INNSPUB

International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 8, No. 3, p. 38-51, 2016

RESEARCH PAPER OPEN ACCESS

Assessment of the efficiency of essential oils of *Ocimum gratissimum* L. (Lamiaceae) and *Hyptis suaveolens* (L.) Poit. (Lamiaceae) in the biological control against sclerotinia of *Abelmoschus esculentus* (L.) Moench (Malvaceae) or okra (cultivars Volta and Hiré)

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Article published on March 26, 2016

Key words: Okra; Sclerotinia; essential oil; biological control; Côte d'Ivoire.

Abstract

A study was conducted in order to determine the most sensitive cultivar of okra [*Abelmoschus esculentus* (L.) Moench], to fungus *Sclerotium rolfsii* Sacc. on the one hand, and on the other hand to compare the antifungal activity of two essential oils from *Ocimum gratissimum* L. and *Hyptis suaveolens* (L.) Poit., on this telluric mycopathogen from most vegetable crops grown in Côte d'Ivoire. Essential oils from dried leaves of these two plants were extracted by steam distillation for 3 hrs. The essential oil yield was very low with 0.29 % and 0.06 % respectively for *O gratissimum*. and *H. suaveolens*. The fungicidal properties of these essential oils were evaluated *in vitro* on the radial mycelial growth of S. *rolfsii* through five concentrations (125, 500, 1000, 2000 and 4000 ppm). After 7 days of incubation at 12-hour photoperiod at 25 ± 2 °C on PDA medium supplemented with essential oil, the radial mycelial growth of S. *rolfsii* was completely inhibited with 4000 ppm essential oils of *O. gratissimum* and *H. suaveolens*. The *in vivo* study showed a higher sensitivity of the Volta cultivar (83.33 %) to *S. rolfsii* than Hiré (16.67 %). The essential oils of *O. gratissimum* and *H. suaveolens* can be used in biological control against *S. rolfsii*, causal agent of Sclerotinia in okra crops. The Hiré cultivar is the best cultivar to be advised to farmers faced with this disease

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Introduction

Abelmoschus esculentus (L.) Moench, commonly called "okra" originates from Africa and Asia and belongs to the Malvaceae family. It is one of the most consumed vegetables in Côte d'Ivoire where two species were identified, *Abelmoschus esculentus* and *Abelmoschus caillei* (Fondio *et al.*, 2007). The cultivation of okra is a vital lucrative business for many producers in rural, urban and suburban areas. It is grown for its fruit (eaten fresh or dried) over the entire Ivorian territory. The annual yield is estimated at about 100 000 tons per year (Fondio *et al.*, 2007). However, productivity is greatly limited by abiotic and biotic stresses. Among all the quantitative losses due to the action of harmful microorganisms, *Sclerotium rolfsii* Sacc., which causes sclerotinia, is now poised to become the most formidable land-based fungal parasite in market gardening throughout the country. It is a ubiquitous filamentous fungus that causes white rot on different hosts in tropical and subtropical regions (Punja, 1985). The conservation of this fungus as sclerotia in the soil, makes it difficult to control (Boisson & Renard, 1967; Blancard, 1988); which would explain the misuse of synthetic pesticides by producers. The risks caused by excessive and uncontrolled use of these synthetic fungicides are real for consumers' health and environmental pollution (Harman, 1992). Biological control through the use of natural substances such as essential oils, is potentially the most effective and most environmentally friendly (Zollo *et al.*, 1988). Indeed, extracts and essential oils of various species of aromatic plants have achieved positive results on mycoses (Zirihi *et al.*, 2003; Camara *et al.*, 2007). The use of essential oils is therefore a promising alternative for the control of certain fungal diseases. But to date, very few studies have examined the efficiency of such biological control methods against the fungus that causes sclerotinia in okra. The main objective of this study is to highlight the fungistatic or fungicidal properties *in vitro* and *in vivo* of essential oils of *Ocimum gratissimum* L. and *Hyptis suaveolens* (L) Poit. on *Sclerotium rolfsii* Sacc., causal agent of Sclerotinia in *Abelmoschus esculentus*. The first issue will be to assess the effect of essential oils of *Ocimum gratissimum* and *Hyptis suaveolens* on the radial mycelial growth of S. *rolfsii* firstly and secondly to study varietal sensitivity of okra cultivars Volta and Hiré, vis-à-vis S. *rolfsii* and the *in vivo* activity of the essential oil *O. gratissimum* on okra seedlings inoculated with S. *rolfsii*.

Materials and methods

Biological material

Two cultivars of okra *Abelmoschus esculentus* (L.) Moench, were used for this study. They are commercial cultivars selected by Tropicasem for cultivation in hot and humid climate. They include "Volta" from the seed company Semivoire and "Hiré" cultivar originating from Côte d'Ivoire. These two cultivars have distinct agronomic characteristics both in terms of their cycle and the color of their stems and leaves. They are also distinct in their capsule (fruit). The essential oils used to fight *in vitro* and *in vivo* against the fungal strain of *Sclerotium rolfsii* Sacc., were extracted from the leaves of *Ocimum gratissimum* L. and *Hyptis suaveolensis* L. Poit. Originating from Asia, *Ocimum gratissimum* is a medicinal and aromatic plant that has many virtues (Zhiri et Baudoux, 2005). *Hyptis suaveolens* is an aromatic herb, originating from Tropical America. It was classified as weeds whose development and spread must be controlled (Thiombiano *et al.*, 2009). Both plants belong to the Lamiaceae family. The fungal strain of *Sclerotium rolfsii* Sacc., used in this study, was isolated from the seeds of okra cultivar Clemson Spineless collected in the vegetable production area of Rubino in Côte d'Ivoire, showing symptoms of collar wilt and dry rot (Koné *et al.*, 2010). The strain was stored at the fungus culture collection of the Plant Physiology Laboratory at the University Felix Houphouët-Boigny in Abidjan (Côte d'Ivoire).

The soil used was taken at the National Floristics Center of the University Felix Houphouët-Boigny in Abidjan (Côte d'Ivoire). It is a sandy soil rich in organic matter. The cultivation was carried out under a roof consisting of a concrete table which dimensions were 1 m high, 1 m wide and 1.5 m long. All

surrounded by wooden rafters and covered with transparent plastic film enabling to protect the seedlings and facilitate the control of water, temperature and relative humidity.

Methods and experimental design

The experimental methodology was distributed in two sections that were the *in vitro* and *in vivo* studies.

For *in vitro* studies, the leaves of *Ocimum. gratissimum* were harvested on the experimental plot of the Plant Physiology Laboratory at the University Felix Houphouët-Boigny in Abidjan (Côte d'Ivoire). As for the *Hyptis suaveolens* leaves, they were harvested in Bouaké (Côte d'Ivoire). The harvested material was transported to the laboratory. The leaves were cut, weighed and spread in thin layers on a black plastic film on the ground for drying at room temperature during 72 h. The essential oils from dry leaves of *Ocimum gratissimum* L. and *Hyptis suaveolens* (L.) Poit. were obtained by steam distillation of water using a Clevenger type distiller for 3 h (Ketoh *et al*., 2000; Oussou *et al*., 2010) at the Biological Organic Chemistry Laboratory of the University Felix Houphouët-Boigny in Abidjan (Côte d'Ivoire). The yields (R_e) in percentage (%) were calculated from the mass of the oil (m_H) obtained and that of the dry matter (m_S) prior to extraction according to the following formula:

$$R_e = (m_H / m_S) \times 100$$

To assess the *in vitro* effect of essential oils on the mycelial growth of *Sclerotium rolfsii*, five concentrations (125, 500, 1000, 2000 and 4000 ppm) were selected. These essential oils were previously dissolved in Tween 20 (intermediate solution), before being added to the culture medium under magnetic stirring. Thus, each concentration of essential oil was emulsified into the PDA culture medium at 45 °C, using a sterile pipette, just before it was distributed in Petri dishes (Attrassi *et al*., 2007). Two perpendicular lines were drawn on the back of each petri dish prior to distribution of the culture medium in a sterile condition. Their intersection point indicating the center of the dish (Amadioha et Obi, 1999). The Petri dishes containing 20 mL of frozen culture medium (PDA + essential oil or PDA alone) were seeded in the center, with a mycelial disc of 5mm diameter taken from the front of growth of a culture of *Sclerotium rolfsii* that spent seven days on PDA medium. Six Petri dishes were subcultured by concentration and essential oil as well as for the control. The Petri dishes were sealed with paraffin and incubated at 25 ± 2 ° C at 12 hours photoperiod. Measurements in millimeters of radial mycelial growth of the fungus were performed, following the two straight lines drawn previously on the back of the Petri dishes, using a graduated ruler. These measurements were made every 24 hours, over seven days, before the mycelial strand reach the edge of the control Petri dish (Hibar *et al*., 2005). If no mycelial growth was observed for a given concentration, the plates were opened and the mycelial disk was transplanted into a new Petri dish containing PDA medium alone. The set of these boxes was incubated for 7 days at 25 ± 2 °C and at 12 h photoperiod. At the end of the 7th day, the essential oil was evaluated as a fungicide if there was no mycelial regrowth; otherwise it was declared fungistatic (Neri *et al*, 1991; Oxenham *et al*., 2005). This experiment was repeated 3 times. The inhibition rate of mycelial growth was calculated using the following formula (Attrassi *et al*, 2007; Assiri *et al*, 2009; Soro *et al*., 2010):

with:

$$I(\%) = [(D_O - D) / D_O] \times 100$$

D_O : average diameter of control colonies,
D : average diameter of treated colonies.

The inhibitory concentration by 50 % (IC_{50}) and 90 % (IC_{90}) of the mycelial growth were determined graphically from the linear relationship between the logarithm of the concentration of essential oils on the abscissa and probit values from inhibition percentages of mycelial growth in ordinates (Attrassi *et al*., 2007).

For inoculum production as part of *in vivo* studies, a

strain of *Sclerotium rolfsii* aged ten days on PDA medium Petri dish was used. The inoculum used for the bioassay was prepared in millet bran (*Pennisetum glaucum* L.) disinfected 3 times by autoclaving for 1 hour at 121 °C under a pressure of 1 bar at 24 hours apart. Before the first autoclaving, this millet bran was soaked in water for 24 h and partitioned in 500 mL jars at 100 g per jar. After complete cooling of the millet bran, 5 mycelial pellets of 5 mm *Sclerotium rolfsii* were added to the millet bran in the jar. The jars were sealed with paraffin and incubated in the dark at 25 ± 2 °C for two weeks with regular stirring. The prepared inoculum was used and a new inoculum was performed for each trial. The 2 % inoculum dose was tested in each trial with four concentrations of essential oil of *Ocimum gratissimum* (500, 1000 and 2000 ppm) and the soil previously obtained. The soil was first sterilized twice in an autoclave at 121 °C for 1 hour at 1 bar and 24 h apart. The inoculum and essential oil were mixed manually on the ground for 5 min. Three kinds of mixture were made:

- sole soil that will be used to inoculate healthy control seedlings,
- soil-inoculum that will be used to inoculate positive control seedlings, and
- soil- inoculum-essential-oil for inoculation of treated seedlings.

Each mixture had a final volume of 500 ml and enabled to inoculate a pot of seedlings. The content of the 12 pots per basic treatment was spilled into non-transparent polyethylene plastics and incubated at room temperature for one week before distributing again the resulting mixture into the pots.

The seeds of each cultivar were superficially disinfected after soaking in absolute ethanol for 5 minutes, then thoroughly rinsed three times with sterile distilled water to remove the remnants of pesticides used in seed treatment (Benhamou *et al.*, 1997). After drying, the seeds were sown in sterile trays of 40 cm x 25 cm x 15 cm containing the substrate compound of 25 % pig dung, 15 % refined wood sawdust and 60 % earth extracted from the National Floristics Centre of the University Felix Houphouët-Boigny in Abidjan. This substrate was previously sterilized twice in an autoclave at 121 °C for 45 min at 1 bar and 24 hours apart. The sown seeds were uniformly distributed over the entire surface with two seeds per hole. Once the seedlings produced, the trays were placed in an enclosure made with clear plastic film under a temperature of about 30 ° C and the whole was watered regularly with tap water. The transplanting of seedlings of each okra cultivar was performed when they reached 15 days after sowing that is to say, at the stage of two well spread leaves (Woo *et al.*, 1996). The transplantation of seedlings was carried out in 500 mL pots filled with mixtures prepared beforehand. Twelve seedlings per basic treatment were used in this trial. Seedlings of each okra cultivar, transplanted into the autoclaved ground and inoculated by *Sclerotium rolfsii* or in the previously autoclaved and non-inoculated soil were used respectively as untreated non-inoculated control and healthy control. The seedlings thus transplanted were put for growth in the shelter. These plants were watered once a day with tap water. The assessment of mortality, number of living leaves and leaf and root biomass, were performed 30 days after transplantation of seedlings. To avaluate the incidence of Sclerotinia, the method used was the counting of living and dead plants, 30 days after transplantation. It was based on a rating scale of dead plants whose adjusted schedule was as follows (Djidji *et al.*, 2010):

- 0: healthy seedlings;
- 1: 1 to 10 % of dead seedlings;
- 2: 11 to 25 % of dead seedlings;
- 3: 26 to 50 % dead seedlings;
- 4: 51 to 75 % of dead seedlings;
- 5: 76 to 100 % of dead seedlings.

Based on these ratings, the disease index was calculated from the average ratings assigned to the 12 seedlings of each cultivar according to the following formula:

with:

$$I_S = \sum (N_i \times I_i) / N_t$$

I_S : Disease severity index,

N_i : number of seedlings class i,

I_i : index class i,
N_t : total number of seedlings

The living leaves of *Abelmoschus esculentus* seedlings were counted on each seedling per treatment and per cultivar, 30 days after transplantation. The average number of living leaves on seedlings was calculated by treatment and by cultivar on the total number of seedlings for the experiment according to the following formula:
with:

$$N_m = \sum N_i / N_t$$

N_m : Average number of living leaves per treatment and per cultivar,
N_i : Number of living leaves on each seedling per treatment and per cultivar,
N_t : total number of seedlings for the experiment.

Thirty days after transplantation, height growth of the seedlings was estimated by measurement in centimeters (cm) of the epicotyl. The measurement was performed using a measuring tape from the cotyledon leaves until the "V" formed between the last leaf not yet blooming and the penultimate fully blooming leaf. The average height was determined by treatment and cultivar over the total number of seedlings for the experiment according to the following formula:

$$H_m = \sum H_i / N_t$$

H_m : Average height per treatment and per cultivar,
H_i : Height of each seedling per treatment and per cultivar,
N_t : total number of seedlings for the experiment.

Two hours after the last watering, the seedlings were carefully uprooted and the root system excised and rinsed at the cotyledon scar. They were then weighed using an electric balance branded "Jump 1000" at 10^{-1} g precision. The average leaf and root fresh biomass was calculated by treatment and by cultivar over the total number of seedlings for the experiment according to the following formula:

with:

$$B_m = \sum B_i / N_t$$

B_m : Average fresh leaf or root biomass per treatment and cultivar,
B_i : Fresh leaf or root biomass of each seedling per treatment and cultivar,
N_t : total number of seedlings for the experiment.

Experimental design

The main factor studied was the treatment with 5 modalities (healthy control, Inoculated and untreated, Inoculated and treated with 500 ppm, Inoculated and treated with 1000 ppm, Inoculated and treated with 2000 ppm) and the secondary factor, 2-level cultivar (Volta and Hiré). The experimental unit was the okra foot. For each modality, the adopted experimental design was the Fischer block with three repetitions. Each category included two plots to which were allocated cultivars. The trial therefore comprised two plots per modality. Each plot had a cultivar represented by twelve seedlings (12 repetitions of the experimental unit). In total, the test included 24 okra seedlings per modality. The modalities of the treatment were:

- Healthy control,
- Inoculated and untreated,
- Inoculated and treated with 500 ppm,
- Inoculated and treated with 1000 ppm,
- Inoculated and treated with 2000 ppm.

The incidence of sclerotinia was assessed by measuring five variables: the percentage of mortality, the disease severity index, plant height growth and number of living leaves and the fresh leaf and root biomass.

Statistical analysis of the results

The data collected relating to parameters (yield of essential oils, inhibition of the mycelial radial growth of *Sclerotium rolfsii* and effect of *Ocimum gratissimun* essential oil on plant height growth, appearance of leaves and biomass) have suffered a

variance analysis with two classification criteria (ANOVA 2), followed by comparison of averages by the Newman-Keuls test using the Statistica 7.1 software. Differences were considered significant at the 5 % threshold.

Results

In vitro study

Essential oil yield

The results concerning the essential oil yields extracted from these seedlings are given in Table 1. All these species have a very low yield of essential oil. However, *Ocimum gratissimum*, with 0.29 %, had a yield statistically higher than that of *Hyptis suaveolens* (0.06 %).

Table 1. Essential oil yield of dry biomass of Ocimum gratissimum L. and Hyptis suaveolens (L.) Poit.

Species	Quantity of dry biomass (g)	Quantity of Essential oil (g)	Yield (%)
Ocimum gratissimum L.	800	2.32	0.29 ± 0.02 a
Hyptis suaveolens (L.) Poit	800	0.48	0.06 ± 0.00 b

In the same column the concentrations followed by the same letters are not significantly different according to the Newman-Keuls test at 5 % threshold.

Inhibition of radial mycelial growth of Sclerotium rolfsii and resumption of mycelial pellet

Fig. 1 and 2 show that the essential oils of *Ocimum gratissimum* and *Hyptis* suaveolens have fungitoxic activity on *Sclerotium rolfsii*. The inhibition of mycelial growth increased with the concentration of essential oils. The essential oils of *Ocimum gratissimum* and *Hyptis suaveolens* have proven moderately fungitoxic at 125 ppm and 500 ppm, with respective inhibition rates of 17.86 % and 9.91 at 125 ppm and respectively at 42.5 % and 34.33 at 500 ppm. These two essential oils were highly fungitoxic at 1000 ppm with inhibition rates of 73.41 and 73.5 % respectively with the essential oil of *Ocimum gratissimum* and that of *Hyptis suaveolens*. At 2000 and 4000 ppm, both essential oils showed a complete inhibition of fungus mycelial growth. Moreover, the transfer of mycelial fragments treated with these two essential oils at 2000 and 4000 ppm, in a new PDA culture medium was not followed by growth resumption.

Table 2. Concentrations of essential oils of *Ocimum gratissimum* and *Hyptis suaveolens* reducing by 50 (IC_{50}) and 90 % (IC_{90}) the mycelial growth of *Sclerotium rolfsii* at the 7th day of incubation.

	Inhibiting	Concentration (ppm)
Essential oils	IC_{50}	IC_{90}
Ocimum gratissimum	825 b	1093 a
Hyptis suaveolens	828 b	1095 a

In the same column the concentrations followed by the same lettters are not significantly different according to the Newman-Keuls test at 5 % threshold.

On the 7th day of measurement, the concentration of essential oil of *Ocimum gratissimum*, which inhibits 50 % of mycelial growth (IC_{50}), was 825 ppm and the one that inhibits 90 % of mycelial growth (IC_{90}) was 1093 ppm (Table 2). However with the essential oil of *Hyptis suaveolens*, IC_{50} and IC_{90}, on the 7th day, were respectively 828 and 1095 ppm.

In vivo study

Description and evolution of Sclerotium rolfsii symptoms

Transplantation was carried out with 15-days old nursery seedlings. After two weeks, concentric necrotic spots (like burns) on the end leaves of the plant were observed. After the onset of these early

symptoms, we observed a convergence of spots and the fall of all the leaves of the plant which resulted in a browning of the terminal bud after infection. This browning ended up in extending to the entire stem and was followed by the melting of the plant. Such plants contaminated with fungus eventually collapsed and died. On the dead plant, we observed a brown alteration at the neck. We also found that apart from these symptoms, stunted height growth was observed in plants inoculated and untreated and in those inoculated and treated compared to healthy controls (no symptoms of sclerotinia) in both okra cultivars studied.

Table 3. Effect of the essential oil of *Ocimum gratissimum* L. on the incidence of the disease at 30 days after transplantation.

Treatments	Mortality index		
	Concentrations of the essential oil in ppm	Cultivars	
		Volta	Hiré
Healthy witness		0.00 (0.00) e	0.00 (0.00) d
Inoculated and untreated		4.17 (83.33) a	0.33 (16.67) a
Inoculated and treated	500	2.17 (54.17) b	0.08 (8.33) b
	1000	1.50 (50.00) c	0.08 (8.33) b
	2000	0.33 (16.67) d	0.04 (4.17) c

Effect of the essential oil of Ocimum gratissimum on the incidence of the disease

Table 3 shows the effect of the essential oil of *Ocimum gratissimum* on the incidence of sclerotinia in okra cultivars Volta and Hiré. Thirty days after planting okra seedlings on the soil substrate inoculated with *Sclerotium rolfsii* and treated with different concentrations of the essential oil of *O. gratissimum*, we observed a reduction in attacks compared to seedlings planted in the inoculated and untreated soil substrate. Thus, the two okra cultivars undergoing treatment with essential oil showed a significant decrease in mortality as a result of increasing concentrations. For each of the two cultivars, significant differences were observed between the different treatments with the essential oil except for 500 ppm and 1000 ppm treatments whose mortality was similar in cultivar Hiré. A high concentration of 2000 ppm, the mortality index respectively switched from 4.17 (inoculated and untreated) to 0.33 (inoculated and treated) in cultivar Volta and from 0.33 to 0.04 in cultivar Hiré. The cultivar Volta presented the highest sensitivity to sclerotinia with 83.33 % mortality against 16.67 % in cultivar Hiré.

Effect of the essential oil of Ocimum gratissimum on seedlings height growth, leaf emission and biomass

Height growth of the inoculated and untreated seedlings was slowed down, by 12.31 and 29.04 cm respectively in cultivars Volta and Hiré compared to the healthy control (respectively 36.40 cm and 39.52 cm), 30 days after transplantation (Table 4). Infected plants subjected to treatment with the essential oil of *O. gratissimum*, recorded a less severe reduction of their height growth. Thus, at the concentration of 2000 ppm, the cultivars Volta and Hiré, with respectively 28.81 and 33.63 cm, showed significant heights compared to the untreated inoculated seedlings in both cultivars (12.31 cm in Volta and 29.04 cm in Hiré). A significant difference was observed between the different treatments with essential oil in each cultivar except for treatments at 500 ppm and 1000 ppm in cultivar Hiré where seedlings heights were statistically similar. Cultivar Volta was more sensitive to the disease by a reduction in height growth.

Effect of treatment with essential oil on leaves emission

Table 4 shows the average number of living leaves. The treatments had no effect on foliar emission in

cultivar Hiré. The number of living leaves in the healthy control (3.96 leaves) and in inoculated and untreated seedlings did not differ statistically from that of the seedlings inoculated and treated with the essential oil of *O. gratissimum* (3.75; 3.50 and 3.92 leaves respectively for concentrations 500, 1000 and 2000 ppm). However, in cultivar Volta, the infection with *S. rolfsii* generated a decrease in the average number of living leaves to 1.42 compared to the healthy control (4.50). A clear improvement of the emission of the number of leaves was observed at concentrations of 500, 1000 and 2000 ppm of the essential oil with respectively 2.63; 2.42 and 3.25 compared to the inoculated and untreated control (1.42). Cultivar Volta presented greater sensitivity to *S. rolfsii* at leaf emission.

Table 4. Effect of the essential oil of *Ocimum gratissium* on plant height growth, leaves emission and leaf and root fresh biomass, 30 days after transplantation.

Parameters	Cultivars	Treatments				
		Healthy control	Inoculated and untreated	Inoculated and treated with the essential oil (Concentrations in ppm)		
				500	1000	2000
Height of the epicotyl (cm)	Volta	36.40 ± 1.37 a	12.31 ± 3.37 c	14.65 ± 3.43 c	19.05 ± 4.11 b	28.81 ± 2.86 ab
	Hiré	39.52 ± 2.12 a	29.04 ± 2.15 b	29.28 ± 3.10 b	29.35 ± 2.70 b	33.63 ± 2.92 a
Number of living leaves	Volta	4.50 ± 0.27 a	1.42 ± 0.40 c	2.63 ± 0.64 b	2.42 ± 0.53 b	3.25 ± 0.47 ab
	Hiré	3.96 ± 0.30 a	3.46 ± 0.36 a	3.75 ± 0.41 a	3.50 ± 0.31 a	3.92 ± 0.37 a
Fresh leaf biomass (g)	Volta	26.96 ± 1.71 b	16.91 ± 3.18 c	14.85 ± 3.99 c	20.03 ± 6.21ab	32.28 ± 4.62 a
	Hiré	33.57 ± 2.30 b	24.66 ± 1.36 c	26.47 ± 3.07 c	35.22 ± 1.76 a	37.24 ± 2.88 a
Fresh root Biomass (g)	Volta	6.26 ± 0.48 b	3.01 ± 0.60 b	2.03 ± 0.55 b	3.46 ± 1.06 b	9.33 ± 1.36 a
	Hiré	7.78 ± 0.36 b	4.03 ± 0.23 c	4.00 ± 0.60 c	9.65 ± 0.59 a	10.8 ± 0.92 a

Effect of essential oil on the leaf and root fresh biomass

Table 4 shows the leaf and root fresh biomass of cultivars Hiré and Volta. The biomass produced varied according to cultivars and treatments. At the concentration of 2000 ppm, the essential oil of *O. gratissimum* applied to the inoculated seedlings significantly induced an increase in leaf fresh biomass of cultivars Volta and Hiré with respectively 32.28 and 37.28 g. Their root biomass was also improved with 9.33 g for Volta and 10.8 g for Hiré compared to healthy controls and inoculated and untreated seedlings. Inoculated and untreated seedlings for cultivars Volta and Hiré respectively produced a fresh leaf (16.91 g and 24.66 g) and root (3.01 g and 4.03 g) biomass significantly reduced compared with healthy controls (26.96 g and 33.57 g for leaves; 6.26 g and 7.78 g for roots). The leaf and root fresh biomass shows that a plant may not show visual symptoms and be affected.

Discussion

Essential oil yield

The results of this study show that the plant species *Ocimum gratissimum* and *Hyptis suaveolens* have a very low yield in essential oil. The best yield was obtained with the leaves of *Ocimum gratissimum* (0.29 %). The low essential oil content could probably be due to the age of the plants, to the period and place of harvest, the organ used without neglecting the very nature of these aromatic plants. Similar results were obtained by Tonzibo *et al.* (2009) who showed that the yield in essential oil of *H. suaveolens* ranged between 0.04 and 0.12 % depending on the origin of the plant and the organ used. Similarly, Merghache *et al.* (2009) who worked on the essential oil yield *Ruta chalepensis* L., have shown that the variation of the yield depended on the portion of the plant used, the time and place of plant harvesting.

These authors also showed that essential oil was two (2) times concentrated in the flowers than in the leaves when using the same quantities of fresh material. Furthermore, Fellah *et al.* (2006) after a comparative study on essential oil yield of the aerial part of *Salvia officinalis* L., collected from two sites in Tunisia, have reported that the yield differed depending on the place of seedlings harvesting.

The largest amount of essential oil was obtained on the 3rd day of drying of the leaves of both plants (*O. gratissimum* and *H. suaveolens*). This drying time is consistent with the works of Camara (2009), who showed that beyond 3 days of drying the leaves of *O. gratissimum*, essential oil quantities decreased, probably due to evaporation. However, Bourkhiss *et al.* (2009) found that the drying in the shade and sun of adult leaves of *Tetraclinis articulata* (Vahl) increased the concentration in essential oils. For these authors, when the leaves were exposed to direct sunlight, the maximum content was obtained on the seventh day (that is 0.76 %), while the phenomenon kept on until the ninth day of drying in the shade, that is 0.81 %. The content dropped steadily before stabilizing, beyond these periods. The optimum drying time would range between 6 and 9 days and the increase of the essential oil concentration expressed over the weight of dry matter during the first days of drying would be explained by an important physiological activity (enzymatic reactions). Indeed according to Bourkhiss *et al.* (2009) the biosynthesis of essential oils continues and accelerates after the harvest of plant material in response to water stress. Its decrease after the drying time would be due to the reduction or discontinuation of enzyme activity causing cell death due to severe dehydration.

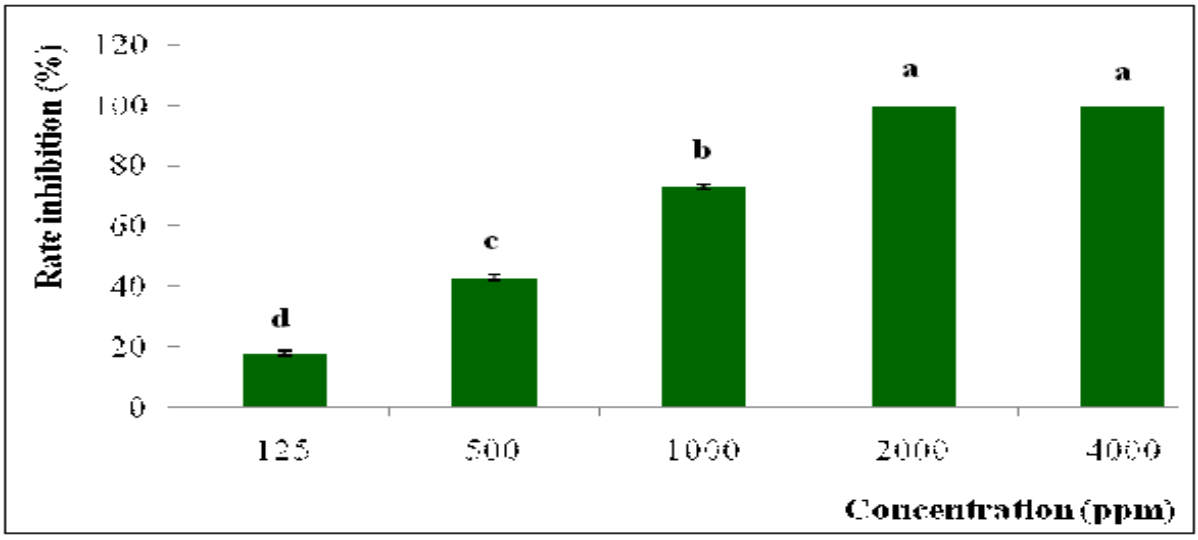

Fig. 1. Inhibition of the radial mycelial growth of *Sclerotium rolfsii* Sacc. with the essential oil of *Ocimum gratissimum* L.

Histograms affected by the same letter indicate that there is no significant difference between the rates of reduction of mycelial growth at concentrations tested (Newman-Keuls at 5 % threshold).

Inhibition of the radial mycelial growth of Sclerotium rolfsii and resumption of the mycelial pellet

The *in vitro* inhibitory effect of essential oils of *Ocimum gratissimum* and *Hyptis suaveolens* proved efficient on the telluric fungal strain of *S. rolfsii*. Indeed, the radial mycelial growth of the fungus was inhibited by the essential oil of *O. gratissimum* as well as by that of *H. suaveolens*. The positive correlation observed between the inhibition rates and the different concentrations for each essential oil demonstrates the inhibitory action (antifungal) of the latter on the fungus. The maximum inhibition threshold (100 %) was reached with the minimum inhibitory concentrations at 2000 ppm for both essential oils. Thus, once incorporated into the culture medium, these essential oils block the development of the fungal colonies. This would be in relation with their compounds which act to neutralize all the mechanisms and processes involved in the fungus cell multiplication. The complete inhibition of the radial mycelial growth of the fungus at certain doses after transfer on PDA medium expressed a fungicidal activity of the essential oils tested.

The excellent efficiency of the essential oil of *Ocimum gratissimum*, even at the smallest concentrations, might be due to the activity of its two main compounds that are thymol and gamma-terpinene. Indeed, Shapiro and Guggenheim (1994), showed the strong inhibitory activity of thymol on the way of ATP generation (adenosine triphosphate). Therefore, its effect on the reduction of the membrane potential and impact intracellular pH on the potassium flux (intra and extracellular) thus damaging the cytoplasmic membrane. The high phenolic compound content (thymol) associated with monoterpene gamma-terpinene, known for its effect on biological membranes (Yoshimura *et al.*, 2010), would explain the fungitoxicity of the essential oil of *Ocimum gratissimum* even at minimal doses. These results confirm those of Khanna *et al.*, (1991) who showed that the extracts of *Ocimum gratissimum* completely inhibited the growth of *Sclerotium rolfsii* at concentrations ranging between 50 and 500 ppm and those of Doumbouya *et al.*, (2012) that have also shown that at the concentration of 100 ppm, the essential oil of *O. gratissimum* strongly inhibited the mycelial growth of *F. oxysporum* f. sp. *radicis lycopersici* and that of *Pythium* sp.

Effect of the essential oil of Ocimum gratissimum on the incidence of sclerotinia

The essential oil of *O. gratissimum* brought in amendments to the growing substrate of okra seedlings, proved efficient in treating sclerotinia. The efficiency revealed in vitro with this essential oil of *O. gratissimum* was observed in vivo. Thus, the transplantation of the seedlings of both okra cultivars (Hiré and Volta) in the soil mixture inoculated with *S. rolfsii* and treated with the essential oil of *O. gratissimum* at three (3) concentrations (500, 1000 and 2000 ppm) generated a low attack of the latter. For the seedlings thus treated, the mortality index was lower with the increase in essential oil concentration in both cultivars and the seedlings of the healthy control (not inoculated and untreated) of both cultivars showed no characteristic sign of sclerotinia during the different observations and no mortality was noted. This confirms the virulence of the fungal strain and the effect of the essential oil of *O. gratissimum* on the reduction of such virulence. Thus, the state of transplanted crops overall (essential oil-mycopathogen-soil) compared to that of the healthy control (not inoculated and untreated) of both cultivars, showed that the seedlings treated with the essential oil of *O. gratissimum* have a more or less similar crop development.

The amount of inoculum brought to seedlings of the two (2) cultivars being the same, cultivar Volta proved more sensitive to *S. rolfsii* and cultivar Hiré with lower mortality index would be less sensitive oil of *O. gratissimum* would then be explained by an activation of the plant defense system to it.

Thus, the severity of Sclerotinia observed in cultivar Volta could therefore be explained by its sensitivity or the aggressiveness of *S. rolfsii*. The strong sensitivity of cultivar Volta vis-à-vis *S. rolfsii* would probably be related to its genome. Indeed, according to Abo *et al.* (2005), the majority of improved plants concerning productivity genes would be more sensitive to pathogens. Less sensitive plants would have a fairly efficient resistance or defense mechanism to inhibit the pathogenesis of fungal strain. The resistance of plants treated with the essential, an increase in chitinase and peroxidase activity and an increase in enzyme activity in leaves inducing thus a systemic resistance in these plants (Colpas *et al.*, 2009). Drysdale (1982) showed that the polyphenol oxidases and peroxidases involved in plant defense would be destroyed by toxins secreted by the fungus. The essential oil of *O. gratissimum* would inhibit the activity of these toxins.

Effect of the essential oil of Ocimum gratissimum on height growth, leaf emission and foliar and root biomass

The efficiency on biomass by treatments with amendment of the essential oil of *O. gratissimum* at the concentration of 2000 ppm would be explained by better protection and health of the root system of okra seedlings. Indeed, the roots of seedlings on substrate amended by essential oil at this concentration would

react by activating the plant defense system especially by secretion of secondary metabolites and increase in chitinase and peroxidase activity (Colpas *et al.* 2009). The essential oil of *O. gratissimum* would induce a reduction in virulence of *S. rolfsii* by inhibiting or delaying the germination of spores. This treatment would cause then a better development of the root system and a good regulation of plant growth factors. The essential oil of *O. gratissimum* has thus proved to have antifungal properties. This beneficial effect was obtained in the presence of the pathogen at the different concentrations of the essential oil. Similar results were obtained by Awuah (1994), who reported that the application of the essential oil of *O. gratissimum* in vivo enabled the control of *Phytophthora palmivora*, a fungal agent of cocoa. Doumbouya *et al.* (2012) also showed that the essential oil of *O. gratissimum* in curative treatment of tomato seedlings generally proves efficient on *F. oxysporum* f. sp. *radicis lycopersici* and *Pythium* sp. The efficiency of this essential oil against black sigatoka in banana in Côte d'Ivoire was also proven by Camara (2011).

Conclusion

This study enabled to identify the essential oil yield of two plant species of the ivorian flora (*O. gratissimum* and *H. suaveolens*). The study showed that the essential oil content is dependent on the plant species used. The best yield (0.29%) is obtained with *O. gratissimum*, 72 h after the drying of leaves.

In vitro and *in vivo* studies have shown that essential oils of *Ocimum gratissimum* and *Hyptis suaveolens* reveal a significant antifungal potency against *S. rolfsii*. The two essential oils inhibited in vitro the radial mycelial growth of *S. rolfsii* at the concentration of 2000 ppm. The essential oil of *O. gratissimum* brought in amendment to okra seedlings of the two culivars (Volta and Hiré) inoculated with *S. rolfsii* significantly reduced sclerotinia. These results show an interest to the use of these two essential oils for phytosanitary applications as an alternative to chemical control against *S. rolfsii*. Cultivar Volta proved more sensitive to *S. rolfsii* compared to cultivar Hiré. Cultivar Hiré would therefore be appropriate on soils having a history linked to this telluric mycopathogen and can be recommended to farmers in order to maximize okra crops yields on these soils.

Acknowledgement

This work was carried out at the Laboratory of Plant Physiology at the University Felix Houphouet-Boigny in Abidjan with outside support including the National Agricultural Research Centre (CNRA). The study which was conducted falls within the general framework of the search for alternative control methods against fungal pathogens of banana and vegetable crops other than the use of fungicide syntheses.

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