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Essential oils as control agents against *Fusarium oxysporum* f. sp. *lycopersici* in *Lycopersicon esculentum* used under *in vitro* and *in vivo* conditions

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ABSTRACT

Tomato (*Lycopersicon esculentum*) is one of the world's cash crops particularly in Cameroon. However, in Cameroon, yield declines are caused by the attack of *Fusarium oxysporum* f. sp. *lycopersici*. In this study, four essential oil extracts including *Cupressus sempervirens*, *Chenopodium ambrosioides*, *Azadirachta indica* and *Capsicum annuum* leaves were evaluated for their suppression ability on this pathogen, under *in vitro* and *in vivo* conditions. *In vitro* tests of the antifungal activity of essential oils at different concentrations showed inhibition effects on the growth of *F. oxysporum* f. sp. *lycopersici* in different rates. The essential oils of *C. sempervirens* and *C. ambrosioides* were the most effective at the concentration of 20 µL/mL whereas *A. indica* was also effective only at the concentration of 100 µL/mL. The most *in vitro* effective essential oils (*C. sempervirens*, *C. ambrosioides* and *A. indica* oil) were then included in an *in vivo* assay to evaluate their suppression activities on the pathogen growth on tomato seedlings. The essential oils were applied to tomato seedlings as two groups; i) preventively; ii) curatively. The preventively treated plants showed an influence of the essential oils on the slowing of the development of disease with a rate of reduced disease leaves and the stem growth, in comparison to the control plants. In the curatively treated group of plants, there was almost an ineffective action of essential oils on both stem growth and disease progression. Consequently, the essential oil obtained from *C. ambrosioides* was most effective *in vivo* at a low concentration (20 µL/mL) against *F. oxysporum* f. sp. *lycopersici*.

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INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops in the world (Mfombep et

al., 2016; María et al., 2019). In Africa, it is the vegetable species most cultivated because it is part of many diets.

This vegetable plays an antioxidant role in human nutrition and the prevention of certain cancers (Nguyen and Schwartz, 1998; Giovannucci, 1999). Its economic importance as well as the availability of important genomic and genetic resources for this plant make it a model for the study of *Solanaceae* and for studies on the development of fleshy fruit (Stevens et al., 2007). In Cameroon, vegetable trade is increasing due to rising urban demand and growing importance of inter-regional markets with neighbouring countries such as Gabon, Equatorial Guinea, Republic of Congo, Chad, Central African Republic and Nigeria (Achancho, 2013). However, tomato production is still lower than consumers demand due to parasitic attacks.

Tomato growers face significant pressures from bio-aggressors in tomato plantations. Damages as well as the negative economic impacts on tomato production are leading to some producers abandoning production or using intensive insecticides on their plantations. Although pesticide applications are recommended, farmers face obstacles in the fight against these pests, thus being unable to reduce their losses (Deguine and Ferron, 2008). In Cameroon, despite numerous genetic improvements in the development of tomato varieties resistant to *Fusarium oxysporum* f. sp. *lycopersici*, tomato is still susceptible to *F. oxysporum* wilt diseases.

The disease would cause great losses in tomato production globally, but especially in warmer climates (Blancard, 1994), with symptoms such as progressive chlorosis in part or all of the plant, stunting and wilting that often become observable at the maturity of the plant after flowering and initiation of fruit is set. *Fusarium* wilt losses in tomato can be severe, sometimes leading to complete loss of green house and field yield (Benhamou et al., 1989).

Fusarium oxysporum f. sp. *lycopersici* is a pathogenic fungus that infects hundreds of plant species (Dilay et al., 2018). *F. oxysporum* f. sp. *lycopersici* is widely distributed in nature and in various environments, causing huge yield losses in plants. It is mainly found in the tropical sub-regions of the world and can cause diseases such as *Fusarium* wilt (Glenn et al., 2008). *Fusarium* wilt can remain viable in the soil for up to 30 years and cause destruction of plants (Thangavelu et al., 2003). The fungus multiplies abundantly throughout much of the root tissue, causing severe damage, including cellular disruption through root-colonizing *Fusarium* hyphae and cell wall alteration leading to plant chitinase production (Fisher et al., 2012; Bing et al., 2019).

After infection of the plant, leaves, twigs or even the whole plant become brown, dry out and die later (Khan et

al., 2002). The death of the plant is caused by the failure of infected xylem to meet the water requirements of the plant (Burgess and Dawson, 2008).

Fusarium wilt is manifested by the development of yellowing of the lower leaves on plants and the defoliation of older leaves. Affected leaves wither and die and the fungus progresses on the stem until the foliage is killed and the stem disintegrates by vessels occlusion (Lund et al., 1998; Burgess and Dawson, 2008). *Fusarium* is one of the most serious diseases affecting the yield of tomatoes and its loss due to *Fusarium* wilt is of the order of 47.94%. *F. oxysporum* f. sp. *lycopersici*, like all phytopathogenic fungi, represents a threat to agriculture since antiquity (Doehlemann et al., 2017; Gautam et al., 2019). However, the use of chemical fungicides, although widely used, is expensive, polluting, presents a risk of toxicity to the planter and therefore does not constitute a crop management process for sustainable development (Heydari, 2007; Bisen, 2014; Keswani et al., 2014). *Fusarium* affects yields also the sanitary quality of the crop. This fungal problem causes significant damage and yield losses of up to 89% (Ma et al., 2012; Talas et al., 2012). The economic damage of the disease is attributed to the considerable yield losses and the deterioration of tomato quality (Lacroix, 2008; Lori et al., 2009). Therefore, it is important to find new strategies to control such fungal plant pathogens (Dita et al., 2018; Gautam et al., 2019).

Beyond curative treatments through the use of pesticides, there is a method of preventive control based on the search for harmful agents. The method consists of using essential oils as insecticides in Cameroon (Ngamo et al., 2007). Njan Nlôga et al. (2007) have proven the essential oils of *Laggera pterodonta* and *Ocimum canum* as an insecticide against *Anopheles gambiae*. These oils from plants are known for their defense properties against insects and fungi (Tatsadjieu et al., 2007; Adjou et al., 2013). In this context, essential oils used to control the growth and proliferation of fungi could become alternatives to synthetic residual chemicals. The plants that produce these oils are readily available, easily biodegradable and relatively less toxic.

The present work aims to contribute to the improvement of tomato tolerance against the attack of *F. oxysporum* f. sp. *lycopersici* through the use of essential oils of *Azadirachta indica* (Neem), *Chenopodium ambrosioides* (Ambrosine), *Cupressus sempervirens* (cypress) and *Capsicum annuum* (Chilli pepper). The results revealed the best concentrations of the most effective essential oils which significantly inhibit the growth of the *F. oxysporum* f. sp. *lycopersici* strain in petri dishes and verified the effectiveness of these oils by comparing them with a chemical fungicide on tomato seedlings against the attack of the pathogen in order to set up a sustainable and effective control technique.

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MATERIALS AND METHODS

Ecological conditions

The trial was conducted at the Research Institute for Agricultural Development (IRAD) in Nkolbisson, Yaoundé-Cameroon (3° 51' North and 11° 40' East, 759 m above sea level) in the Phytopathology Laboratory and in a greenhouse. The average annual temperature is 24.7°C. The relative humidity varies between 50-80% in the dry season and 70-90% in the rainy season. The soils are acidic and belong to the Acrisol group according to the global referential base of soil classification (Kaho et al., 2011).

Plant material and pathogen isolate

The plant material consisted of tomato seeds of the variety Royal Master (vigorous plant with high productivity; good flavor with good fruit size and shape; uniformity throughout the plant), purchased from a private company selling phytosanitary products. The pathogen isolate of *F. oxysporum* f. sp. *lycopersici* was obtained from tomato leaves at the Phytopathology Laboratory of IRAD Nkolbisson.

Methods

Isolation of the fungus

The pathogen isolate was obtained from leaf fragments exhibiting the characteristic symptoms of the disease *Fusarium* wilt. The isolation was performed under aseptic conditions where fragments of diseased leaves were cut into small fragments separately using a sterile scalpel. Subsequently, they were disinfected by soaking in 5% water-bleach for 5 min and rinsed in distilled water for 5 min and then dried on sterile blotting paper. These fragments were deposited in petri dishes, containing the PDA medium. The dishes were incubated at a temperature of 28°C for 4 to 7 days (Bouakaz and Oussaid, 2013).

Purification and microscopic identification of the fungus

The purification was carried out by transfer of the typical colonies grown in the PDA dishes. This culture medium considered as a favourable medium for the development (rapid development) of fungi, as well as for the production of spores (Botton et al., 1990). The incubation was carried out at a temperature of 28°C for 4 to 6 days. This method was repeated until pure colonies were obtained. Using a microscope connected to a computer, we observed characteristic structures of the fungus *F.*

oxysporum f. sp. *lycopersici*.

Extraction of essential oils

Extraction of *A. indica* oil

Before extraction, the seeds were cleaned manually, all the leaves and dirt were removed by winnowing and the seeds were heated to 55-60°C so that all moisture can be removed. After the preparation steps of the seeds were completed, the seeds were crushed to extract the oil. Extraction was carried out using the Soxhlet extractor (coupled with the heating mantle) and extraction solvents.

Extraction of *C. sempervirens* oil

Leaf samples of *C. sempervirens* were obtained in the field. The branches were cut into small pieces (100 g) and distilled by vapours for 3 h in a device called Clevenger. Then the oil was collected and the weight of fresh mass of the sample was measured (3.15 and 4.70 mL/100 g of fresh weight). The oil was kept dry in sealed Eppendorf tubes and stored at 4°C.

Extraction of *C. ambrosioides* oil

The extraction of the essential oils was carried out by hydrodistillation of 100 g of the dry vegetable matter in 1.5 L of distilled water at 100°C in a Clevenger type essencier (Clevenger, 1928). The distillation lasts three hours after recovery of the first drop of distillate. The essential oils were dried over anhydrous sodium sulphate and stored at 4°C in the dark. The yield of each essential oil was expressed in relation to its dry matter.

In vitro Antifungal tests

Antifungal tests in petri dishes

The realization of these antifungal tests between the essential oils and the fungus *F. oxysporum* f. sp. *lycopersici* required the use of the technique of amendment of the medium by the different oils. This method of amendment consists in introducing a defined volume of oil into the medium ready to be poured, homogenized and poured directly into the petri dishes. The medium solidifies by enclosing the volume of oil. The different volumes were taken using a micropipette. Two different concentrations were used for the different oil (5 and 10 µL/mL).

Greenhouse tests

Field preparation

The field was weeded and levelled and then covered by a transparent tarpaulin to let in the light and prevent the growth of grass. The field was fenced with mosquito nets to reduce the sabotage caused by animals and insects. Before the introduction of our seeds in greenhouse they are first passed by the germoir built near the weed field. The experiment was conducted in the fields to evaluate the impact of the antifungal activity of essential oils on the growth of *F. oxysporum* f. sp. *lycopersici* in tomato.

Preparation of pots and greenhouse

The pots of 5 L were used for planting and growing tomato seedlings. These pots were drilled with a heated diameter iron. Then the pots were filled with about 80% soil and 20% chicken droppings. The pots formed were left at rest for at least a week to allow the droppings to mineralize well. Before placing the pots in the greenhouse, it was cleaned and disinfected by sweeping and spraying with a chemical antifungal solution. This operation aimed to eliminate any other pathogen attack.

Sowing

The seeds were first sown in a sprouter. The sprouter was made of soil and droppings and seeds. The seeds were kept 3 to 4 weeks in the germoir before putting in greenhouse. In the greenhouse the batches were separated into three groups and each group contained all the batches of plants. Groups 2 and 3 served as rehearsals to check the first results. The seedlings were transplanted into single lines 1.5 m apart, with a spacing of 0.4 m in the greenhouse.

Maintenance of the greenhouse and transplanting seedlings

At the beginning, watering was done at the base of the tomato plant, then at the level of the leaves. The watering of the leaves was done every two days to promote the development of the pathogen. Transplanting consisted of removing the seedling from where it was grown for planting at a definitive location.

Tomato treatments

Preventive treatments

The specificity of this type of treatment is that the plants

received their treatments before the inoculation of the fungus, specifically two treatments for two weeks before inoculation (one treatment per week). After 3 weeks in the shade, the tomato plants were transplanted into the pots (two plants per pot). Each pot was assigned a particular treatment. Eight treatments were performed namely; the control (T0); *Cupressus* oil treatments at 10 and 20 $\mu\text{L/mL}$ (T1 and T2); treatments with *Chenopodium* oil at 10 and 20 $\mu\text{L/mL}$ (T3 and T4); *Azadirachta* oil treatments at 40 and 50 $\mu\text{L/mL}$ (T5 and T6); and chemical fungicide (T7) treatment.

Curative treatments

This process consisted of applying the treatments to the plants only after inoculation of the fungus. The plants were treated once every two weeks. The treatments used are identical to the treatments used during the preventive process at the same concentrations.

Plants inoculation with the pathogen

Preparation of the inoculum

The fungus strains were subcultured in many petri dishes at least 7 days in advance and were then stored in the shade and at room temperature. Quantification of the inoculum was performed using a microscope connected to a computer and the malassez cell counting method cell.

Inoculation of plants and experimental design

The petri dishes containing the inoculum were collected, the mycelium was withdrawn and their culture medium was inserted in a crushing machine. Finally, a paste containing *Fusarium* conidia was obtained. The tomato seedlings from three weeks-old were removed from the germoir and rinsed with sterile distilled water. Then, the root system of the seedlings was cut to 7 mm below with the scalpel (Si Mohammed et al., 2016). Pots were made in the pots prepared at least a week beforehand and then two spatulas of inoculum paste were introduced into each of these pockets followed by transplanting seedlings. Both spatulas were used to support the seedling during its growth.

The test was conducted according to a block device with total randomization to a factor organized into treatments, T0: control; T1: Cup at 10 $\mu\text{L/mL}$; T2: Cup at 20 $\mu\text{L/mL}$; T3: Cheno at 10 $\mu\text{L/mL}$; T4: Cheno at 20 $\mu\text{L/mL}$; T5: Azad at 40 $\mu\text{L/mL}$; T6: Azad at 50 $\mu\text{L/mL}$; T7: chemical fungicide. The number of repetitions per treatment was 3, making a total of 24 pots containing 2

Figure 1. Inhibitory effect in function of essential oils and their different concentrations: **T0**, Control; **T1**, Cupressus; **T2**, Chenopodium; **T3**, Capsicum; **T4**, Azadirachta.

plants each (Djebbour and Kebala, 2017).

Parameter settings

The parameters in petri dishes were taken using a graduated rule and consisted in measuring the growth diameter of the fungus in petri dish. The parameters in the plants as length of the stem, diameter of the base, total number of leaves and number of diseased leaves were measured with a tape measure, of a grouting foot and by visual counting.

Statistical analyzes

Descriptive and quantitative analyzes were performed during this study. Descriptive analyzes allowed to graphically illustrate the results obtained, while analysis of variance (ANOVA) was to evaluate the variation in the growth diameters of the pathogen according to the parameters studied at the 5% threshold. The relationships between the different parameters were measured through linear regression. Microsoft Excel was used for descriptive statistics (histograms and linear regression), while SAS-JMP software was used to compare the growths and the inhibitory effects of essential oils depending on the treatments.

RESULTS

In vitro evaluation of the antifungal activity of essential oils

Evaluation of the diameters and inhibition percentage of essential oils

The development of diameters of *Fusarium* wilt in petri dishes in the presence of essential oils was used to evaluate their antifungal activities. The growth diameter of the fungus differs in function of treatments. Indeed, the T0 treatment (control) was significantly different ($P < 0.05$) from all other treatments. This treatment was characterized by the average of the highest diameter (6.13 cm). T1 treatments (10 µL/mL); T2 (10 µL/mL) and T4 (100 µL/mL) have the smallest diameters close to zero (0.32, 0 and 0.26 cm, respectively) (Figures 1 and 2).

A significant difference ($P < 0.05$) was also observed between treatments with essential oils. The T0 treatment forms a distinct group (group a) of the others with an average diameter greater than 6.13 ± 0.5 cm and the T2 treatment (10 µL/mL) which also forms a distinct group (group g) of the others with an average diameter zero. However, the similarities were observed between the T3 (5 µL/mL), T3 (10 µL/mL), T3 (50 µL/mL), T4 (5 µL/mL) and T4 (10 µL/mL) treatments (Figures 1 and 2). The diameters of the necrosis obtained were used to calculate

Figure 2. Different growths of mycelium in function of treatments: **A**, Control; **B**, Cup 5 $\mu\text{L/mL}$; **C**, Cup 10 $\mu\text{L/mL}$; **D**, Cheno 5 $\mu\text{L/mL}$; **E**, Cheno 10 $\mu\text{L/mL}$; **F**, Cap 5 $\mu\text{L/mL}$; **G**, Cap 50 $\mu\text{L/mL}$; **H**, Cap 100 $\mu\text{L/mL}$; **I**, Azad 5 $\mu\text{L/mL}$; **J**, Azad 50 $\mu\text{L/mL}$; **K**, Azad 100 $\mu\text{L/mL}$.

Table 1. Inhibition percentage of essential oils.

Concentrations of essential oils ($\mu\text{L/mL}$)	In percentages (%)			
	T1	T2	T3	T4
5	64.62	75.64	17.56	29.48
10	82.02	100	23.08	38.46
50	/	/	37.18	61.54
100	/	/	92.69	92.82

T1, Cupressus; T2, Chenopodium; T3, Capsicum; T4, Azadirachta.

Figure 3. Effects of essential oils on the length of treated stems during preventive conditions: **T0**, Control; **T1**, Cup 10 $\mu\text{L/mL}$; **T2**, Cup 20 $\mu\text{L/mL}$; **T3**, Cheno 10 $\mu\text{L/mL}$; **T4**, Cheno 20 $\mu\text{L/mL}$; **T5**, Azad 40 $\mu\text{L/mL}$; **T6**, Azad 50 $\mu\text{L/mL}$; **T7**, Chemical fungicide.

the inhibition rates of the different oils and their different concentrations (Table 1). Treatments 1 and 2 were most effective at low concentrations because the inhibition percentage was greater than 60% at the first concentration followed by an increase at the 2nd concentration. In the case of T3 and T4 treatments, at low concentrations, the inhibition percentage does not reach 40%. For this purpose, it was necessary to use higher concentrations to obtain a very large inhibition percentage. T2 treatment at 10 $\mu\text{L/mL}$ was most effective with maximum inhibition percentage and at the same concentration, T3 treatment was most ineffective. However, at high concentrations, the T3 and T4 treatments had a percentage of inhibition close to that of T2 at 10 $\mu\text{L/mL}$.

***In vivo* evaluation of the activity of essential oils**

Evaluation of the effects of the treatments on the length of the stem during preventive conditions

The results show a significant difference ($P < 0.05$) between treatments. These results show the influence of essential oils on stem growth of tomato plants treated preventively (Figure 3). Indeed, it is distinguished that the average stem length of the control is slightly below all other treatments except T6 treatment. However, the

plants of T6 treatment were mostly dead after wilting. The results also show a singularity in the T0 treatment (group d) which has the smallest average length of the stem if we exclude the T6 treatment group that was decimated. T2 treatment (group a) has the average of the lengths of the highest stem of all. Similarity has also been observed between the T1, T3, T4 and T5 treatments which form three groups (abc; ab and bcd) with values approaching the global average of 35 cm (Figure 3).

Evaluation of the effects of treatments on the stem length during curative conditions

The results also show a significant difference ($P < 0.05$) between treatments. The influence of essential oils on the length of the stem of tomato plants treated curatively is different from the case of preventive treatment (Figure 4). Indeed, on these plants one obtains averages of the length of the stems much smaller than those observed in the preventive treatment. The T0 treatment has the average of the smallest length, making it a separate group (group e) of the others. T2 treatment is the one with the average of the highest length and also making it a separate group (group a) of the others. However, a similarity was also observed between the T3 and T6 treatments, which form two groups (bc and abc), approaching the global average of 19 cm (Figure 4).

Figure 4. Effects of essential oils on the length of treated stems during curative conditions: **T0**, Control; **T1**, Cup 10 $\mu\text{L/mL}$; **T2**, Cup 20 $\mu\text{L/mL}$; **T3**, Cheno 10 $\mu\text{L/mL}$; **T4**, Cheno 20 $\mu\text{L/mL}$; **T5**, Azad 40 $\mu\text{L/mL}$; **T6**, Azad 50 $\mu\text{L/mL}$; **T7**, Chemical fungicide.

Figure 5. Diameter of the base of the stem and number of diseased leaves over time during preventive conditions.

Evaluation of the evolution of the diameter of the base of the stem and the number of disease leaves over time during preventive conditions

The results show the evolution of the diameter of the base of the stem and the number of disease leaves of tomato plants that have undergone preventive treatment in function of time (Figure 5). These results show the

appearance of the disease after 3 weeks and the number of disease leaves which increases with time. The diameter of the base of the stem shows a gradual increase in the average from the first week to the third. However, in the fourth week, there is a gradual decrease in the average until the sixth week. It therefore appears that as soon as the disease appears, the growth of the diameter is inversely proportional to the growth of the

Figure 6. Diameter of the base of the stem and number of diseased leaves over time during curative conditions.

Figure 7. Diameter of the base of the stem and number of diseased leaves in function of treatments during preventive conditions: **T0**, Control; **T1**, Cup 10 $\mu\text{L/mL}$; **T2**, Cup 20 $\mu\text{L/mL}$; **T3**, Cheno 10 $\mu\text{L/mL}$; **T4**, Cheno 20 $\mu\text{L/mL}$; **T5**, Azad 40 $\mu\text{L/mL}$; **T6**, Azad 50 $\mu\text{L/mL}$; **T7**, Chemical fungicide.

number of diseased leaves (Figure 5).

Evaluation of the evolution of the diameter of the base of the stem and the number of disease leaves over time during curative conditions

The presence of affected leaves observed from the first week reflects the development of the disease in tomato (Figure 6). The number of diseased leaves increases

during the first two weeks and then decreases during the third week, so that it starts to grow again during the last week. The regression in the third week coincides with the second treatment with essential oils that took place the same week. The diameter of the plants shows an average that remains substantially constant from the first week to the last (Figure 6). The average diameters of plants treated curatively were lower than those treated preventively. The average number of diseased leaves in the curative treatment was higher than that of preventive

Figure 8. Diameter of the base of the stem and number of diseased leaves in function of treatments during curative conditions: **T0**, Control; **T1**, Cup 10 $\mu\text{L/mL}$; **T2**, Cup 20 $\mu\text{L/mL}$; **T3**, Cheno 10 $\mu\text{L/mL}$; **T4**, Cheno 20 $\mu\text{L/mL}$; **T5**, Azad 40 $\mu\text{L/mL}$; **T6**, Azad 50 $\mu\text{L/mL}$; **T7**, Chemical fungicide.

treatment (Figure 6).

Evaluation of the effects of the treatments on the diameter of the base of the stem and the number of diseased leaves during preventive conditions

The results also showed a significant difference ($P < 0.05$) between treatments. The diameter of the base of the stem and the number of diseased leaves evolve differently in function of different treatments. Indeed, in the case of diameter, a low average was not observed in the T6 treatment (Azad at 50 $\mu\text{L/mL}$). Taking into account the high mortality rate of the T6 treatment plants, it can be said that the treatment with the lowest average was the T0 treatment while that with the highest average was the T5 treatment (Figure 7).

For the parameter number of diseased leaves, while taking into account the mortality rate of the T6 treatment (Figure 7), it was found that the treatment with the largest number of diseased leaves was the T0 treatment, while the T7 treatment (Chemical fungicide) was the one with the least disease leaves. Among the essential oils, it was found that the T2 treatment was the most effective against the disease with a diseased leaf rate that was half as small as that of T0 (Figure 7).

Evaluation of the effects of treatments on diameter and number of diseased leaves during curative conditions

The results also show a significant difference ($P < 0.05$)

between treatments. In curative conditions, the T5 and T6 treatments (Azadirachta at 40 and 50 $\mu\text{L/mL}$) had the highest disease leaf averages of all other treatments (even exceeding the T0 control). The treatment with the lowest average of leaves was the treatment T4 (*Chenopodium* at 20 $\mu\text{L/mL}$). The action of the T4 treatment was substantially similar to the T7 treatment (chemical fungicide). As for the other treatments, their respective averages were quite close to each other (Figure 8). The average diameter of the T0 treatment (control) was the lowest of all and was quite close to the average of the T5 treatment (*Azadirachta* at 40 $\mu\text{L/mL}$). The treatment with the largest diameter was T2 treatment (*Cupressus* at 20 $\mu\text{L/mL}$). The other treatments had average diameters quite close to each other.

General averages of diameters of plants treated curatively were lower than those of pre-treated plants. Regarding the number of diseased leaves, the overall average of plants treated curatively was higher than the average of plants treated preventively.

Evaluation of the effects of treatments on the number of leaves during preventive conditions

The results obtained show no significant differences ($P > 0.05$) between treatments apart from the T4 treatment which shows a slight difference. The number of leaves of the plants treated in preventive conditions showed a certain similarity between the different treatments, except the T0 treatment which was below the general average. However, the T6 treatment (*Azadirachta* at 50 $\mu\text{L/mL}$) was the one with the lowest average because of the very

Figure 9. Number of leaves in function of treatments of plants treated during preventive conditions: **T0**, Control; **T1**, Cup 10 $\mu\text{L/mL}$; **T2**, Cup 20 $\mu\text{L/mL}$; **T3**, Cheno 10 $\mu\text{L/mL}$; **T4**, Cheno 20 $\mu\text{L/mL}$; **T5**, Azad 40 $\mu\text{L/mL}$; **T6**, Azad 50 $\mu\text{L/mL}$; **T7**, Chemical fungicide.

large number of dead plants. A similarity was observed between the treatments T1, T2, T3, T5 and T7 marked by belonging to the group (a). The singularity of the treatments T0 and T6 was highlighted by their isolation in their respective groups; group (b) for treatment T0 and group (c) for treatment T6 (Figure 9).

Evaluation of the effects of treatments on the number of leaves during curative conditions

The results show a significant difference ($P < 0.05$) between treatments. The number of leaves of plants treated curatively was characterized by the low average leaf size of the T0 treatment (control), which was the lowest and was below the general average, forming an isolated group (group c). T2 treatment (*Cupressus* at 20 $\mu\text{L/mL}$) was the one with the highest average, thus forming a distinct group (group a). A similarity was observed between the T1 and T6 treatments which had values close to 5 sheets forming the ab group while the T3, T4, T5 and T7 treatments had values close to 4 leaves, forming the bc group (Figure 10).

However, the average overall leaf of plants treated curatively was much lower than the average overall leaf of plants treated preventively.

DISCUSSION

***In vitro* evaluation of the antifungal activity of essential oils**

Evaluation of the diameters obtained in function of different treatments

Differences in sensitivity or resistance were noted in function of different essential oils and the concentrations tested. Only T2 treatment was observed to be the most effective against the *F. oxysporum f. sp. lycopersici* strain. The control showed negative results compared to the others, this implies that the antifungal activity was due solely to the essential oils. The results obtained from the various concentrations of the essential oils revealed that the inhibitory activity of each essential oil increases as the concentration increases. These results corroborate those of Salhi et al. (2015) and Hanana et al. (2014) who showed the inhibitory effect of essential oils on the growth of mushrooms in petri dishes, during their work respectively on evaluation of the *in vitro* antifungal activity of essential oils of *Laurus nobilis* on the growth of *Fusarium sporotrichoide* and on the study of the biological activities of the essential oils of pines on several strains of *Fusarium*.

Figure 10. Number of leaves in function of treatments of plants treated during curative conditions: **T0**, Control; **T1**, Cup 10 $\mu\text{L/mL}$; **T2**, Cup 20 $\mu\text{L/mL}$; **T3**, Cheno 10 $\mu\text{L/mL}$; **T4**, Cheno 20 $\mu\text{L/mL}$; **T5**, Azad 40 $\mu\text{L/mL}$; **T6**, Azad 50 $\mu\text{L/mL}$; **T7**, Chemical fungicide.

Evaluation of the inhibition percentage of essential oils

The percentage of inhibition varied in function of oils and concentrations tested. The maximum inhibition percentage was observed at T2 treatment at 10 $\mu\text{L/mL}$. Treatments T1 and T2 at two different concentrations showed high percentages of inhibition above 50%, which was not the case for treatments T3 and T4 at the same concentrations. To obtain results similar to those obtained with T1 and T2 treatments, the concentrations of the essential oils of treatments T3 and T4 were readjusted to 50 and 100 $\mu\text{L/mL}$. These results support the results of petrel diameter measurements of those of Hanana et al. (2014) and Amri et al. (2014) who found similar inhibition percentages at the same concentrations during their work on the antifungal properties of *Biota orientalis* essential oils against several strains of *F. oxysporum* f. sp. *lycopersici*.

However, these results differ from those of Si Mohammed et al. (2016) who obtained the percentages of the inhibition of *F. oxysporum* f. sp. *lycopersici* fungus growth on tomato plants with *Juniperus phoenicea* essential oil. Indeed, the quantities of essential oils used were less important to obtain the same results as those obtained by Si Mohammed et al. (2016) who used large

quantities of essential oils that were not applicable by farmers.

In vivo evaluation of the activity of essential oils

Evaluation of the effects of treatments on the length of the stem

In the case of tomato plants treated preventively, an overall effect of the essential oils on the growth in tomato stem length was observed, except for the T6 treatment whose plants died before the end of the setting of the parameters. Apart from the T6 treatment, the T0 treatment was the only treatment where the average length of the stems was below the overall average. This result tends to prove that essential oils have an effect on the growth in length of tomato stems. However, in the curative method, the results showed that essential oils were less effective since the overall average of stem length was lower than the overall average stem length of plants treated preventively. These results show that the preventive treatment with essential oils allows a better growth in length of the tomato stems and confirms the work of Hanana et al. (2014) who showed that essential oils acted on the aerial parts of plants indiscriminately

during the study of pine essential oils on three plant species *Sinapis arvensis*, (wild mustard) *Phalaris canariensis* (canary grass) and *Triticum turgidum* (durum wheat).

Evaluation of the activity of essential oils on the leafing

Tomato plants treated preventively showed great similarity in leafing. Only the T6 treatment was below average with a large number of dead plants. The curatively treated tomato plants showed a difference in sensitivity depending on the different treatments. T2 treatment was most effective with very high leaf average. Other treatments except T0 had more or less similar effects. Only the T0 treatment was found to be well below the overall average and therefore the least effective. These results confirm that the essential oils had a positive effect on the flowering of tomato plants after fusarium infection. These results confirm those of Hassan II and Rabat (2014) who identified an action of essential oils on the flowering of wheat plants during the study of the preventive control of *Helminthosporian* blem spot by the essential oils of *Thymus satureioides* and *Origanum compactum*.

Evaluation of the antifungal activity of essential oils under in vivo conditions

The susceptibility of tomato plants to disease, differs in function of treatments. In the case of preventive treatment, essential oils have had a positive effect in the fight against *Fusarium*. These results are similar to those obtained by Sharma et al. (2017) who demonstrated that essential oils could be used as biofungicide for the control of *F. oxysporum* f. sp. *lycopersici* in preventive conditions. These results are also similar to those obtained by Laila et al. (2018) who showed that antagonistic bio-agents are important source of compounds that are effective against some fungi and could be good replacements of fungicides. Indeed, the T0 treatment that is the control has the high number of diseases and proves that the essential oils by their antifungal properties have helped the plants in the fight against the disease. Among the other treatments, while taking into account the mortality rate of the T6 treatment plants, the most effective treatment was the T4 treatment (Cheno 20 µL/mL). The T4 treatment activity was superior to the T7 treatment (chemical fungicide) because the average of T4 treatment diseased leaves was much lower than that of the T7 treatment. One of the factors that influenced the antifungal activity of essential oils was concentration. Indeed, in the case of treatments T1 (Cup 10 µL/mL) and T2 (Cup 20 µL/mL), the

antifungal effect was better for the highest concentration. This remark was also observed for T3 treatments (Cheno 10 µL/mL) and T4 (Cheno 20 µL/mL), where the highest concentration was also the most effective. In the case of plants treated curatively, the overall average of diseased leaves was much higher than overall average of diseased leaves of the preventive case. The effect of the essential oils was totally different from the first case because the least effective treatments were the treatments T5 (Azad 40 µL/mL) and T6 (Azad 50 µL/mL) which had an average of disease leaves greater than that T0 treatment (control). The most effective treatment was T4 treatment (Cheno 20 µL/mL). In this healing case, the T4 treatment was more effective than the T7 treatment (chemical fungicide). Concentration was also a determining factor because for each essential oil, the highest concentration was more effective than the lowest concentration. These results corroborate the results of those observed by Si Mohammed et al. (2016), who proved the antifungal effect of essential oils on *Fusarium* by assessing the influence of variation in the concentrations of these essential oils in the fight against *Fusarium* and the results of Amri et al. (2014) who demonstrated the antifungal power of essential oils on *F. oxysporum* f. sp. *lycopersici*.

CONCLUSION

The present work focused on the use of four essential oils as biological control agents for the tomato pathogen *F. oxysporum* f. sp. *lycopersici*. The antifungal activity of essential oils was verified firstly, by *in vitro* tests which allowed us to distinguish the most effective essential oils to fight against the growth of the fungus and secondly, by *in vivo* tests on tomato plants treated with the best essential oils applied on the one hand in preventive conditions and on the other hand in curative conditions. In these *in vivo* tests, the activity of essential oils was compared to that of a chemical fungicide. The most effective essential oil was *C. ambrosioides*, which had the highest inhibition percentages among the oils included *in vitro* tests. Two essential oils of *A. indica* and *C. annuum* were distinguished by their inefficiency at the same concentrations of 5 and 10 µL/mL with inhibition percentages of less than 50%. The oil considered to be the least effective was the oil of *C. annuum* because, at a high concentration (100 µL/mL), it has the lowest inhibition percentage. Regarding to *in vivo* preventive tests, the oil showing the greatest antifungal activity was *C. ambrosioides* oil. On the other hand, in curative conditions, although essential oils slowed the evolution of the disease, tomato plants all developed the disease whatever the treatment. However, these *in vivo* tests have allowed to realize that the essential oils have an effect on the physiology of the tomato plants during the study of the growth parameters of this plant.

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