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Free and nanoemulsified essential oils of *Cymbopogon winterianus* and *Cymbopogon flexuosus* as an emerging alternative for the control of phytopathogens in stored soybean seeds

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Abstract: When soybean seeds are stored, they are susceptible to pathogens, so phytosanitary treatment is necessary. The use of essential oils has emerged as a promising alternative for storing agricultural products. The aim of this study was to evaluate the effect of *Cymbopogon winterianus* (citronella) and *Cymbopogon flexuosus* (lemongrass) essential oils on the control of phytopathogenic fungi in stored soybean seeds. The experiment was conducted in a completely randomized design, with 20 treatments and three replications. The oils were extracted by hydrodistillation and nanoemulsified using the high-energy method. The applications took place at concentrations of 25%, 50%, 75% and 100%, as well as negative (untreated and water + Tween® 80) and positive (Vitavax-Thiram®) controls. Lemongrass essential oil in nanoemulsion form, especially at a concentration of 25%, showed the best results for vigor and germination, outperforming the positive control. Citronella in its free form performed best in terms of germination (50%), while the nanoemulsified form stood out in terms of vigor (25%). In terms of antifungal activity, all the treatments were similar against *Fusarium sp.,* except for the 100% citronella nanoemulsion, which reduced the incidence to 11.66%, outperforming the chemical treatment. Against *Aspergillus sp.,* the lemongrass nanoemulsion was the most effective at all concentrations. It can be concluded that essential oils, especially in nanoemulsion form, have the potential to replace chemical seed treatments, promoting effective phytosanitary control and improving physiological performance.

Keywords: alternative control; citronella; Glycine max (L.) Merrill; lemongrass; phytopathogens; seed treatment.

Abbreviations: EOs_Essential Oils; N-CEO_Nanoencapsulated Citronella Essential Oil; RS_Rio Grande do Sul; HURB_Rogério Bueno University Herbarium; MCT_ Medium-chain triglycerides; NOT_No treatment; POC_Positive control; TWE_Water and emulsifier; C. flexuosus_Cymbopogon flexuosus; C. winterianus_Cymbopogon winterianus.

Introduction

Soybean (*Glycine max (*L.*) Merrill*) is considered one of the most important agricultural crops worldwide, mainly due to its high nutritional value and its use as a source of vegetable oil and protein in human nutrition (Yang et al., 2021; Deng et al., 2022). The increasing demand for high-quality soybean seeds has driven the expansion of areas dedicated to its cultivation (Deng et al., 2022).

Among the criteria used to assess seed quality, health stands out, referring to the absence of pathogenic agents capable of compromising germination and the early development of plants. Pathogens such as fungi, bacteria, viruses, and nematodes can be transmitted through seeds, leading to crop losses (Ramdan et al., 2021). In the case of soybeans, fungal contamination is particularly concerning, as it can reduce germination rates, cause root system diseases, and seedling damping-off (Hosseini et al., 2023). During storage, the most commonly found fungi in seeds are species from the *Aspergillus* and *Penicillium* genera, which lead to economic losses and reduced nutritional value of the grains (Deligeorgakis et al., 2023).

Among the fungi with the greatest economic relevance is the *Fusarium* genus, known for its ability to cause plant diseases and contaminate food and feed with mycotoxins that pose risks to human and animal health. Species of this genus produce various toxins, such as trichothecenes, zearalenone, fumonisins, moniliformin, fusarins, and enniatins. Trichothecenes, in

particular, are significant due to their protein synthesis inhibition effect, which can lead to serious illnesses in both humans and animals (Hafez et al., 2021).

Although synthetic chemical products such as fungicides are widely used in disease control, their continuous use raises concerns about toxicity, environmental impact, and the emergence of resistant microorganisms. As a result, interest is growing in more sustainable and safer natural alternatives. Essential oils (EOs) stand out in this context for containing compounds with antifungal, antibacterial, and antioxidant properties (Khaleil et al., 2021).

To improve the efficacy of EOs and overcome limitations such as low water solubility, nanoemulsification technology has been widely employed. This technique allows for the production of emulsions with nanometric-sized particles, offering greater stability, low viscosity, and a translucent appearance, which favors their application in the food industry and agriculture (Nie et al., 2023).

The species *Cymbopogon winterianus*, has main chemical components include geraniol and citronellal, which contribute to its therapeutic and functional applications. Its essential oil is mainly composed of monoterpenes, including alcohols and aldehydes, and has been associated with beneficial effects such as antimicrobial, antioxidant, and anti-inflammatory action (Rathore et al., 2023; Dangol et al., 2023). *Cymbopogon citratus*, is a plant with recent research that highlights its therapeutic potential, including antiviral and antifungal (Dangol et al., 2023). The essential oil of this plant, particularly rich in citral, has also been studied as a natural alternative for controlling pathogenic microorganisms, functioning as a plant-based pesticide with significant antifungal activity (Antonioli et al., 2020).

The present study highlights the importance of researching new substances with antifungal potential, extracted from aromatic plants, as there is little research on the application of essential oils for antifungal treatment of soybean seeds. Therefore, the objective was to evaluate the effect of *Cymbopogon winterianus* and *Cymbopogon flexuosus* essential oils in the treatment of phytopathogenic fungi in stored soybean seeds.

Results and discussion

Analysis of essential oil nanoemulsions

Physicochemical parameters (mean diameter, PDI, zeta potential, and pH) were monitored for both control nanoemulsions (blank) and those containing *C. flexuosus* and C*. winterianus* essential oils under three storage conditions: room temperature (RT), refrigeration (REF), and climatic chamber (CC).

Formulations composed only of emulsifiers (blank) showed an initial mean diameter of 154.2 nm, PDI of 0.243, pH of 5.86, and zeta potential of -6.6 mV. When stored under refrigeration and room temperature, particle size remained stable throughout the 90 days of analysis, with significant differences occurring only under the climatic chamber condition. In the latter, as well as at room temperature, an increase in PDI and pH values was observed, indicating the onset of system instability after 30 days.

Nanoemulsions containing *C. flexuosus* essential oil presented an initial mean diameter of 175.5 nm, PDI of 0.249, zeta potential of -7.0 mV, and pH of 4.61. Among the tested formulations, lemongrass exhibited the greatest instability, with signs of phase separation after 45 days in the climatic chamber, while remaining visually stable at RT and REF. According to Pereira et al. (2021), the relatively high water solubility of citral can trigger the destabilization of nanoemulsions rich in this terpene, a prominent characteristic of this species.

Formulations with *C. winterianus* essential oil showed an initial mean diameter of 134.0 nm, PDI of 0.221, pH of 5.36, and zeta potential of -6.5 mV. It was found that the mean diameter underwent statistical variation only in the climatic chamber starting from the 30th day. The PDI remained stable for the same period only under refrigeration. Zeta potential was the least affected parameter, with significant changes only on the 15th day (REF) and 45th day (RT). Conversely, pH was the most sensitive parameter during the 90 days, with significant differences detected within the first 15 days of storage.

The polydispersity index (PDI) indicates the homogeneity of nanoparticle size distribution in dispersion, ranging from 0 to 1. Values below 0.3 represent monodisperse systems with a homogeneous distribution (Kumar and Dixit, 2017; Khan et al., 2019). Zeta potential is used to predict colloidal stability; the higher its absolute value, the greater the stability, as electrostatic repulsion between particles prevents aggregation (Laouini et al., 2012; Mariano et al., 2011).

In the study by Pereira et al. (2021), a low-energy method was used to produce nanoemulsions of Pectis elongata Kunth, an oil also rich in citral, similar to *C. flexuosus*. Using polysorbate 80 and sorbitan trioleate, the authors obtained droplets of 116 nm, PDI of 0.215, and zeta potential of -2.80. After 90 days, the size increased to 193.9 nm, with a PDI of 0.082 and zeta potential of -0.662, indicating instability. These data differ from the present study, which demonstrated greater stability and preservation of its major constituents.

Analysis of essential oil treatments on soybean seeds

In the present study, two technologies were analyzed (free and nanoemulsified essential oils) across four concentrations, totaling 20 treatments. The comparison of treatment means and the analysis of variance results (Table 1) revealed distinct responses across essential oil doses and essential oil technology (free and nanoemulsified) when evaluating seed germination and vigor. The analysis showed that essential oil treatments significantly influenced germination, vigor, and the proportion of normal, abnormal, and dead seeds, highlighting the combined effect of formulation type (free or nanoemulsified) and applied concentration.

Table 1. Analysis of variance of results of germination and vigor of soybean seeds subjected to different treatments.

Test	Statistic	Dead			Normal			Abnormal		
		T	B	R	T	B	R	T	B	R
GER	DF	19	2	38	19	2	38	19	2	38
GER	Sum Sq	3052.93	13.63	330.36	8383.91	45.30	906.03	2240.0	30.4	647.6
GER	Mean. Sq	160.68	6.81	8.69	441.25	22.65	23.84	117.89	15.20	17.04
GER	F value	18.48	0.78	-	18.50	0.94	-	6.91	0.89	-
GER	Pr(F)	<0.0001	0,46	-	<0.0001	0.39	-	<0.0001	0.41	-
VIG	DF	19	2	38	19	2	38	19	2	38
VIG	Sum Sq	5386.98	47.10	913.56	9653.91	94.03	1388.63	1455.25	3.03	604.30
VIG	Mean. Sq	283.52	23.55	24.04	508.10	47.02	36.54	76.59	1.52	15.90
VIG	F value	11.79	0.97	-	13.90	1.28	-	4.81	0.09	-
VIG	Pr(F)	<0.0001	0,38	-	<0.0001	0.28	-	<0.0001	0.90	-

GER: Germination; VIG: Vigor; T: Treatment; B: Block; R: Residuals; DF: Degrees of Freedom; Sum Sq: Sum of Squares; Mean. Sq: Mean Squares; Pr(F): P-value for the F-statistic $p<0.05$.

The data demonstrate that the nanoemulsified lemongrass essential oil exhibited results superior to its free form, showing a better response at the 25% dose and surpassing the positive control in germination rates. Conversely, the free lemongrass essential oil yielded its best results at concentrations of 25% and 50% for vigor and germination; while these results were superior to the positive control but inferior to the negative control, as shown in Table 2. The aforementioned results highlight the superiority of the nanotechnology of essential oils, as nanoemulsions, due to their greater stability and better dispersion of bioactive compounds, improve the efficacy of the product and the uniformity of seed coverage (Preeti et al., 2023).

As observed for the nanoemulsified essential oil in the present study, Hillen et al. (2012) reported that the use of such oils in more technological formulations can enhance their antifungal action. In contrast, our results indicated that high concentrations (100%) of essential oil compromised germination, a phenomenon previously described by Liu et al. (2006), who observed the inhibition of seed germination when exposed to high doses of plant-derived volatile compounds. These data reinforce the potential of essential oils as alternative agents in seed treatment, provided they are used in formulations and concentrations appropriate to the plant species and agronomic objective (Daronco et al., 2015). The decrease in germination may be an indicator of phytotoxicity, as previously addressed in the study by Rolli et al. (2016), where pure essential oil from *Cryptocarya massoy* caused complete inhibition of tomato and cucumber seed germination. In the present study, these specific phytotoxicity variables were not analyzed, which represents a limitation of the research.

Furthermore, according to Table 2, it can be seen that the results obtained with citronella essential oil were inferior to those of lemongrass for vigor and germination. The free citronella essential oil showed better results at a dose of 50% for germination, but with inferior performance for vigor. In contrast, the nanoemulsified essential oil exhibited results lower than the free form for germination but higher for vigor, with the concentration of 25% providing the best results.

Table 2. Comparison of means of percentages of normal, abnormal and dead seedlings, in germination and vigor tests of soybean seeds subjected to different treatments with essential oils and controls.

Treatment	Dose / control	Germination (%)			Vigor (%)		
		As	Ns	Ds	As	Ns	Ds
Free Lemongrass EO	100%	22.66 a	57.00 de	20.33 ab	20.33 a	46.66 g	33.00 a
Free Lemongrass EO	75%	22.00 ab	49.00 e	29.00 a	19.00 a	59.66 efg	21.33 abc
Free Lemongrass EO	50%	7.00 c	79.00 abc	14.00 bc	13.66 ab	69.66 cdef	16.66 bcd
Free Lemongrass EO	25%	12.33 abc	81.33 abc	6.33 cd	14.33 ab	70.66 bcdef	15.00 cd
Nano Lemongrass EO	100%	4.67 c	92.66 a	2.67 d	8.66 ab	87.00 abcd	4.33 d
Nano Lemongrass EO	75%	5.33 c	90.00 ab	4.67 d	5.33 b	90.66 a	3.66 d
Nano Lemongrass EO	50%	6.33 c	90.00 ab	3.67 d	11.00 ab	85.33 abcd	4.33 d
Nano Lemongrass EO	25%	6.00 c	92.33 a	2.00 d	11.00 ab	84.66 abcd	4.33 d
Free Citronella EO	100%	14.67 abc	76.00 bc	9.33 cd	6.33 b	60.66 efg	33.00 a
Free Citronella EO	75%	21.33 ab	69.33 cd	9.33 cd	14.66 ab	69.00 def	16.33 bcd
Free Citronella EO	50%	3.67 c	92.66 a	3.67 d	15.00 ab	74.33 abcde	10.66 cd
Free Citronella EO	25%	5.33 c	89.33 ab	3.67 d	9.33 ab	72.66 abcde	14.66 cd
Nano Citronella EO	100%	5.33 c	92.66 a	2.67 d	4.33 b	88.00 abc	4.33 d
Nano Citronella EO	75%	5.67 c	88.33 ab	6.00 cd	5.33 b	90.33 a	4.33 d

Treatment	Dose / control	Germination (%)			Vigor (%)		
		As	Ns	Ds	As	Ns	Ds
Nano Citronella EO	50%	16.00 abc	79.00 abc	5.00 cd	3.66 b	87.66 abcd	8.66 cd
Nano Citronella EO	25%	9.67 bc	81.33 abc	9.00 cd	6.33 b	84.66 abcd	9.00 cd
CTRL	Pure soy	3.67 c	69.66 cd	21.67 ab	15.33 ab	53.66 fg	31.00 ab
CTRL	Water + Tween	10.67 abc	82.00 abc	7.33 cd	14.00 ab	73.66 abcde	12.33 cd
CTRL	No treatment	11.67 abc	84.33 abc	4.00 d	15.00 ab	74.00 abcde	11.00 cd
CTRL	Vitavax	6.00 c	89.00 ab	5.00 cd	5.66 b	88.66 ab	5.00 d

As: Abnormal seeds; Ns: Normal seeds; Ds: Dead seeds; EO: Essential Oil; Nano: Nanoemulsified; CTRL: Controls. Means followed by the same lowercase letter in the columns do not differ by Tukey HSD test at $p \leq 0.05$.

Regarding the incidence of phytopathogens, all essential oils tested, whether in nanoemulsified or free form, showed similar responses, including the control, for the fungus *Fusarium sp.* On the other hand, for *Aspergillus sp.,* the nanoemulsified lemongrass essential oil was superior at all doses compared to the other treatments. The nanoemulsified citronella oil also showed significant results. It is noteworthy that the 100% nanoemulsified oil contained a concentration of 5% essential oil. Complete data are presented in Table 3.

Table 3. Incidence of phytopathogens in soybean seeds stored for four months as a function of the application of different treatments.

Treatment	Concentration	*Fusarium* sp. (%)	Aspergillus sp. (%)
Free Lemongrass EO	100%	16.66 a	9.66 abcd
Free Lemongrass EO	75%	25.00 a	20.00 a
Free Lemongrass EO	50%	17.66 a	6.00 bcd
Free Lemongrass EO	25%	20.33 a	9.33 abcd
Nano Lemongrass EO	100%	15.66 a	0.00 d
Nano Lemongrass EO	50%	18.33 a	2.00 cd
Nano Lemongrass EO	25%	16.33 a	1.00 d
Free Citronella EO	100%	16.33 a	21.33 a
Free Citronella EO	75%	16.33 a	9.33 abcd
Free Citronella EO	50%	12.66 a	11.33 abcd
Free Citronella EO	25%	23.66 a	15.66 ab
Nano Citronella EO	100%	11.66 a	1.66 cd
Nano Citronella EO	75%	16.33 a	2.33 cd
Nano Citronella EO	50%	18.33 a	2.33 cd
Nano Citronella EO	25%	21.66 a	6.00 bcd
CTRL	Pure soy	17.00 a	5.00 bcd
CTRL	Water + Tween	12.66 a	13.66 abc
CTRL	No treatment	24.66 a	20.33 a
CTRL	Vitavax	14.66 a	12.00 abcd

Nano: Nanoemulsified; CTRL: Controls. Means followed by the same lowercase letter in the columns do not differ by Tukey HSD test at $p \leq 0.05$. * 75% lemongrass EO nanoemulsions were excluded due to analytical errors.

The control of *Fusarium sp.* in seeds is challenging due to its ability to persist in the environment and the presence of resistant structures (Ekwomadu and Mwanza, 2023). Daronco et al. (2015) also verified the efficacy of essential oils in the phytosanitary treatment of soybean seeds, noting that the treatment with Vitavax + Thiran® had the best performance, but the EOs of *E. globulus* (20% and 30%) and *C. flexuosus* (20%) also demonstrated good performance in controlling this fungus, with incidence rates ranging from 48% to 87%, varying according to the oil tested and the concentration, all of which were lower than the positive control that showed no contamination. In the present study, the free essential oil of lemongrass was more efficient, achieving a lower incidence of *Fusarium sp.* in the treated seeds. Furthermore, the data obtained in this work demonstrate that nanoemulsified formulations further enhance the antifungal effect of the EOs, specifically, the treatment with N-CEO at 100%, which resulted in an incidence of only 11.66% of *Fusarium sp*., even surpassing the performance of the control treatment (Vitavax).

For *Aspergillus* sp., the results showed that the essential oils in nanoemulsified form, especially lemongrass, were more effective in inhibiting fungal growth. The study by Yaouba and Essola Etoa (2017) also investigated the essential oil of

Cymbopogon citratus in the treatment of soybean seeds stored for 40 days, using concentrations of 0, 2000, 4000, and 6000 ppm. The authors observed a significant reduction in the incidence of fungi such as *Aspergillus niger, A. flavus, Fusarium oxysporum, F. moniliforme, F. solani, and Cercospora kikuchii.* Although the studies followed distinct methodologies and concentrations, both demonstrate the antifungal potential of lemongrass in its free form; however, the present study expanded the analysis by testing the nanoemulsified formulation, which proved to be even more efficient in reducing *Aspergillus sp.* compared to the free form, an aspect not addressed in the research by Yaouba and Essola Etoa (2017), which is a distinguishing feature of the present research.

The antifungal efficacy observed in this study may be associated with the chemical constituents found in the EOs studied, specifically the terpenoids. These constituents present in the oils of *C. citratus* and *C. winterianus* were also found in the studies by Dangol et al. (2023), Jin et al. (2024), and Hu et al. (2022). The antifungal action of the mentioned constituents occurs mainly through the destruction of the cell membrane, induction of oxidative stress, and negative regulation of vital genes for the fungus, causing inhibition of essential enzymes for fungal metabolism (Tang et al., 2018).

In this context, the use of nanoemulsions containing essential oils represents a promising strategy to enhance this antifungal action. According to Padrilah et al. (2024), the small size and large surface area of the droplets of nanoemulsions associated with essential oils, such as lemongrass, improve the dispersion of active compounds, increasing their bioavailability and antifungal efficacy, as it is an alternative that has the capacity to encapsulate, protect, and deliver a variety of compounds.

Fig. 1 evaluates the distance between treatments, revealing two main subgroups, the first subgroup, characterized by inferior results, is composed of the pure soybean, free lemongrass essential oil at concentrations of 75% and 100%. Another subgroup formed consisted of Vitavax® and nanoemulsified citronella essential oil at concentrations of 75% and 100%. A third group with similar results included nanoemulsified lemongrass essential oil at concentrations of 25%, 75%, and 100%.

When comparing the results presented in the current study regarding citronella essential oil, it is evident that they differ and vary according to the dose and the form used, whether free EO or nano. In the aforementioned figure, which concurrently evaluates all analyses conducted in the study, it can be observed that the nanoemulsified citronella essential oil stood out and was compared to the positive control.

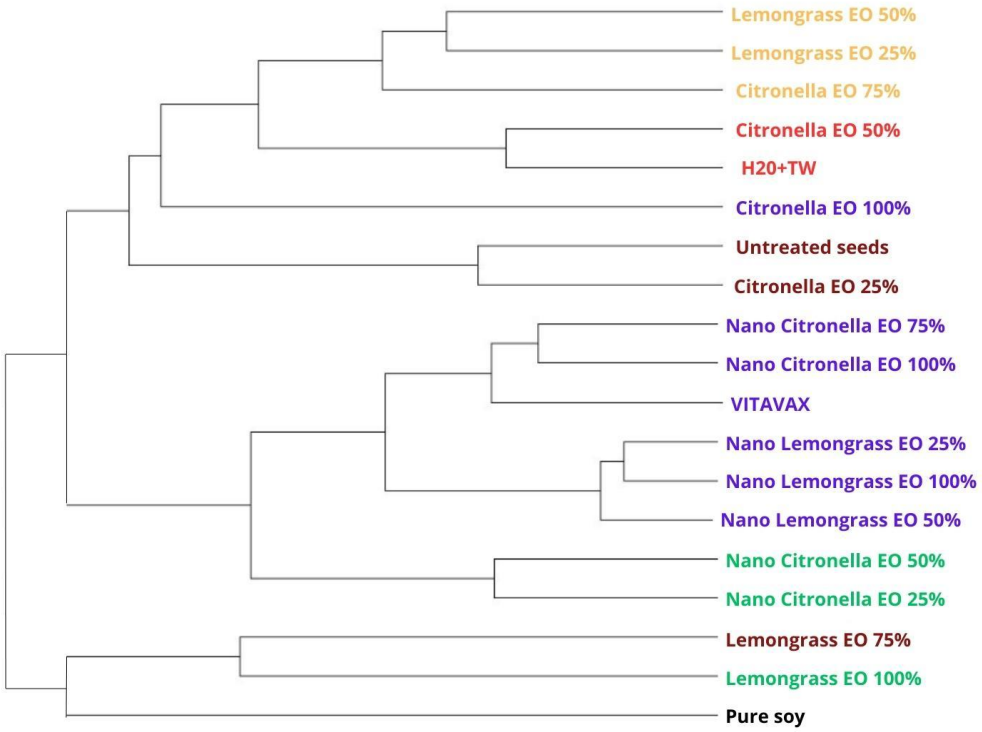


Fig. 1. Distance Analysis between treatments: Identification of subgroups with differentiated performance.

In our study, the 100% nanoemulsified citronella EO proved effective in antifungal activity, consistent with the findings of Fontana et al. (2020). Their study aimed to evaluate the in vitro fungicidal potential of this EO against soil pathogens, indicating high efficacy against *Fusarium spp.* and *S. sclerotiorum*. However, while Fontana et al. (2020) utilized the free essential oil against two pathogens across eight concentrations and ten replicates, the present study tested the nanoemulsified version against *Aspergillus sp*. using fewer concentrations and replicates. The same occurred in the study by Clevelares et al. (2024), which aimed to evaluate the antifungal potential of free citronella essential oil (*Cymbopogon winterianus*) in the *in vitro* and *in vivo* control of *Fusarium oxysporum* in tomato seedlings, and found that the oil caused a significant decrease in fungal sporulation, along with the inhibition of mycelial growth in the *in vitro* assessment. Additionally, the study by Cruz et al. (2015) aimed to identify the components present and evaluate the in vitro effect of *C. winterianus* essential oil against three isolates of *Fusarium solani*, observing that there was a reduction in the mycelial growth of the three fungal isolates where the free oil was applied.

The aforementioned studies demonstrate the important antifungal potential of free *C. winterianus* essential oil against different genera of fungi. In comparison to the researched literature, the present study presents a distinguishing feature with the use of pharmaceutical technology of nanoemulsion of essential oils, utilizing nanoemulsified essential oil, which demonstrated superiority in efficacy, with potential for the development of products that can be used as alternatives in agriculture in place of synthetic products.

Materials and methods

This is an experimental study using a randomized block design with 20 treatments in 3 replicates, totaling 60 experimental units. A 2 x 2 x 4 + 4 triple factorial scheme was employed, consisting of two essential oil types (C. winterianus and C. flexuosus), two delivery technologies (free and nanoemulsified), and four concentrations of each oil. The design included a positive control (synthetic fungicide) and two negative controls: 1) distilled water and 2) water + polysorbate 80. The experiment was conducted from May to September 2024 at the Seed Analysis Laboratory of UNIJUÍ, Ijuí Campus, using seeds of the Brasmax Lance® soybean cultivar. In the present study, it was decided not to inoculate the seeds based on previous data published by the group (Baiotto et al., 2023), which indicated no significant differences between inoculated and non-inoculated seeds.

Plant materials

C. flexuosus and *C. winterianus* were used in this study. Species were identified at UNIJUÍ's Botany Laboratory and cataloged in the Rogério Bueno University Herbarium (HURB) under vouchers 8113 (*C. flexuosus*) and 8114 (*C. winterianus*), with scientific names confirmed via Flora do Brasil. The study is registered in SisGen under AE1F391. The soybean seeds used were Brasmax Lance®.

Extraction of essential oils

Essential oils from *C. flexuosus* and *C. winterianus* were extracted from fresh leaves at the Unijuí Oleochemical Center in Três Passos/RS (coordinates: 27°26'02.4" S, 53°57'06.7" W) via hydrodistillation using a modified Clevenger apparatus (LINAX D20 model) for 1.5 hours.

Nanoemulsion production

Nanoemulsions were prepared using a high-energy method without organic solvents and with temperature control, following Giongo et al. (2016). Formulations (n=3) were obtained by injecting the oil phase into the aqueous phase (Tween 80® and ultrapure water) under high agitation with an Ultra-Turrax® disperser (T18, IKA®, Germany) at 10,000 rpm, then increased to 17,000 rpm for 45 minutes. Temperature was controlled (ice bath) to prevent volatilization/degradation. Blank formulations (n=3) used inert medium-chain triglycerides (MCT) oil. All were prepared in triplicate (n=3) and stored protected from light under refrigeration.

Physicochemical characterization of nanoemulsions

pH determinations were performed in triplicate using a potentiometer (Denver Instrument®), previously calibrated with buffer solutions (pH 4.0 and 7.0). The mean particle diameter and polydispersity index (PDI) were determined by dynamic light scattering (DLS) using a Zetasizer® Nano-ZS instrument (Malvern, England). For these analyses, samples were diluted 500-fold (v/v) in ultrapure water and filtered through a 0.22 µm membrane (Millipore®) to ensure optical transparency and prevent laser beam attenuation (Mir-Palomo et al., 2016).

Zeta potential was measured by electrophoretic mobility in the same equipment, with formulations diluted (1:500 v/v) in a NaCl solution (10 mmol L^{-1}). All readings were expressed as the mean of three replicates. The essential oil content was evaluated after 30 days of cold storage. The physical stability of the nanoemulsions, stored in amber glass bottles, was monitored under three conditions: room temperature (25 ± 2 °C), refrigeration (4 ± 2 °C), and a climatic chamber (40 °C; 75% RH), considering variations in diameter, PDI, pH, and zeta potential.

Treatments

The experiment consisted of different treatments, as described in Table 4. To apply the treatments to the seeds, the manual mixing method in transparent plastic bags was used, with the addition of 500 g of Brasmax Lance® soybean seeds. The mixture was shaken for 5 minutes to ensure better coating and homogenization of the seeds with the treatments, which were applied individually with the addition of a syrup at a ratio of 4 mL Kg^{-1} of seed. For the *Cymbopogon winterianus* and *Cymbopogon flexuosus* essential oils, concentrations of 25%, 50%, 75% and 100% were used. The essential oil was diluted in sterile distilled water containing 2% Tween® 80 (Polysorbate-80), ensuring homogenization and better dispersion. Concentrations were prepared in sterile Eppendorf microtubes. Additionally, *Cymbopogon winterianus* and *Cymbopogon flexuosus* essential oils were tested in nanoemulsified forms at the same concentrations.

Three controls were used: a) sterile distilled water in the same proportion used for the essential oils, along with the emulsifying agent Tween® 80; b) no treatment; and for the positive control, the synthetic fungicide Vitavax-Thiram® 200 SC (Carboxin 200 g L^{-1} + Thiram 200 g L^{-1}) was used at a ratio of 4 mL Kg^{-1} of seed, according to the manufacturer's recommended dosage; c) pure soybean, evaluated at the beginning of the experiment without treatment.

Table 4. Description of the treatments used in the experiment to verify the fungal incidence in soybean seeds of the Brasmax Lance® cultivar, not inoculated with the fungus.

Code	Description	Concentration / rate	Application volume (mL kg^{-1})
T1	Untreated seeds	-	4 mL Kg-1
T2	Pure soybean*	-	4 mL Kg-1
T3	Seeds + sterile distilled water with Tween® 80	-	4 mL Kg-1
T4	Seeds + Vitavax-Thiram® 200SC + sterile distilled water	-	4 mL Kg-1
T5	Seeds + EO *Cymbopogon flexuosus*	25%	4 mL Kg-1
T6	Seeds + EO *Cymbopogon flexuosus*	50%	4 mL Kg-1
T7	Seeds + EO *Cymbopogon flexuosus*	75%	4 mL Kg-1
T8	Seeds + EO *Cymbopogon flexuosus*	100%	4 mL Kg-1
T9	Seeds + EO *Cymbopogon winterianus*	25%	4 mL Kg-1
T10	Seeds + EO *Cymbopogon winterianus*	50%	4 mL Kg-1
T11	Seeds + EO *Cymbopogon winterianus*	75%	4 mL Kg-1
T12	Seeds + EO *Cymbopogon winterianus*	100%	4 mL Kg-1
T13	Seeds + EO *Cymbopogon flexuosus* nanoemulsified	25%	4 mL Kg-1
T14	Seeds + EO *Cymbopogon flexuosus* nanoemulsified	50%	4 mL Kg-1
T15	Seeds + EO *Cymbopogon flexuosus* nanoemulsified	75%	4 mL Kg-1
T16	Seeds + EO *Cymbopogon flexuosus* nanoemulsified	100%	4 mL Kg-1
T17	Seeds + EO *Cymbopogon winterianus* nanoemulsified	25%	4 mL Kg-1
T18	Seeds + EO *Cymbopogon winterianus* nanoemulsified	50%	4 mL Kg-1
T19	Seeds + EO *Cymbopogon winterianus* nanoemulsified	75%	4 mL Kg-1
T20	Seeds + EO *Cymbopogon winterianus* nanoemulsified	100%	4 mL Kg-1

EO = Essential Oil; "*" it was not stored and was analyzed at the beginning of the experiment, without treatment.

Experiment

After treatments, soybean seeds were dried at room temperature in an open aseptic container for 4 hours in the shade, then stored in labeled polypropylene bags in a room adjacent to UNIJUÍ's Plant Production Laboratory (Department of Agrarian Studies) for 4 months at 18°C ± 2°C and <70% relative humidity. Initial seed germination and moisture content analyses were performed on field samples before fungal inoculation and treatments at UNIJUÍ's Seed Analysis Laboratory to assess storage-end conditions. Seed humidity was measured on the first and last storage days using a Gehaka portable moisture meter. After 120 days, samples underwent germination, vigor, and pathology tests in the same laboratory.

For the germination test, 200 field soybean seeds were tested in four replicates of 50 seeds, following the Rules for Seed Analysis (Brasil, 2009a). Seeds were incubated on Germitest® paper rolls moistened with distilled water (3x paper weight) in a germination chamber at 25°C ± 2°C with an 8-hour photoperiod. On day 7, normal (perfect structures), abnormal (imperfect structures), and dead (non-germinated) seedlings were counted.

For the vigor test (accelerated aging), treated samples were placed in Gerbox® boxes with 40 mL distilled water, exposed in a BOD chamber at 41°C for 24 hours (Ferreira and Borghetti, 2004). Four replicates of 200 seeds per treatment were then subjected to the germination test per Rules for Seed Analysis (Brasil, 2009a).

For the pathology test, 100 seeds were used per treatment, with four replications of 25 seeds. The seeds were distributed in Gerbox® boxes, equidistantly on three sheets of filter paper, previously moistened with 15 mL of distilled water. The boxes with the seeds were incubated in the chamber at a temperature of 25ºC (±2ºC) with a 12-hour photoperiod for seven days. After this period, each seed was individually evaluated with the aid of stereoscopic and optical microscopes, identifying the fungi through the morphological characteristics of their structures (Henning, 2015; Goulart, 2018), according to the Seed Health Analysis Manual (Brasil, 2009b). The results were expressed as the percentage of contaminated seeds for each fungus.

The 75% lemongrass essential oil nanoemulsions were excluded from the study due to technical errors during the pathological analysis and insufficient remaining sample volume precluded re-analysis.

Statistical analysis

The data obtained with these analyses were entered into a database and subjected to analysis of variance; the analyses of means of qualitative variables were carried out using plus or minus one standard deviation and coefficient of variation, and compared by the Tukey HSD test at $p \leq 0.05$, using the free software R with the aid of the AgroR and ggplot2 packages (R

Core Team, 2023). In the absence of normality, the data were transformed into $\sqrt{x} + 0.5$. Therefore, the data referring to the abnormal and dead seeds were not submitted to the test of means and to the analysis.

Data regarding the variables, phytopathogens, germination, and vigor, were subjected to distance analysis to evaluate the multivariate dissimilarity between treatments. Previously, data were standardized using the z-score method to eliminate the effect of different measurement scales. The distance matrix was then calculated using Euclidean distance as a measure of dissimilarity. The hierarchical clustering methods employed were UPGMA (Unweighted Pair-Group Method with Arithmetic Mean) and WPGMA (Weighted Pair-Group Method with Arithmetic Mean). To ensure the robustness of the clusters and evaluate the reliability of the branches in the dendrograms, bootstrap analysis was applied.

Conclusion

The findings of this study indicate that essential oils have significant potential for soybean seed treatment, aiding in seed quality preservation and fungal control during storage. Among the treatments, lemongrass essential oil (*Cymbopogon citratus)*, especially as a nanoemulsion, showed the best results in seed germination and vigor at 25% concentration, outperforming the traditional chemical approach with Vitavax-Thiram in reducing *Fusarium sp.* Presence.

The nanoemulsified oil was more effective than the free form, likely due to its stability and enhanced release of active compounds, improving antifungal activity and coating uniformity on seeds. Both lemongrass and citronella oils effectively inhibited *Aspergillus sp.,* with nanoemulsified lemongrass oil performing best. While free-form lemongrass essential oil yielded good results at 25% and 50%, it was less effective than the nanoemulsion. Citronella oil showed modest results but positively impacted seed vigor when used as a nanoemulsion at 25%.

In conclusion, essential oils, particularly as nanoemulsions, are promising alternatives to synthetic fungicides for soybean seed treatment. However, several challenges must be overcome for their effective implementation as a treatment technology, including the development of products with long-term stability under adverse temperature conditions and low production costs. Thus, further studies are needed to develop stable, standardized, and safe formulations for effective large-scale application.

Declaration of competing interest

The authors declare that they have no competing interests related to the content of this manuscript. There are no financial or personal relationships that could inappropriately influence the research presented in this study.

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