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In vitro antifungal activity of aqueous extract and essential oil of African basil (*Ocimum gratissimum L.*)

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ABSTRACT

Background: Fruit and vegetables are threatened by several diseases. These diseases are mainly treated with chemicals representing a real danger to human health and the environment. **Aims:** This study aims to develop safe and non-polluting alternatives such as medicinal plants to control fungal phytopathogens. **Material and Methods:** In the present study, the aqueous extract and essential oil of the medicinal plant *Ocimum gratissimum* L. were tested in vitro against *Botrytis cinerea, Colletotrichum gloeosporioides* and *Fusarium oxysporum*. **Results:** The results show that the essential oil of *Ocimum gratissimum* L. has significant antifungal activity on the studied strains. It inhibits completely the growth of *Botrytis cinerea* and *Colletotrichum gloeosporioides* from the concentration of 500 ppm. While at least 750 ppm is required for complete inhibition of the growth of *Fusarium oxysporum*. Regarding the aqueous extract, total inhibition has been observed at the 60% concentration for *Botrytis cinerea* and *Colletotrichum gloeosporioides*. However, no concentration of aqueous extract completely inhibited the growth of *Fusarium oxysporum*. **Conclusion:** This study can be a starting point for research on a promising solution using the essential oil and aqueous extract of *O. gratissimum* L. as alternatives to chemicals to manage anthracnose (caused by *C. gloeosporioides*) and gray mold (caused by *B. cinerea*).

Keywords: Antifungal activity, aqueous extract, essential oil, *Ocimum gratissimum* L.

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1 Introduction

Fruit and vegetables (quantity and quality) strongly influence food security and the global economy as they are an essential source of economic income and nutrients (vitamins, polyphenols, minerals) [1]. However, they are susceptible to heavy attack by a variety of pathogenic microorganisms, including bacteria, viruses, and fungi [2]. These plant pathogens can cause considerable economic damage to plant products. The most important plant pathogens are fungi that cause serious diseases in fruit and vegetables during the different stages of plant growth and after harvest [3]. Various approaches have been put forward to prevent, control, or eradicate plant diseases. Among them, synthetic fungicides are the most common approach used to control several post-harvest plant diseases. However, their continuous or repeated use can disrupt the balance of ecosystems (soil, water, and air), develop pathogens resistant to one or more chemicals, and be toxic to

non-target organisms [4]. In addition, failure to comply with recommended doses results in the bioaccumulation or bioconcentration of residues in the food chain beyond safe limits [5]. This poses a risk to human and animal health and the environment [6]. Therefore, there is a need to develop an alternative control method that is eco-friendly, sustainable, safe, and efficient for plant disease management. Hence, researchers are focused on the biological approach to control many plant diseases and overcome the resistance of new mutants to chemicals [7]. This approach involves balancing a pest population (insects, pathogens) with their natural or antagonistic predators until total elimination of the target pathogen or pest is achieved [8]. However, this approach is less effective, especially when a large number of pests or pathogens has to be eliminated [9]. Although the use of chemicals of plant origins is also chemical control, it is worth exploring because its residues are less harmful than those of synthetic fungicides. Thus, in recent years, the use of medicinal plants (organic or aqueous extracts and essential oils), has proven to be efficient against many plant pathogens and is known to be safe for the consumer and the environment [1, 10, 11]. Scientific studies have also revealed that *Ocimum gratissimum* L. has natural substances that can inhibit or kill a wide variety of fungi, bacteria, and insects [12, 13]. *Ocimum gratissimum* L. commonly known as African basil or clove basil belongs to the family *Lamiaceae* [14]. It is an important aromatic and medicinal plant that grows abundantly in tropical and subtropical regions. The plant has a wide geographical distribution due to its ability to adapt to water deficit [15]; mineral deficiency (K and Ca), and light [16, 17]. The uses of *Ocimum gratissimum* L. are quite varied and diverse and depend on the region. In Africa, African basil is used in traditional medicine to treat diarrhea, vomiting, pregnancy termination, digestive disorders, fever, headaches, abdominal pain, colds and malaria, and convulsions [18, 19]. While in Brazil, it is used as a sedative for children and in the treatment of coughs, bronchitis, and sore throat [20, 21]. In Asia, the plant is used as an anti-inflammatory and in the treatment of influenza [22]. In all regions where *Ocimum gratissimum* L. grows, it is used as a condiment or flavoring in food to give a specific taste or smell to food. The leaves of *O. gratissimum* are traditionally used to flavor foods and prevent spoilage[23, 24]. Extracts of *Ocimum gratissimum* L. leaves have important biological and pharmacological properties. These properties include antioxidants [13, 25], antimalarial [26], antimicrobial [27]; insecticide [28]. However, despite their potent properties, this plant species is almost employed exclusively in medicine and pharmacopoeia. Yet, instead of using synthetic chemicals, their properties can be exploited in agriculture to manage post-harvest microbial diseases such as anthracnose (mango, papaya, and banana) and gray mold (tomato), etc. The present study was carried out to investigate the *"in vitro"* antifungal activity of the essential oil and aqueous extract of *Ocimum gratissimum* L. against *Colletotrichum gloeosporioides, Botrytis cinerea* and *Fusarium oxysporum* which are well known as causal agents of main postharvest diseases.

2 Material and methods

2.1 Plant material

Fresh leaves of *Ocimum gratissimum* L. were collected in Dabou located on the outskirts of Abidjan, Côte d'Ivoire, and sent for identification to the botany laboratory, Department of Natural Sciences, Nangui Abrogoua University. The leaves were carefully washed, shade air-dried (16-18°C) for 7 days and ground into powder. The powder was sealed in polyethylene jars and transferred to the Laboratory of Fruit and Vegetable Physiology, Avignon University, France. The powdered samples were stored at 4 °C until further use. The essential oil of *Ocimum gratissimum* L., commercially known as "Oshadi basil pungent *Ocimum gratissimum*", was purchased from the local supermarket in the city of Avignon, southern France.

2.2 Fungal material

The Three phytopathogenic fungi including *Colletotrichum gloeosporioides, Botrytis cinerea* and *Fusarium oxysporum* constituted the fungal material. *C. gloeosporioides* and *F. oxysporum* were provided by CIRAD, Montpellier and the University of Montpellier, France, respectively. The *B. cinerea* was provided by INRAE Monfavet, Avignon, France. Each fungus was grown in a Potato Dextrose Agar medium for at least 7 days and stored at 4°C until use. We used *C. gloeosporioides* (anthracnose), *B. cinerea* (gray mold), and *F. oxysparium* (vascular wilts diseases) for their widespread impact on various fruits such as mango, banana, avocado, orange, papaya, tomato, apple, etc. In addition, these pathogens can cause losses before and over post-harvest storage.

2.3 Preparation of the aqueous extract

The crude aqueous extracts of *Ocimum gratissimum* L. leaves were reconstituted to a concentration of 10 % (w/v). Thus, 100 g of powder was added to 1000 mL of ultrapure water. The mixture was autoclaved at 121 °C for 20 min. After cooling, three filtrations were performed using Whatman No1 filter paper, sterile cheesecloth folds and cotton. The filtrate obtained was used to evaluate the antifungal activity tests. All filtration equipment was autoclaved to avoid bacterial contamination and aseptic conditions were ensured by a 0.42 m/s airflow extractor hood.

2.4 *In vitro* antifungal activity of aqueous extracts and essential oil against the fungal mycelial growth

The "*in vitro*" antifungal assays were performed using the poisoned food technique as described by Busuman *et* al. [11]

with slight modifications. The extract was added directly to the PDA medium previously prepared following the manufacturer's instructions that recommend 39g of PDA to 1L of water and autoclaved at 121°C for 15 minutes. The concentrations of 30%, 40%, 50%, 60% and 70% (v/v) were constituted for the aqueous extract and the concentrations of 250, 500 and 750 ppm for the essential oil. To facilitate the miscibility of the essential oil in the PDA medium, 0.05% Tween 80 was added. The mixture was then stirred manually to obtain a homogeneous medium. For each concentration, approximately 20 mL of the warm mixture (45-50 °C) was poured into sterile Petri dishes under an extractor hood (0.42 m/s). After solidification, 5 µL of fungal suspension (10^6 cells/mL) was aseptically deposited in the center of each Petri dish. A Bunsen burner was placed in the extractor hood to reinforce the aseptic conditions avoiding all possible contamination during the inoculation. The same procedure was used for the control experiments except the PDA medium was prepared exclusively with ultrapure water (the essential oil control contains Tween 80 (0.05%)). For each concentration, the experiments were performed in five replicates and the experiment was repeated twice. The inoculated Petri dishes were sealed with adhesive parafilm and incubated at 30 °C for seven days. Every 24 hours, pictures of the incubated dishes were taken, and the mycelial growth area was measured using ImageJ software. Fungal susceptibility was calculated as the percentage of inhibition of the surface growth (expansion) of mycelium compared to the control using the following formula [29]:

$$PGI = \frac{C_{moy} - T}{C_{moy}} \times 100$$

Where: PGI = Percentage of growth inhibition (%); C_{moy} = average surface area of pathogen growth in the control plate; T = average surface area of pathogen growth in the treated plate.

2.5 Minimum inhibitory concentration and minimum fungicidal concentration

The nature of the toxicity (fungistatic/fungicidal) of the oil and aqueous extract was determined following Thompson's method [30]. For concentrations where there is no mycelium growth, the inoculum deposited in their plates was re-inoculated into a fresh PDA medium without plant extracts. The renewed growth expresses a fungistatic effect and the lowest concentration which produces that effect is considered as Minimum Inhibitory Concentration (MIC). On the other hand, the absence of growth means that the plant extracts have a fungicidal effect and the lowest concentration inducing that effect is the Minimum Fungicidal Concentration (MFC).

2.6 Effect of essential oil and aqueous Extract on germination

To evaluate the effect of the treatments on spore germination, Potato Dextrose Broth (PDB) was used following the method of Tian *et* al. [31] with some modifications. Different concentrations of essential oil and aqueous extract were prepared as described above except for the PDA medium replaced by PDB. A 5 µL suspension (8.10^6 spores/mL) of each fungus was added separately to 95 µL of each sample in an Eppendorf tube. Five replicates per concentration were performed simultaneously and incubation was performed at 30°C. After 6, 24, 48 and 72 hours, the germination rate was determined using a hemocytometer and a light microscope. Germination was recorded when the germ tube could be identified.

2.7 Statistical analysis

Data were subjected to analysis of variance (ANOVA) to determine any differences between the treatments. The significant difference was assessed using Tukey's adjustment for multiple comparisons at $p \leq 0.05$. For non-parametric data, a Kruskal-Wallis test with Bonferroni adjustment for multiple comparisons was used to determine differences ($p \leq 0.05$) between means. Statistical analysis was performed using RStudio software.

3 Results

A total of 130 questionnaires were administered. Three questionnaires were discarded due to errors and incomplete data. As a result, 127 questionnaires were analyzed giving a response rate of 97.7%.

3.1 In vitro effect of essential oil on mycelial growth of *Colletotrichum gloeosporioides, Botrytis cinerea* and *Fusarium oxysporum*

Figure 1 shows the effect of the essential oil of *Ocimum gratissimum* L., on the three phytopathogenic fungi in the *"in vitro"* test. The results revealed that the essential oil of *Ocimum gratissimum* L. has strong antifungal activity against *Colletotrichum gloeosporioides* (100% at 500 ppm), *Botrytis cinerea* (100% at 500 ppm), and *Fusarium oxysporum* (100% at 750 ppm). Furthermore, the efficacy of the essential oil increases with concentration and decreases with the incubation time.

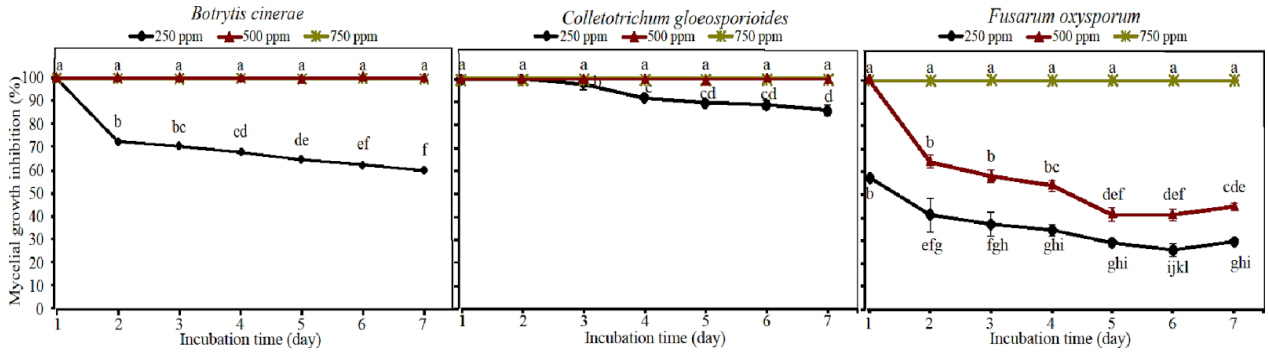

Figure 1. Antifungal activity *Ocimum gratissimum L.*, essential oil on the mycelial growth of *Botrytis cinerea, Colletotrichum gloeosporioides* and *Fusarium oxysporum* during incubation

Means affected by different letter(s) above the bars are significantly different at the 5% level as determined by Tukey HSD or Bonferroni multiple comparison tests based on normal distribution of data and homogeneity of variances.

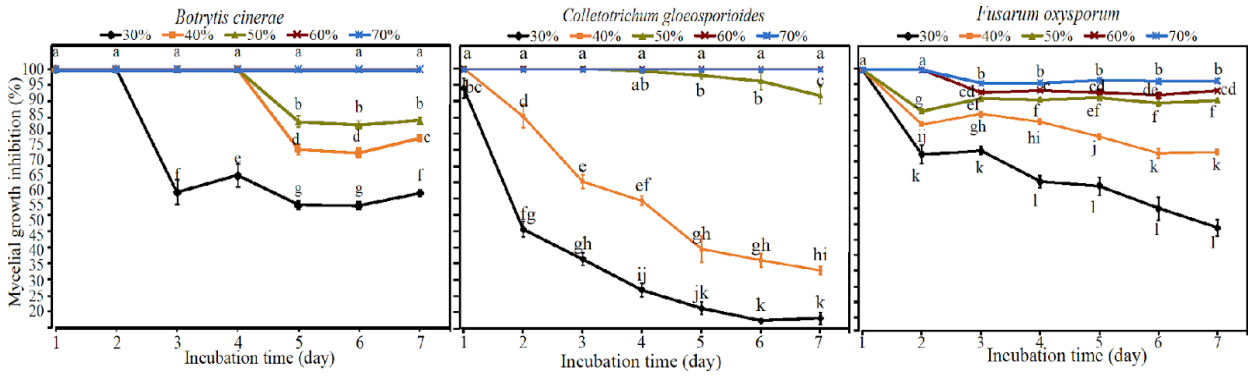

Figure 2. Antifungal activity *Ocimum gratissimum L.*, on the mycelial growth of *Botrytis cinerea, Colletotrichum gloeosporioides* and *Fusarium oxysporum* during incubation

Means affected by different letter(s) above the bars are significantly different at the 5% level as determined by Tukey HSD or Bonferroni multiple comparison tests based on normal distribution of data and homogeneity of variances.

3.2 In vitro effect of an aqueous extract on the growth of mycelia of Colletotrichum gloeosporioides, Botrytis cinerea and Fusarium oxysporum

The aqueous extract caused inhibition of the mycelial growth of *Botrytis cinerea, Colletotrichum gloeosporioides* and *Fusarium oxysporum* (Figure 2). Its activity on the three fungal pathogens varied with the concentrations and decreased with the incubation period. Thus, the inhibition rate of *Botrytis cinerea* varies from 57.98 ± 1.06 to 100 %. While the inhibition rates of *Colletotrichum gloeosporioides* and *Fusarium oxysporum* vary from 22.37 ± 0.66 to 100 % and from 49.37 ± 2.75 to 96.4 ± 0.25 %, respectively. The decrease of effect on *Colletotrichum gloeosporioides* is higher than that of the other pathogens.

3.3 Sensitivity of three pathogens to the aqueous extract and essential oil of *Ocimum gratissimum* L.

For all concentrations, the essential oil reduced the mycelial growth of *Colletotrichum gloeosporioides* and *Botrytis cinerea* by more than 50 % after seven days. In contrast, for *Fusarium oxysporum,* mycelial growth was inhibited to less than 50 % except for the 750-ppm concentration which completely reduced growth (100 %) after 7 days (Figure 3). The aqueous extract completely inhibited the mycelial growth of *Botrytis cinerea* and *Colletotrichum gloeosporioides* from the 60 % (v/v) concentration. No concentration aqueous extract used in this study was able to completely stop the mycelial growth of *Fusarium oxysporum*. Globally, the three pathogens are more sensitive to essential oil than aqueous extract.

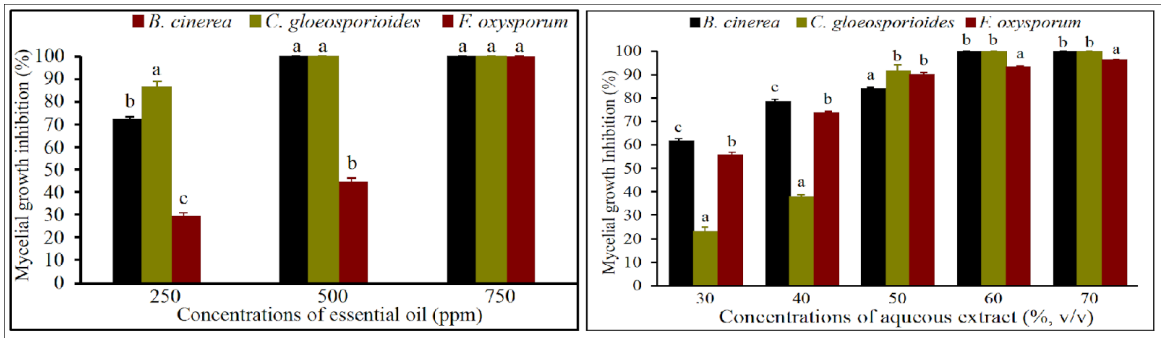

Figure 3. Sensitivity of the three pathogens to essential oil and aqueous extract of *Ocimum gratissimum L.* after seven days of incubation

Means affected by different letter(s) above the bars are significantly different at the 5% level as determined by Tukey HSD or Bonferroni multiple comparison tests based on normal distribution of data and homogeneity of variances.

3.4 Antifungal parameters of the aqueous extract and essential oil of *Ocimum gratissimum* L.

Regardless of the pathogen, the antifungal activity of *Ocimum gratissimum* L. (aqueous extract and essential oil) was strongly correlated with concentration (Table 1). The minimum inhibitory concentration for *Colletotrichum gloeosporioides* and *Botrytis cinerea* was the same for both aqueous extract and essential oil. For the essential oil, the MIC and MFC are the same and equal to 500 ppm for both *C. gloeosporioides* and *B. cinerea*. The same observation was noted with aqueous extract with a concentration of 60 %. These concentrations inhibited and killed *Colletotrichum gloeosporioides* and *Botrytis cinerea* over the incubation time.

3.5 Effect of plant extracts on pathogen germination

Table 2 exhibits the results of the effect of the different extracts of *Ocimum gratissimum* L. on the germination of the three pathogens. The results show that the aqueous extract and the essential oil have inhibitory properties against the germination of the pathogens used in this work. On the germination of *Colletotrichum gloeosporioides*, from the concentration of 40 % (v/v) of aqueous extract and 500 ppm of essential oil, the inhibition is complete (100 %) after 6 h of incubation. However, after 48 h of incubation, the inhibition rate was recorded below 50 % and even null for the 30% and 40% (v/v) concentrations. Only the concentration of 750 ppm completely stopped the germination of *Colletotrichum gloeosporioides*.

Table 1. Antifungal parameters of the aqueous extract and essential oil of *Ocimum gratissimum* L. on the mycelial growth of *Botrytis cinerea*, *Colletotrichum gloeosporioides,* and *Fusarium oxysporum*

		Pathogens		
		B. cinerea	*C. gloeosporioides*	*F. oxysporum*
Aqueous extract	MIC (%, v/v)	60	60	-
	MFC (%, v/v)	60	60	-
	r	0.96	0.93	0.96
Essential oil	MIC (ppm)	500	500	750
	MFC (ppm)	500	500	-
	r	0.84	0.95	0.96

r: Spearman's Rho, MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration.

For *Botrytis cinerea* and *Fusarium oxysporum*, during the first two days (24h and 48h), no germination was observed even in the control tube. Furthermore, the results revealed that the inhibition of germination of *Botrytis cinerea* and *Fusarium oxysporum* was complete during the whole incubation period at all concentrations of essential oil and aqueous extract (except for 30 %).

4 Discussion

Post-harvest diseases control using plant extracts is a promising alternative method for post-harvest product management and ensures Global Health. The fungicidal activity of some plant extracts to control different plant pathogens has been reported by several authors [32, 33]. This work aims to evaluate the antifungal activity of *Ocimum gratissimum* L. (aqueous extract and essential oil) to use as a natural fungicide in the treatment of post-harvest diseases of

Table 2. Influence of aqueous extract and essential oil of *Ocimum gratissimum* L. on the germination of *Botrytis cinerea, Colletotrichum gloeosporioides* and *Fusarium oxysporum*

Plant extracts	Germination inhibition (%)												
		Botrytis cinerae				*Fusarium oxysporum*				*C. gloeosporioides*			
	Dose	6h	24h	48h	72h	6h	24h	48h	72h	6h	24h	48h	72h
Aqueous extract (%, v/v)	30	-	-	-	0,00a	-	-	-	0,00a	26,32±9,12bc	17,51±2,93e	0,00e	-
	40	-	-	-	100b	-	-	-	100b	100a	34,00±3,69d	0,00e	-
	50	-	-	-	100b	-	-	-	100b	100a	48,69±6,97d	4,80±4,76d	-
	60	-	-	-	100b	-	-	-	100b	100a	85,31±4,00c	31,20±3,11c	-
	70	-	-	-	100b	-	-	-	100b	100a	96,70±2,53b	46,80±6,38bc	-
Essential oil (ppm)	250	-	-	-	100b	-	-	-	100b	83,40±3,79b	4,4±2,07f	0,00e	-
	500	-	-	-	100b	-	-	-	100b	100a	97±0,71b	88,40±2,88ab	-
	750	-	-	-	100b	-	-	-	100b	100a	100a	100a	-

Values (mean ± SE) affected by different letter(s) are significantly different at the 5% level as determined by Tukey HSD or Bonferroni multiple comparison tests based on normal distribution of data and homogeneity of variances.

fruit and vegetables such as mango, tomato, banana, and others. The results revealed that *O. gratissimum* L. aqueous extract and essential oil have strong antifungal activity against *Colletotrichum gloeosporioides* (causal agent of mango anthracnose), *Botrytis cinerea* (gray mold in tomato and mango) and *Fusarium oxysporum* (fusarium rot in tomato or mango maturity malformation). The antifungal activity of *O. gratissimum* L. could be associated with its phytochemical composition. According to the literature, the bioactive compounds in the essential oil of *O. gratissimum* L. are mainly thymol, carvacrol, citral, eugenol, geraniol, and linalool [34, 35]. Yet, the antimicrobial properties of some of these compounds have been widely demonstrated such as eugenol [36]; thymol [37, 38] or linalool [39]. Regarding the aqueous extract, it is thought to be composed of monoterpenes and sesquiterpenes which can induce antifungal effects by establishing hydrogen bonds with the active sites of target enzymes [40]. Thus, the antifungal activity of the aqueous extract would be related to monoterpenes and sesquiterpenes that possess aromatic rings and phenolic groups. Moreover, as basil compounds, the inhibition rate is strongly correlated with concentrations of aqueous extract and essential oil of *O. gratissimum* L. The antifungal activity of essential oil and aqueous extract increases with concentration and decreases with the incubation time. This implies that the essential oil and aqueous extract of *O. gratissimum* L. have a dose-response antifungal activity as a function of time. The decrease of the inhibitory effect with time could be due to the volatilization of the active compounds. As the Petri dishes were sealed, this suggests that the essential oil or aqueous of African basil would be a contact-antifungal agent. Even though, other phenomena like the adaptation of the fungus or breakdown of the active molecules may result in decreasing inhibitory effects. The minimum inhibitory concentrations of the essential oil and aqueous extract required to inhibit *C. gloeosporioides* and *B. cinerea*, were equal and estimated at 500 ppm and 60% (v/v) respectively. Yet, Anaruma et al. [41] reported 2000 ppm from Brazil as MIC against the mycelial growth of *Colletotrichum gloeosporioides*. While, for *Fusarium oxysporum,* the minimum inhibitory concentration of the essential oil found in our work is 750 ppm. This disagrees with Soro *et* al. [38] who indicate 250 ppm as the minimum inhibitory concentration of essential oil of *O. gratissimum* L. for mycelial growth of *F. oxysporum*. Nakamura *et* al. [42] have reported 750 and 1500 µg/ml of *O. gratissimum* essential oil as MIC for *Candida albicans* and *Candida tropicalis*, respectively. Furthermore, according to the results reported by Stan (Tudora) *et* al. [43], the inhibitory activity of *O. gratissimum* against *Botrytis cinerea* and *F. oxysporum* is higher than that of *Ocimum basilicum*. These differences can be explained by chemical composition related to the geographical location of the plant. However, this difference can be associated with other factors including the age of the plant, extracting solvent, method of extraction, climatic conditions, harvest time, or plant part collected [10, 44]. However, it has been reported that hydroethanolic and ethanolic extracts derived from *O. gratissimum* have not inhibitory effect on *Candida strain ATCC 90028* [45]. Globally, the activity of essential oil is higher than aqueous extract. This indicates that essential oil is more concentrated in active compounds than aqueous extract leading to the extensive antifungal spectrum and broad uses of essential oil as reported in the literature. In addition, *C. gloeosporioides* and *B. cinerea* are more sensitive to the essential oil and aqueous extract of *O. gratissimum* L. than *F. oxysporum*. This suggests that

essential oil and aqueous extract are more indicated for the treatment of anthracnose (mango) and gray mold (tomato). The results showed that the *O. gratissimum* L. essential oil inhibits the germination of *C. gloeosporioides* at a concentration of 750 ppm. This observation indicates that *O. gratissimum* L. essential oil is fungistatic (at 500 ppm) and fungicide (at 750 ppm). Therefore, this confirms that the essential oil of African basil (*O. gratissimum* L.) can be used as an alternative to chemicals in the postharvest treatment of mango anthracnose. The minimum concentration required to completely inhibit the mycelial growth of *C. gloeosporioides* or *B. cinerea* is equal to that which definitively eliminates the strain with both the aqueous extract and the essential oil.

5 Conclusion

From the results of this study, it can be concluded that the aqueous extract and the essential oil of *Ocimum gratissimum* L. have high antifungal activities against *Botrytis cinerea, Colletotrichum gloeosporioides* and *Fusarium oxysporum*. Furthermore, our results showed that essential oil is more effective as an antifungal agent than the aqueous extract even though the difference in origin might affect these results. This study also shows that *Colletotrichum gloeosporioides* and *Botrytis cinerea* are more susceptible than *Fusarium oxysporum*. Therefore, essential oil and aqueous extract of *Ocimum gratissimum* L. can be recommended as a valid alternative to control mango anthracnose (caused by *Colletotrichum gloeosporioides*) and tomato gray mold (caused by *Botrytis cinerea*).

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Authors' Contribution: AFA and YS: As the lead authors of the study, they were responsible for conceptualizing the research idea, designing the study methodology, collecting, and analyzing the data, and drafting the manuscript. YS also took the lead in coordinating the collaboration among the co-authors, addressing reviewer comments, and finalizing the manuscript for submission, CN and FLL significantly developed the research design and methodology. They contributed to data collection, conducted statistical analyses, and provided valuable insights during the interpretation of results, KT critically reviewed and provided feedback on multiple drafts of the manuscript, enhancing its overall quality. He played a key role in reviewing and editing the manuscript, ensuring its clarity and coherence, FAT contributed to the literature review, gathering relevant references, and synthesizing previous research findings. He actively participated in data analysis discussions and contributed to the interpretation of results. Furthermore, He contributed to the revision process by providing critical feedback and suggestions for improving the manuscript.

Conflicts of Interest: The authors declare that they are no competing interests.

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