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IN VITRO EVALUATION OF ESSENTIAL OILS FROM SENEGALESE AROMATIC PLANTS AGAINST MANGO ANTHRACNOSE

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Keywords: Colletotrichum gloeosporioides, essential oils, biological control, antimicrobial activity, mango anthracnose, Senegal.

Abstract

Mango anthracnose caused by Colletotrichum gloeosporioides is a significant post-harvest disease that affects mango production in Senegal, leading to significant economic losses. The use of synthetic fungicides to control anthracnose has various negative impacts on the environment and human and animal health. Therefore, alternative methods are being developed, such as biological control using essential oils from aromatic plants. In this study, the in vitro antifungal activity of ethanol extracts of Mentha piperita, Ocimum basilicum, Melaleuca quinquenervia, and Eucalyptus camaldulensis essential oils were tested on C. gloeosporioides. The essential oils of M. piperita and O. basilicum exhibited the most potent antifungal activity, showing the highest efficacy in inhibiting mycelial growth, spore production, and spore germination of the fungus. The study findings suggest that these essential oils could offer potential for use in biological control, which could help reduce the reliance on synthetic fungicides and promote sustainable agriculture practices.

Introduction

Mango is an economically significant tropical fruit in Senegal, contributing to the livelihoods of nearly 30,000 people and generating a turnover of more than 10 billion CFA francs per year. However, mango production in Senegal is constrained by various pests and diseases, including anthracnose caused by the fungus C. gloeosporioides. The disease can cause significant losses in yield and quality, resulting in economic losses for farmers and the entire value chain. The control of anthracnose mainly relies on the use of synthetic fungicides, which have various negative impacts on public health and the environment. Therefore, alternative control methods, such as the use of natural products, are being developed to promote sustainable agriculture practices. Essential oils from aromatic plants have been shown to have various antimicrobial properties and are considered potent natural products for controlling plant diseases. In this study, we aimed to evaluate the in vitro antifungal activity of essential oils from Mentha piperita, Ocimum basilicum, Melaleuca quinquenervia, and Eucalyptus camaldulensis on C. gloeosporioides. The study findings could help identify potential natural products for controlling mango anthracnose and promote sustainable and eco-friendly agriculture practices in Senegal.

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Material and methods Fungal material

Strains of *Colletotrichum gloeosporioides* were isolated from infested mangoes of the variety Kent from Casamance.

Essential oils

The steam extraction method was used to obtain the essential oils from the biomass of the several plant species targeted. Therefore, the whole plant of *Mentha piperita*, and the leaves and flowers of *O. basilicum*, were submitted to extraction after 10 days of drying at room temperature. As for *Eucalyptus camaldulensis* and *Melaleuca quinquenervia*, their essential oils were extracted from fresh leaves.

After extraction, the essential oils were stored in tinted vials at room temperature in the laboratory for protection from light.

***In vitro* activity of essential oils on mycelial growth Preparation of fungal cultures**

The essential oils were incorporated into the PDA (Potato Dextrose Agar) culture medium after autoclaving at a temperature of 121 °C, at a pressure of 1 bar for 25 minutes and then poured into Petri dishes of 9 cm in diameter at a rate of 20 ml. per box. For each concentration of essential oils, 3 PDA plates were inoculated with 3 mm diameter of agar disc colonized by a pure culture of *Colletotrichum gloeosporioides* aged of 7 days. An incubation period of 8 days was considered because it corresponded to time interval taken by the fungus to fill the nontreated petri dishes.

Preparation of different concentrations

The essential oils are first mixed with Tween 80 (0.1%) before being incorporated in the PDA culture medium after sterilization and stirred for a few minutes to allow good homogenization.

Concentrations ranging from 1000 to 10000 ppm were tested for all the essential oils tested.

Evaluation of mycelial growth

Mycelial growth was assessed every 48h by calculating the average colony diameter of perpendicular measurements passing through the middle of the fungal colony. Three repetitions are performed for each concentration. Growth inhibition rates (GIR) were determined according to the formula from Doumbouya *et al.* (2012):

GIR (%) = (T-E) * 100 / T; where

GIR = Growth inhibition rate

T = Average diameter of fungal colony in the control treatment (in cm)

E = Average diameter of fungal colony in treated plates (in cm)

The LD50s were subsequently deduced on the basis of the Probit method of Finney (1941) by using the dose.p function of the R software MASS package.

Activity of essential oils on the production of spores of *C. gloeosporioides*

The fungal strain growing in the Petri dishes containing the media amended with the essential oils, 10 mL of sterile distilled water with Tween 80 (0.1%) were added. Three minutes later, the mycelium is scrapped using a scalpel blade and then filtered twice to have a spore suspension free from mycelial fragments. The resulting spore suspension was diluted 100-fold with sterile distilled water. Using a micropipette, 50 µL of this suspension were taken and deposited on a hemocytometer. The number of spores was counted under an optical microscope at X 400 magnification. Five repetitions were made for each concentration as well as with the control treatment. Inhibition of spore production was determined by the formula proposed by Doumbouya *et al.* (2012):

ISP = (SCo0-SCoT) * 100 / SCo0

ISP = inhibition of sporulation performance (in %)

SCo0 = spore concentration in control treatment
SCoT = spores concentration in the treatment considered
The LD50s are subsequently deduced using the Finney Probit method (1941) using the dose.p function of the MASS package of the R software.

Activity of essential oils on spore germination of *C. gloeosporioides*

In a seven-day old culture dish of *C. gloeosporioides*, 10 ml of sterile distilled water with Tween 80 solution (0.1%) was added. The mycelium was collected from petri dish and put into a beaker containing 100 ml sterile distilled water. The whole was shaken during 3 min before filtration over a 4 layers gaze clothe to obtain a spore suspension. The spore suspension was diluted 100 times with sterile distilled water and 50 µl of the suspension were mixed with 50 µl of essential oil previously mixed with 0.1% Tween. A drop of the mixture was then deposited between a slide and coverslip. The preparations were placed in Petri dishes containing moistened tissue to maintain a high relative humidity.

The observations were made every 12 hours for 2 days and three repetitions are performed for each concentration of essential oil which ranged from 100 to 1500 ppm. The number of germinating spores was counted as based on the initiation of the germ tube. The percentage of germination in the different treatment was confronted to spore germination in the control to assess inhibition performance of the essential oils using the formula proposed by Doumbouya *et al.,* (2012):

GR = NGSpT / NGSpC * 100
GR = germination rate
NGSpT = Number of germinating spores in considered treatment
NGSpC = Number of germinating spores in control treatment

The LD50s are subsequently deduced on the basis of the Probit method of Finney (1941) using the dose.p function of the R software MASS package.

Statistical analysis

The data was processed with R.3.2.3 software (R Core Team, 2015). A two-factor analysis of variance (ANOVA) was performed using the aov function of the agricolae package (de Mendiburu, 2015).

Results

Activity of essential oils on mycelial growth of C. gloeosporioides

Mentha piperita had a high efficacy at inhibiting mycelial growth of the fungus with 77% at 1000 ppm and total inhibition at 3000ppm. It is followed by *Ocimum basilicum* where total inhibition was obtained at 6000ppm (Figure 1). The essentials oils of Melaleuca and Eucalyptus were the less effective but still achieve at least 80% inhibition of mycelial growth at 10 000 ppm.

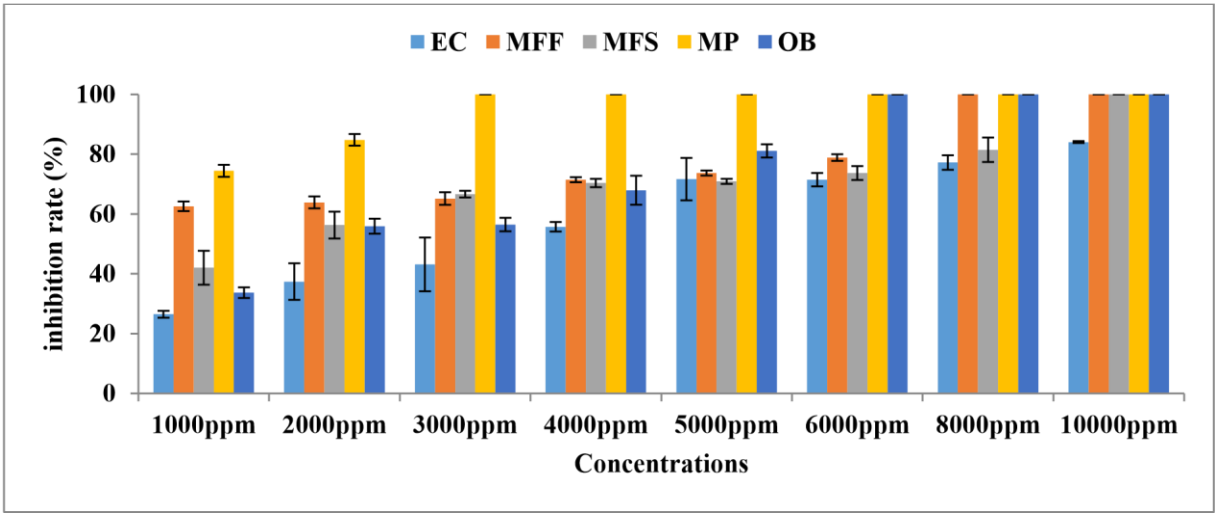

Figure 1: Activity of essential oils on the mycelial growth of *Colletotrichum gloeosporioides* (n = 120, P = 2e-16).

EC = Eucalyptus camaldulensis, MFF = Melaleuca quinquenervia (fresh leaves), MFS = Melaleuca quinquenervia (dry leaves), MP = Mentha piperita, OB = Ocimum basilicum

Activity of essential oils on the sporulation of *C. gloeosporioides*

The essential oils inhibited significatively the sporulation of the pathogen. On that parameter also, the mint oil was still the most effective with a reduction of 60% of fungal sporulation at 1000 ppm and total inhibition achieved at 3000ppm (Figure 2). With the basilic oil, fungal sporulation was reduced at 40% at 1000 ppm and with 6000ppm, sporulation was inhibited completely. The essentials oils of Melaleuca and Eucalyptus were the less effective but still achieve total inhibition of fungal sporulation at 10 000 ppm.

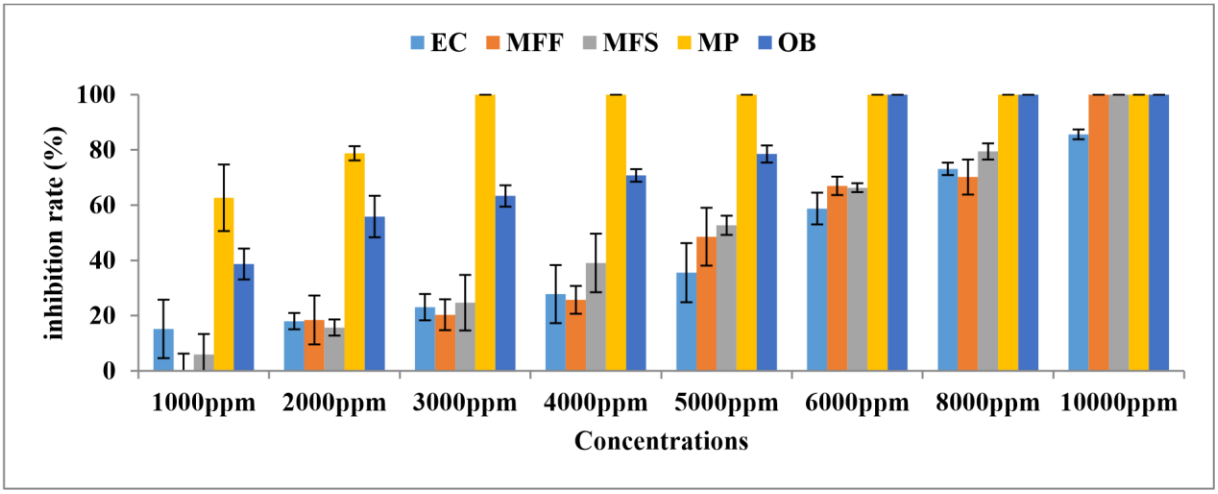

Figure 2: Activity of essential oils on the production of spores of *C. gloeosporioides* (n = 120, P = 2e-16).

EC = Eucalyptus camaldulensis, MFF = Melaleuca quinquenervia (fresh leaves), MFS = Melaleuca quinquenervia (dry leaves), MP = Mentha piperita, OB = Ocimum basilicum **Activity of essential oils on spore germination of *C. gloeosporioides***

Spore germination of ***C. gloeosporioides*** was about 70 to 80% without treatment (Figure 3). It was reduced down to 20% and 30% through treatment with respectively 100 ppm of essential oil of *Mentha piperita* and *Ocimum basilicum*. Spore germination was further totally inhibited at 500ppm of the essential oil of *M. piperita* while with *O. basilicum* total suppression of spore germination was observed at 1500ppm. The essential oil of *E. camaldulensis* reduced spore germination down to 21.66 ± 14.37% at 1500 ppm.

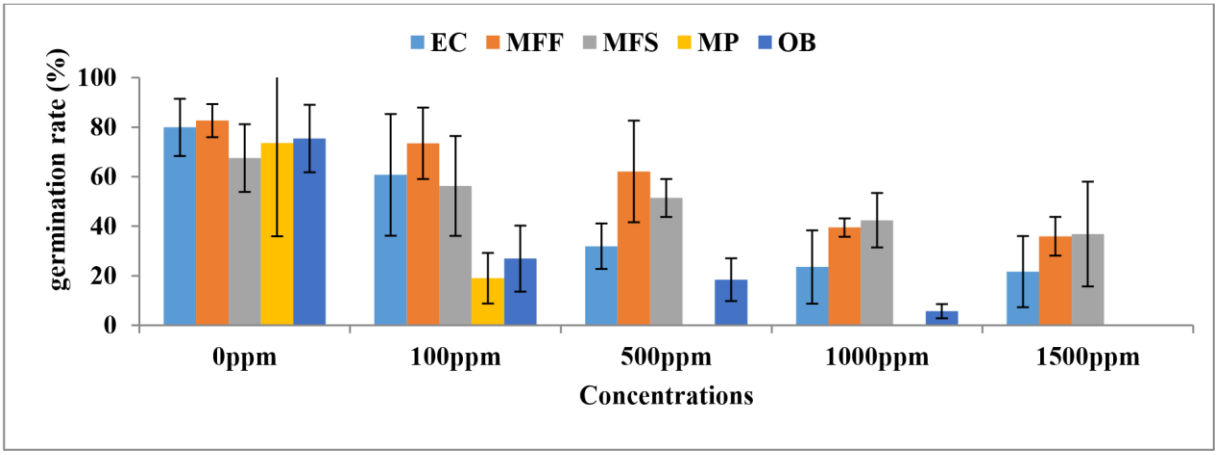

Figure 3: Activity of essential oils on the germination of spores of Colletotrichum gloeosporioides (n = 75, P = 2e-16)

EC = Eucalyptus camaldulensis, MFF = Melaleuca quinquenervia (fresh leaves), MFS = Melaleuca quinquenervia (dry leaves), MP = Mentha piperita, OB = Ocimum basilicum

Determination of the lethal concentration of essential oils

For the essential oil of *Mentha piperita* the LC50 for spore germination was 48.12 ppm (Table 1). The value raised up to 156.34 ppm for mycelial growth and was about 749.12 ppm for spore production. For *O. basilicum*, the LC50 reached 85.02 ppm for spore germination and was at 156.34 for mycelial growth, while 2242.93 ppm was the LC50 for inhibition of sporulation.

Table 1: Lethal Concentration 50 (LC50) in ppm of Essential Oils on Measured Parameters

	Spore germination	Mycelial growth	Sporulation
Eucalyptus camaldulensis	376,12	3752,42	5762,79
Melaleuca quinquenervia	322,45	416,57	4067,06
Melaleuca quinquenervia	594,84	1780,81	4899,18
Mentha piperita	48,12	156,34	749,12
Ocimum basilicum	85,02	2243,47	2142,93

Discussion

Essential oils could be an important source of solutions towards the environment while being consumer and user friendly alternatives for chemical fungicides. Their performance against the mycelial growth, spore germination and spore production of *C. gloeosporioides*, was very important. Among the 5 tested oils, the antifungal effect of *M. piperita* essential oil was the effective. It allowed to obtain a total inhibition of mycelial growth and spore production at 3000 ppm while spore germination was totally suppressed at 500 ppm. The essential oil of *M. piperita* is composed mostly of menthol which is a monoterpene (Mahboubi and Kazempour, 2014), menthone and limonene (Iscan *et al*., 2002). These molecules are suspected of causing disruption of cell membranes of fungi (Ghosh *et al.*, (2005), which could be one of the mechanisms of action leading to the observed performance of

the essential oil of *M. piperita*. The menthol and menthone are also speculated to initiate an alteration of cell metabolism of fungi that leads to complete destruction (Zani *et al*., 1991). Those 2 mechanisms linked to the active molecules in the essential oil of *M. piperita* could be at the origin of the efficiency observed in mycelial growth as well as sporulation and spore germination of *C. gloeosporioides*.

The essential oil of *O. basilicum* was highly effective against the pathogen with 100% inhibition of mycelial growth and spore production at 6000 ppm and spore germination at 1500ppm. A strong inhibitory activity of this essential oil of *O. basilicum* on mycelial growth, spore germination and spore production of plant pathogens such as *Phytophthora capsici*, *Phytophthora dreschleri* and *Phytophthora melonis* has already been reported (Amini, *et al*., (2016). The essential oil of *O. basilicum* was reported to contain of more than 20 chemical compounds including terpenic compounds such as linalool (Amini *et al*., 2016), 1,8 cineol and methyl eugenol (Govindarajan *et al*., 2013). It has antimicrobial properties (Sokovic *et al*., 2010, Rao *et al*., 2011, Matiz *et al*., 2012), antioxidant activity (Trevisan *et al*., 2006) and antifungal futures (Edris and Farrag, 2003, Amini *et al*., 2016). Some terpenic compounds are known to be able to penetrate into the phospholipid double layer of the membrane of the cell and induce its rupture. The cytoplasmic contents are thus discharged outside the cell involving its destruction (Tsuchiya *et al*., 1996). They are also reported to induce a chemo-osmotic disruption and intra-cytoplasmic potassium leakage, followed by release of nucleic acids, ATP, and inorganic phosphate (Mokaddem, 2011). Those mechanisms might be at the basis of the efficiency of the essential oil of *O. basilicum* on the inhibition of fungal growth but also on spore germination and the sporulation of fungi.

The essential oil of *M. quinquenervia* is composed of various chemical compounds including terpinene-4-ol and 1,8-cineol, which have a broad spectrum of antimicrobial activity, including antibacterial, antiviral and antifungal properties (Funery *et al*., 2006, Shelton *et al*., 2004). According to Doumbouya *et al.* (2012), the essential oil of *M. quinquenervia*, causes total inhibition of mycelial growth, spore germination and spore production of *C. gloeosporioides* at 8500 ppm. The antifungal capacity of this essential oil on *C. gloeosporioides* was confirmed by the present study showing but total inhibition of the fungus was obtained at a higher concentration. Variability in the composition and concentration of the essential oils of the same species according to location and time of harvest of biomass as well as the genotype of fungi tested might be the cause of this variation.

With the essential oil of *Eucalyptus camaldulensis* spore germination of *C. gloeosporioides* was reduced down from only 80% to 21.66 % at a concentration of 1500 ppm, which ranked medium among the tested oils. Its activity on mycelial growth and spore production where the respective the least effective. *Eucalyptus camaldulensis* is one of the most widespread plant species worldwide (Gil *et al*., 2010). Its oil contains 1,8 cineol, p-cimene (Shahwar *et al*. ., 2012), ethanol, eucalyptol and carvacrol (Akin *et al*., 2012). The essential oil of *E. camaldulensis* had a moderate efficiency against *C. gloeosporioides* despite the insecticidal, nematicidal (Shahwar *et al*., 2012), antibacterial and fungicidal properties of its chemical compounds (Bamayi *et al*., 2004).

The essential oils of *M. piperita* and *Ocimum basilicum* are rich in terpene molecules such as 1,8-cineol and terpinene-4-ol, (Funery *et al.,* 2006) (Akin *et al*., 2012). Arras and Usai (2001) showed that these aromatic molecules increase the cell permeability, induce a degradation of nucleic acids and cause an inhibition of mitochondrial energy metabolism (Lambert *et al*., 2001) in microorganisms. These alterations result in major disruption of physiological and biochemical processes in the cell leading therefore to the inhibition of the microorganisms (Yoshimura *et al*., 2010).

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