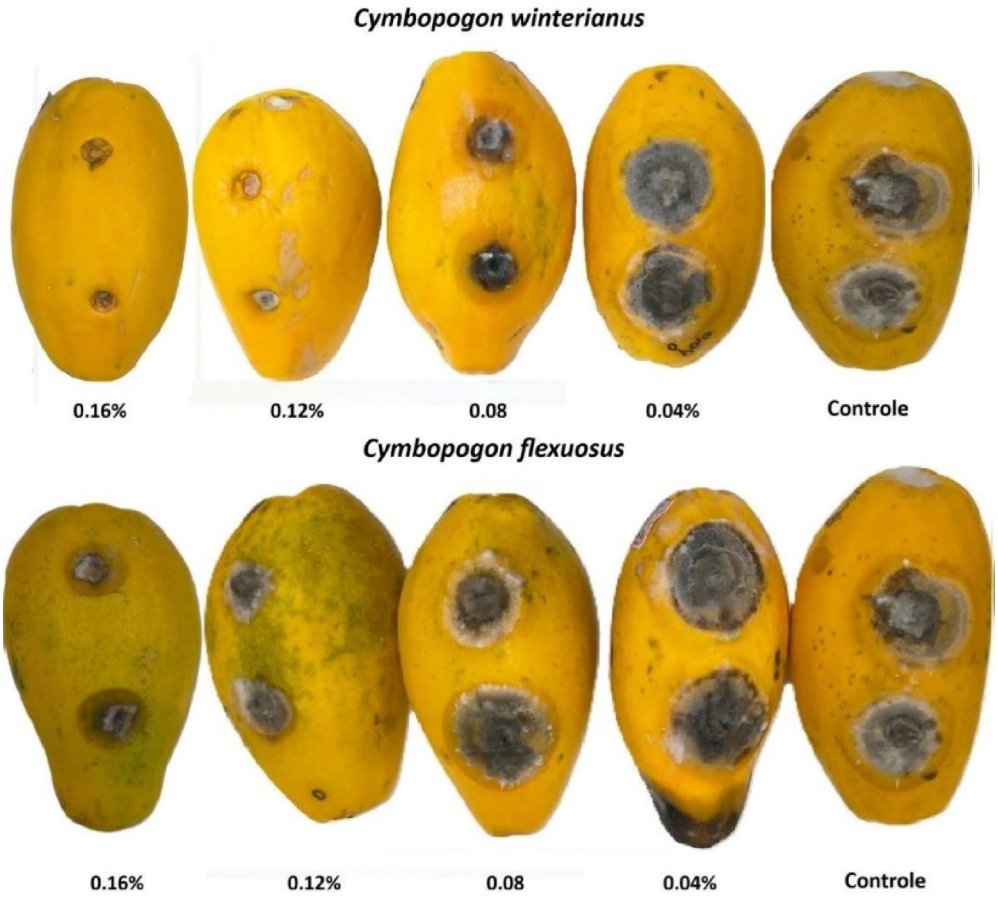

Fig. 7 In vivo effect of *C. winterianus* and *C. flexuosus* nanoemulsions on the control of *Colletotrichum plurivorum* in papaya fruits at different concentrations.

Table 4 Effect of *Cymbopogon* essential oil nanoemulsions on the physicochemical parameters and mycelial growth of *Colletotrichum musae* in banana fruit.

Colletotrichum musae					
Treatment (%)	**Initial weight**	**°Brix**	**Refractive index (nD)**	**pH**	**Mycelial growth (mm^2)**
Cymbopogon winterianus					
0.04	109 ± 20a	24.55 ± 2.56a	1.37a	4.77a	88.33 ± 8.97a
0.08	95.25 ± 19a	24.89 ± 1.28a	1.37a	5.00a	82.55 ± 13.75a
0.12	106.25 ± 24a	23.88 ± 0.82a	1.37a	5.10a	91.28 ± 2.86a
0.16	107.5 ± 17a	22.08 ± 0.74a	1.37a	4.98a	88.36 ± 5.58a
control	98.00 ± 13a	23.86 ± 1.50a	1.37a	5.23a	82.21 ± 13.83a
Cymbopogon flexuosus					
0.04	88.75 ± 12a	26.26 ± 1.21a	1.38a	5.03a	93.14 ± 4.53a
0.08	107.25 ± 20a	24.49 ± 1.66a	1.37a	5.19a	81.87 ± 7.87a
0.12	100.75 ± 21a	23.97 ± 2.44a	1.37a	5.36a	78.5 ± 12.90a
0.16	109.25 ± 22a	26.25 ± 1.54a	1.37a	5.25a	73.6 ± 12.08a
control	98.00 ± 13a	23.86 ± 1.50a	1.37a	5.23a	82.21 ± 13.83a

Values in the same column followed by the same letter do not differ significantly according to Tukey's test ($p < 0.05$).

The nanoemulsion had droplet sizes predominantly below 200 nm, low PdI, and negative zeta potentials, consistent with sterically stabilized colloidal systems, although droplet growth was observed in some formulations during storage. Such characteristics may favor spreading and adhesion on fruit surfaces; however, whether they improve efficacy relative to bulk essential oil was not tested directly in this study and remains to be demonstrated.

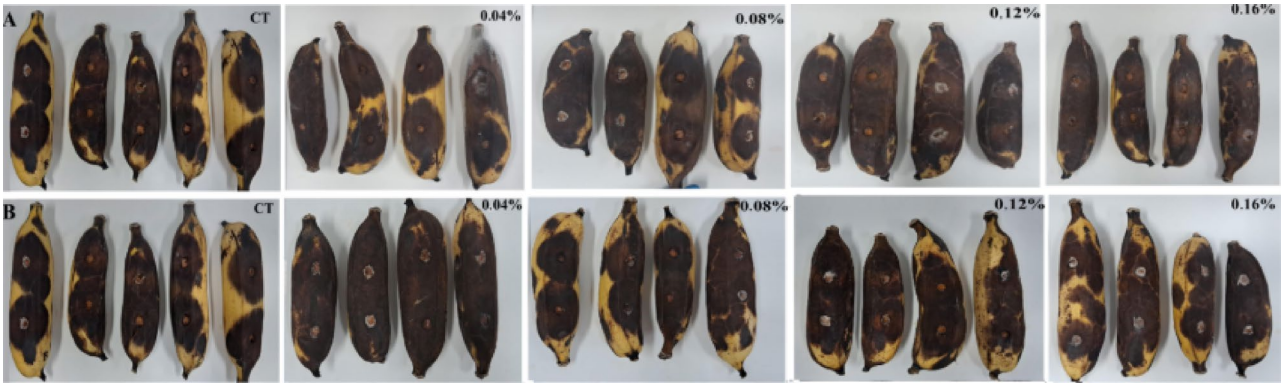

Fig. 8 Evaluation of the antifungal efficacy of *Cymbopogon* essential oil nanoemulsions against *Colletotrichum musae* in bananas. Images in row A: *C. winterianus*; In row B: *C. flexuosus*.

Efficacy varied between host–pathogen systems: formulations that controlled *C. plurivorum* on papaya were less effective against *C. musae* on banana. This discrepancy may reflect differences in pathogen susceptibility, inoculation and host surface properties. The host-dependent responses, together with the small in vivo sample size ($n=4$ fruits per treatment) and the limited set of quality parameters measured, indicate that the results should be interpreted as an initial screening and not extrapolated to other oils, pathogens, fruit hosts, or to commercial storage conditions.

The increase in soluble solids at 0.16% in papaya is consistent with normal ripening in fruits protected from disease, whereas the low values in control fruits reflect advanced decay. Effective application will therefore require balancing antifungal effect against the maintenance of fruit ripening parameters across storage.

Several limitations should be considered when interpreting these results. The in vivo assays used four fruits per treatment; although the fruit rather than the individual wound was taken as the experimental unit, this remains a small sample for a biologically variable trait such as lesion development, and the fruit assays should therefore be regarded as an initial screening rather than as confirmation of efficacy. No commercial fungicide treatment was included as a comparator in the present fruit assays; a comparison between an essential oil nanoemulsion and a standard postharvest fungicide had previously been established by our group in a related in vivo model against *C. plurivorum* on papaya[30], and the present study prioritized the comparison among the three candidate essential oils. The minimum fungicidal concentration was not determined, so complete absence of visible growth is reported as growth inhibition and is not interpreted as fungicidal activity. The postharvest quality evaluation was restricted to °Brix, pH, and mass loss; future studies should incorporate comprehensive physicochemical assessments, including firmness, peel color evolution, titratable acidity, and respiration rates. Additionally, to fully validate the robustness of this technology for commercial supply chains, subsequent research must evaluate the nanoemulsions under simulated commercial cold storage and shelf-life conditions, exploring the physiological defense responses (e.g., antioxidant enzyme activities) elicited in the host tissues.

This study developed and characterized essential oil nanoemulsions of *C. flexuosus*, *C. winterianus* and *Z. officinale* and evaluated their activity against *C. plurivorum* and *C. musae*. The *C. flexuosus* and *C. winterianus* nanoemulsions inhibited both pathogens in vitro at low concentrations. In fruit, the *C. winterianus* nanoemulsion controlled *C. plurivorum* in papaya in a concentration-dependent manner, reaching no visible lesion at 0.16% without deterioration of the physicochemical parameters measured. Under the same conditions, the formulations did not control *C. musae* in banana, and the *Z. officinale* nanoemulsion showed low activity throughout. The *C. winterianus* nanoemulsion should be regarded as a potential candidate for postharvest anthracnose management in papaya rather than as a confirmed alternative to conventional fungicides. Establishing its practical value will require comparison with registered commercial fungicides, larger numbers of fruits, an extended set of quality attributes, and evaluation under commercial storage conditions.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-026-67933-9.

Acknowledgements We also thank the Centro Multiusuário para o Desenvolvimento Tecnológico e Inovação de Vila Velha (CMVV) for assistance with chemical analyses and dynamic light scattering measurements.

Author contributions PFAR: Conceptualization, methodology, investigation, data curation, writing-original draft. FSM: Methodology, investigation. IZS: Methodology, investigation. JAV: Resources, supervision. AGGA: Methodology, investigation. MPSC: Methodology, investigation. HSF: Conceptualization, methodology, supervision, project administration, funding acquisition, writing-review and editing.

Funding The authors acknowledge the Federal University of Espírito Santo (UFES) and the Federal Institute of Education, Science and Technology of Espírito Santo (IFES, Campus Vila Velha; PRODIF call) for institutional support. This study was financed in part by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES), Finance Code 001. Marcos P. S. Câmara acknowledges the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq) for the research productivity (project 303221/2024-0). Ana Gabriele Gurgel Amaral acknowledges the Foundation for Support of Science and Technology of the State of Pernambuco (Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco - FACEPE) for the postdoctoral fellowships (project BFP-0099-5.01/23). Foundation for Support of Science and Technology of the State of Espirito Santo (Fundação de Amparo à Ciência e Tecnologia do Estado do Espirito Santo – FAPES) award number 22/2023.

Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. The fungal isolates (Colletotrichum spp.) evaluated in this study are preserved and maintained in the fungal collection of the Plant Pathology Laboratory at the Capixaba Institute for Research, Technical Assistance, and Rural Extension (Incaper), Espírito Santo, Brazil. The datasets generated and/or analysed during the current study are available in the GenBank repository (NCBI)The deposited sequences are as follows:• Isolate 892:GAPDH: PZ531034 - https://www.ncbi.nlm.nih.gov/nuccore/PZ531034TUB2: PZ531035 - https://www.ncbi.nlm.nih.gov/nuccore/PZ531035GS: PZ531037 - https://www.ncbi.nlm.nih.gov/nuccore/PZ531037APMAT: PZ531039 - https://www.ncbi.nlm.nih.gov/nuccore/PZ531039• Isolate 898:TUB2: PZ531036 - https://www.ncbi.nlm.nih.gov/nuccore/PZ531036GAPDH: PZ531038 - https://www.ncbi.nlm.nih.gov/nuccore/PZ531038.

Declarations

Competing interests The authors declare no competing interests.

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