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Antifungal effect of natural extracts on fungal contamination in the *vitro* culture of *Calodendrum capense* (L.f.) Thunberg

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Abstract

In order to overcome endogenous *in vitro* contamination, we have studied the effect of essential oils as an alternative to conventional chemical treatments. The objective of this study is to enhance the use of essential oils to inhibit the development of these fungi. The essential oils extracted from four Moroccan aromatic plants, Oregano - three chemotypes (*Origanum compactum*), Thyme (*Thymus vulgaris L.*), Eucalyptus (*Eucalyptus camaldulensis*) and Fenugreek (*Trigonella foenum graecum L.*) were use. The antifungal activity of these essential oils was studied against two fungal strains *Cylindrocarpon spp* and *Verticillium spp* identified from *Calodendrum capense in vitro* plants. The results showed a significant inhibitory effect on both fungi. This activity is manifested by the total inhibition of mycelial growth, with a low concentration of essential oils. The best inhibitory effect was obtained by the use of Oregano (MIC-0.15 µl/ml for chemotype 3, MIC-0.25 µl/ml for chemotype 2 and 0.5 µl/ml for chemotype 1) and Thyme (MIC- 0.25µl/ml). In addition, the Eucalyptus essential oil showed an incomplete inhibition (74.37%) of *Verticilium spp.* and (51.28%) of *Cylindrocarpon spp.* However, fenugreek extract has the lowest activity compared to the other oils. The chemical composition of these essential oils was analyzed by mass spectrometric chromatography (GC-MS). The analysis revealed the dominance of two compounds (Thymol and Carvacrol) in the most effective oils, and can represent the principal active ingredient in the pathogen control. Therefore, the essential oils of Oregano and Thyme or their major compounds could be investigated to solve the problem of contamination during *in vitro* culture.

1. Introduction

Tissue culture is preferable for fast multiplication of healthy plants. Nevertheless, endogenous microbial or fungal contamination is known to be one of the most serious problems in tissue culture [1]. This contamination can occur in several ways, either through defective laboratory procedures or through the transfer of microorganisms to the surface or inner tissues of explants. Plant surfaces are areas where micro-organisms can live [2]. To solve this problem, we use organic products such as essential oils, which are natural compounds extracted from plants. From a physiological point of view, these products are secondary metabolites produced by plants to cope with various hazards encountered in nature such as hydric stress or phytopathogenic parasites. In addition, these natural compounds have a powerful fungicide and bactericide potential that enables them to be a good candidate to replace the use of chemical fungicides and antibiotics in plant tissue culture [3], [4]. Essential oils of plants have already

found their place in aromatherapy, perfumery, pharmacy, food preservation and cosmetics. Their use is linked either to their wide spectrum of biological activities or to very specific targeting [5].
Nikkhah et Hashemi (2020) proved antifungal effect of some essential oils to control postharvest spoilage and preserving the jujube fruit quality [6]. Xie et al. (2017) tested Antifungal activity of several essential oils and major components against wood-rot fungi [7], and Hadizadeh et al. (2009) analyzed antifungal potential of five essential oils against *Alternaria alternate* [8]. Thyme covers a wide range of biological activities: antiseptic, antibacterial, antifungal, antispasmodic and antioxidant [9]. The essential oil of *O. compactum* contains a high level of carvacrol, which gives this oil powerful antibiotic and antiseptic property, as well as an antifungal capacity [10], [11]. Several authors have discussed the antimicrobial and antifungal activity of Eucalyptus essential oil [12], [13]. The biological activity of Fenugreek is employed mainly in human medicine [14], [15]. In addition, it was reported that this species exhibited a strong insecticidal activity against the small pests *Tribolium castaneum* and *Acanthoscelides obtectus* [16].
Many authors have reported that chemical composition of essential oils can vary with extraction methods, plant genotype, and growing conditions, also at different seasons of the year [17]–[19]. For this reason, it has been assessed using Gas Chromatography – Mass Spectrometry (GC-MS). This determination facilitates comparisons with other essential oils with an already known antifungal activity. This study is part of the scientific program of the botanical garden of Rabat, The aim of study was to evaluate the efficiency of some essential oils (3 chemotypes of *O. compactum, T. vulgaris, E. Camaldulensis,* and *T. foenum-graecum*) in controlling the contaminations of *Calodendrum capense* tissue cultures, to determine the minimum inhibitory concentration of essential oils needed to control pathogen growth (MIC), and to analyze the essential oils by GC-MS.

2. Material and Methods

2.1. Plant materials and essential oil extraction

The following medicinal plants: Oregano *(O. compactum),* Thyme *(T. vulgaris),* Eucalyptus *(E. camaldulensis),* and Fenugreek (*T. foenum graecum L),* were collected from their natural habitat at different locations in Morocco in July 2016 (Table 1). Air-dried plant materials (leaves, 200 g) were placed in a 5 l round-bottom distillation flask and 3 l distilled water was added. The essential oils were obtained by steam distillation for 3 h using Clevenger-type apparatus. The isolated fractions of plant parts exhibited two distinct layers: an upper oily layer and the lower aqueous layer. Both the layers were separated.

Table1: List of the essential oils used in this study and their origins.

Essential oils	Plant origin	Family	Origin	Local name
Oregano (chemotype 1)	*Origanum compactum*	*Lamiaceae*	Ouazzane (commune Zoumi)	Z'itrah
Oregano (chemotype 2)	*Origanum compactum*	*Lamiaceae*	Ouazzane (commune Brikcha)	Z'itrah
Oregano (chemotype 3)	*Origanum compactum*	*Lamiaceae*	Ouazzane (commune Mokrisset)	Z'itrah
Thyme	*Thymus vulgaris*	*Lamiaceae*	Marrakech	Zaâtar
Eucalyptus	*Eucalyptus camaldulensis*	*Myrtaceae*	Khouribga	Calibtus
Fenugreek	*Trigonella foenum graecum L*	*Fabaceae*	Settat	Halba

Fixed oil of Fenugreek was obtained using a soxhlet extraction, it was performed using a solvent extractor. For each extraction, dried seeds (100 g) were packed in a thimble and extracted with ethanol (500 mL). The immersion, washing, and recovery steps lasted for 4 h, with 17 cycles. The Soxhlet

method was used to extract the fixed oil of fenugreek and compare it with other essential oils extracted by hydrodistillation. The essential oils were then stored at 4°C in dark until use. The yields were calculated as the ratio of extracted oil mass to initial plant sample weight.

2.2. Antifungal activity assays in vitro

Fungal strains were selected on the basis of their frequency *in vitro* contaminations that are purified by successive subculturing. The purification steps were done in the laboratory of tissue culture on Biotechnology Unit in INRA- Rabat. The identification of these fungi was performed using fungal species identification keys [20]. The fungus (*Cylindrocarpon spp* and *Verticillium spp*) were multiplied on Potato Dextrose Agar medium (PDA) and incubated at 25°C in dark for 7 days.

The antifungal activity assays were performed on PDA medium amended with different oils at the following concentrations: C_0: 0% (control), C_1: 0,0015% - C_2: 0,0025%- C_3: 0,005% - C_4:0,015% - C_5: 0,025% - C_6: 0,05% and C_7:0,15%. For essential oil of Thyme and Oregano, and at higher concentrations: C_1:0,025% - C_2: 0,05% - C_3:0,15% - C_4: 0,25% - C_5 :0,35% - C_6: 0,45% - C_7: 0,55% - C_8:1% for Eucalyptus and Fenugreek. Essential oils were prepared by dissolving them in Tween 20 (0.5 %, v/v) and added to PDA immediately before pouring into 80 mm Petri dishes. The fungus was inoculated immediately by plating in the center 5 mm plugs from actively growing cultures. The Petri dishes were incubated at 25°C in the dark. Radial growth of colonies was measured every day during 10 days.

For each concentration, four replicate plates were used. The mean growth values were obtained and then converted in to the inhibition percentage of mycelial growth in relation to the control treatment by using the formula [13]:

$$MGI\ (\%) = ((dc-dt)/dc) \times 100$$

Where dc and dt represent mycelia growth diameter in control and treatment, respectively.

The minimum inhibitory concentration (MIC) that produced a 100% growth reduction was estimated for each compound after 10 days of inoculation.

2.3. Components separation and identification of essential oils composition

Agilent Technologies 7890A gas chromatograph was used to analyze the chemical composition of the essential oils. It's equipped with a Mass Selective Detector (MSD) and an HP-5MS capillary column (30 m long; 0.25 mm diameter). Helium was the carrier gas (1 ml/min flow rate). The initial temperature in the column was 50°C, increased to 150°C (+3°C/min) and maintained at 250°C (after an increase of +10°C/min). Essential oils were volumetrically diluted to a thousand times in ethyl acetate prior to gas chromatography (GC) injection.

A volume of 1 µl of each sample was injected in split mode. The mass percentage of the different constituents of essential oils is given in relative peak area. The fragmentation is carried out in a 70 eV electric field. The fixed fenugreek oil obtained is used to carry out a preliminary phytochemical screening, which is a qualitative analysis based on coloring and/or precipitation reactions in order to highlight the major chemical groups. For this purpose, several types of reagents were used. We used the analytical techniques described in [21]–[24].

3.3. Statistical analyses

Data were subjected to analysis of variance (ANOVA) using GenStat Procedure Library Release PL23.1. The significance of differences among treated samples was evaluated using Duncan's multiple range tests.

3. Results

1.1.Antifungal activity of essential oils in vitro

Two fungal species were isolated from *in vitro* infected roots. The identification of the fungus was done in the laboratory of phytopathology in INRA- Settat, Morocco. Based on morphological characteristics observed by optical microscopy, isolates were identified, as *Cylindrocarpon spp* and *Verticillium spp* according to Barnett et al. (1998) [20]. The results will be published in another paper.

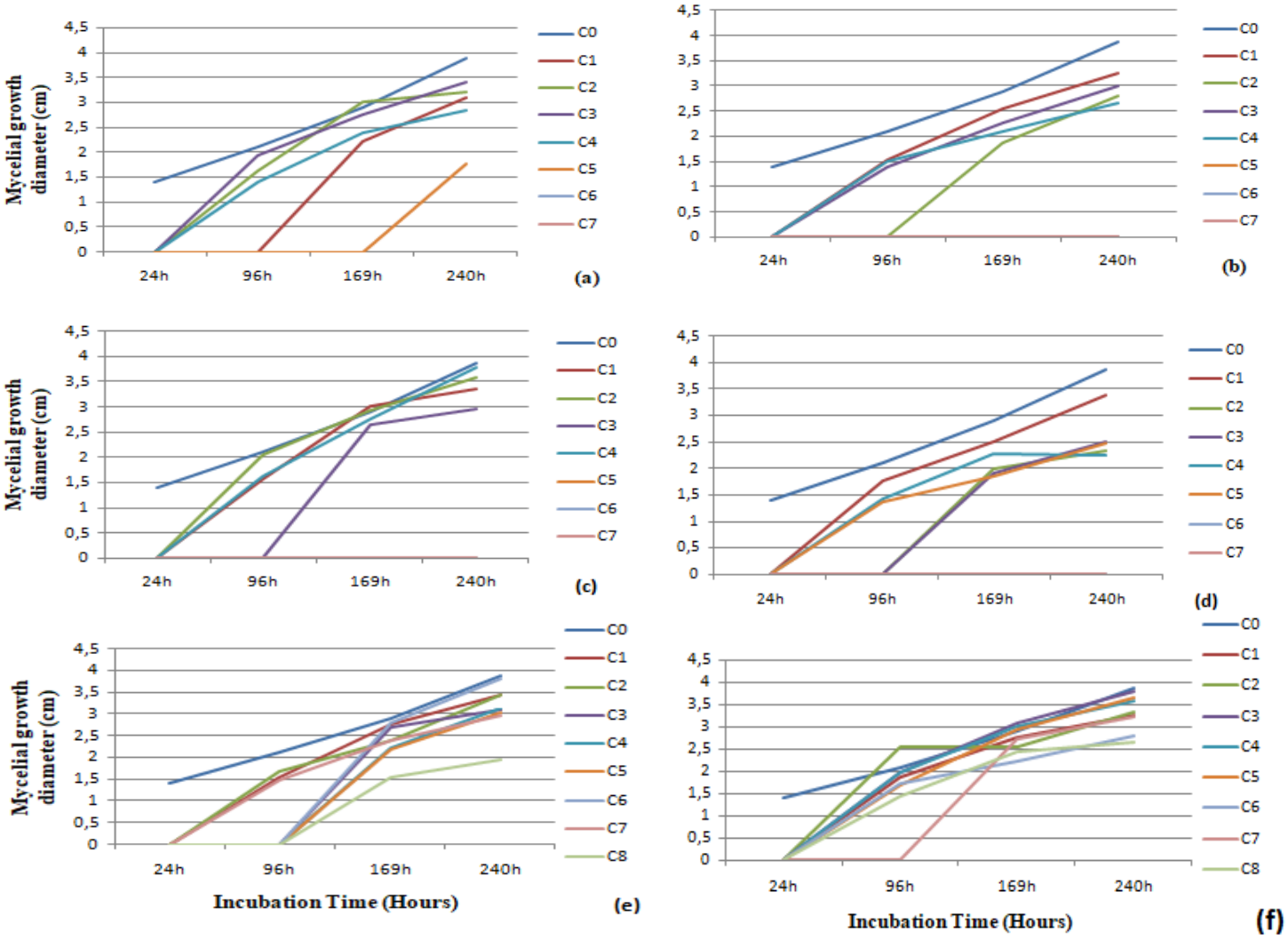

Figure 1: Effect of essential oils on mycelial growth of *Cylindrocarpon spp. in vitro*: Oregano-ch1 (a), Oregano-ch3 (b), Thyme (c), Oregano-ch2 (d), Eucalyptus (e) and Fenugreek (f)

The action of essential oils on mycelial growth of both *Cylindrocarpon spp.* and *Verticillium spp.* are illustrated in Figure 1 and 2. Mycelium growth of the pathogen was measured within the first 24 hours of incubation. The essential oils tested (three chemotypes of Oregano and one of Thyme) have a significant activity against fungal growth at low concentrations (0,0015% - 0,05%). However in the case of Eucalyptus and Fenugreek oils, there's no significant antifungal activity compared to the control (Figure 1, 2).

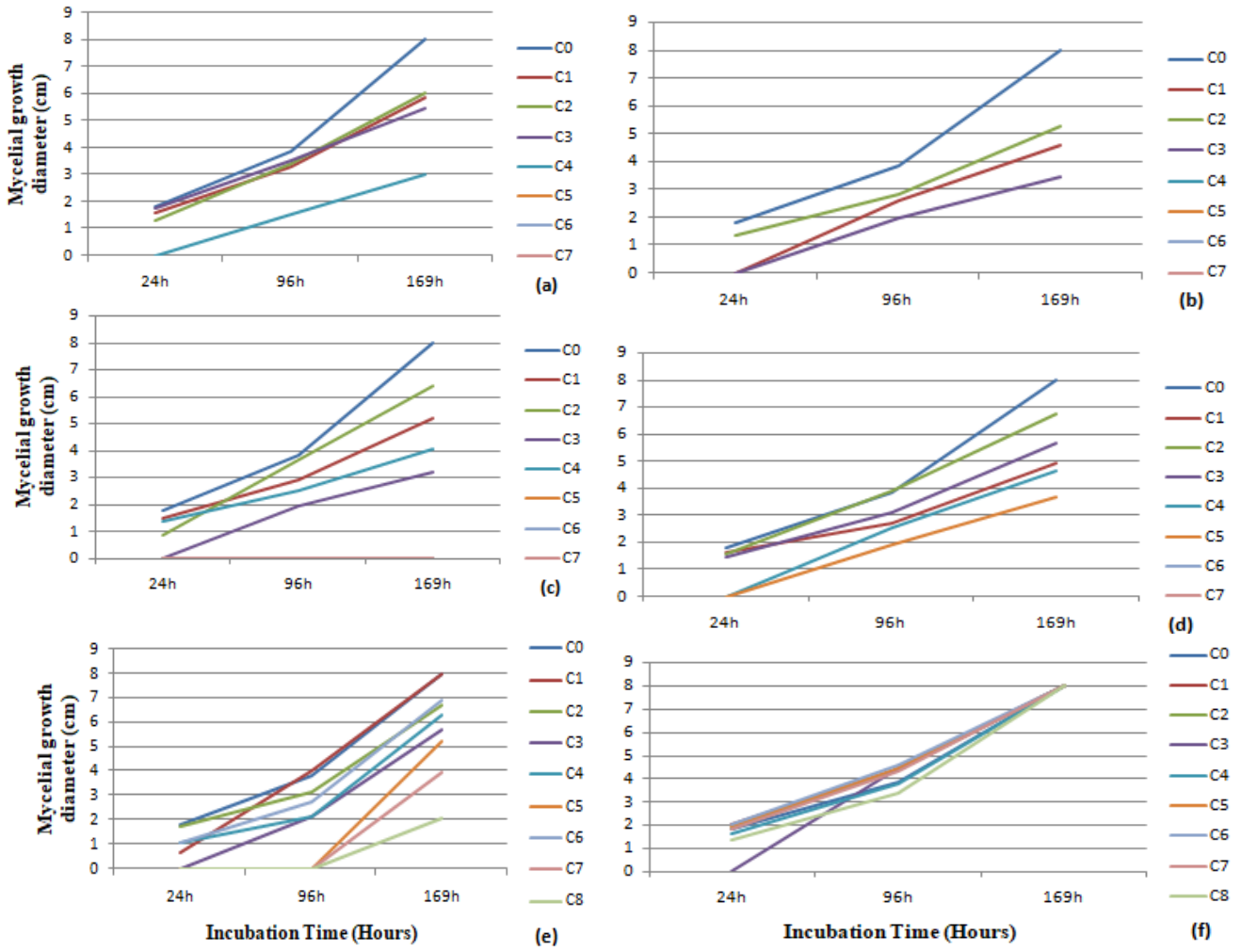

Figure 2: Effect of essential oils on the mycelial growth of *Verticillium spp. in vitro*: Oregano-ch2 (a), Oregano-ch3 (b), Thyme (c), Oregano-ch1 (d), Eucalyptus (e), and Fenugreek (f).

The minimum inhibitory concentration (MIC) was determined for all essential oils when radial growth in the control is maximal, and it is presented in Figure 3 and 4. Four essential oils completely suppressed pathogen growth (MGI= 100%) within 10 days of incubation. These oils were Oregano chemotype 1, chemotype 2, chemotype 3, and Thyme.

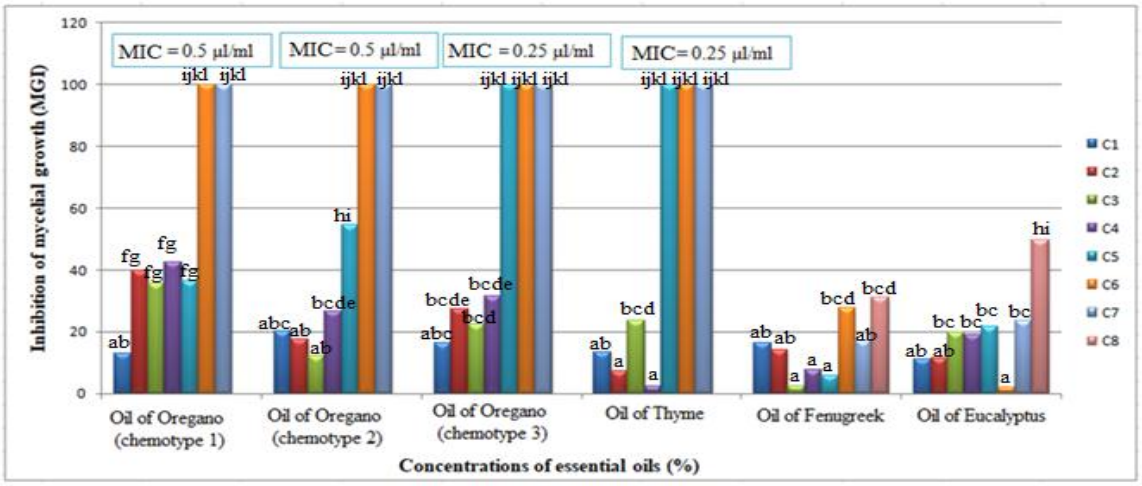

Figure 3: Effects of six essential oils: Oregano (chemotype 1, 2 and 3), Thyme, Fenugreek, and Eucalyptus; at different concentrations on mycelial growth inhibition of *Cylindrocarpon spp*. Concentrations with the same letters represent values that are not significantly different (P≤0.05).

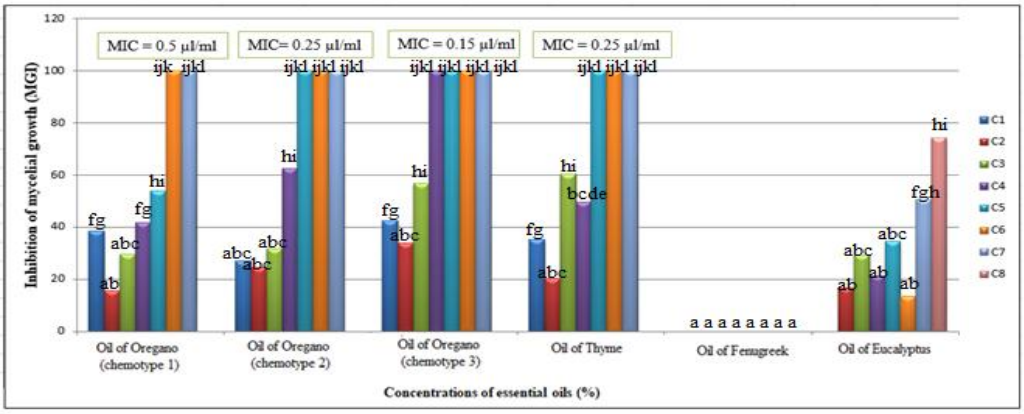

Figure 4: Effects of six essential oils: Oregano (chemotype 1, 2 and 3), Thyme, Fenugreek, and Eucalyptus; at different concentrations on mycelial growth inhibition of *Verticillium spp.* Concentrations with the same letters represent values that are not significantly different (P≤0.05).

1.2.Extraction and chemical compositions of essential oils

The yield of essential oils isolated from the aerial parts of *O. compactum* populations varied considerably from sample to the other. Yields were (2.2 - 1.78 - 1.89%) of all three samples depending on the origin of accession. The oils yields of thyme, eucalyptus and fenugreek are (1.42 - 1.2 - 17.2%) successively. The analysis of *O. compactum* essential oils showed great chemical variability and three chemotypes were distinguished for this Oregano species in Ouazzan Morocco region. Chemotype 1: The predominant constituents were carvacrol (38.67%), thymol (25.90%), and γ -terpinene (17.56%) that presented approximately 82% of the oil.; chemotype 2: This chemotype is characterized by a thymol content of 46% and p-cymene content of 30%; and chemotype 3: the sample classified in this chemotype was 30% thymol, 45% carvacrol and 14,79% p-cymene. This chemical identification allowed us to distinguish the three following chemotypes: carvacrol/thymol/γ -terpinene chemotype; thymol/p-cymene/γ -terpinene chemotype and carvacrol/thymol/ p-cymene chemotype. In Thyme oil, 24 compounds were identified with the dominance of 3 components: Thymol (41,39%), γ-terpinene (22,25%) and p-cymen (15,59%). The major compounds of Eucalyptus oil were 1,8-cineole (34.22%), followed by cedrol (16.13%) and myrtenal (11.34%). Furthermore, to determine the chemical composition of fenugreek's extract, several procedures have been completed, the results showed that fenugreek seeds are rich in phenols, alkaloids, flavonoids and tannins.

4. Discussion

C. capense is a species of the Rutaceae family. It is rich in polyunsaturated fatty acids and in metabolites such as terpenoids, limonoides or coumarin. It also contains many substances used in traditional medicine and cosmetics. The seeds of *C. capense* produce oil that could be used as a FAME biofuel. It was introduced to North Africa in the twentieth century, where it is an ornamental tree, grown for its highly aesthetic, fragrant pink flowers. It is known to have a shallow root system. Calodendrum has never been grown *in vitro*, probably because of its high availability but also because of its low growth rate. In our work, a preliminary study of the culture of juvenile explants of the species achieved green sprouts, but the subcultures were contaminated by the persistent presence of endogenous fungi in the media. The fungal contaminant found associated with tissue culture plants includes *Cylindrocarpon spp* and *Verticillium spp*. In order to overcome this problem, we experimented with the addition of natural extracts to the culture medium in order inhibit *in vitro* infectious agents. We tested essential oils from oregano (3 chemotypes), thyme, and Eucalyptus at different concentrations, in addition to fenugreek's absolute extract.

Table 2. Chemical composite on percentage of essential oils of Oregano (three sample), Thyme, and *Eucalyptus* from Morocco by gas chromatography/mass spectroscopy (GC/MS).

Compound [a]	RT [b]	RI [c]	Oregano [d]	Oregano [e]	Oregano [f]	Thyme	Eucalyptus
			% peak area				
Anisole	3.10	923	-	-	-	-	1,31
α-thujene	3.28	925	-	-	-	1,76	-
Tricyclene	3.41	926	-	-	-	-	1,14
α-pinene	3.58	931	-	-	-	-	-
β-pinene	6.58	974	-	-	-	1,63	-
α-terpinene	8.50	1015	-	-	-	3,25	-
p-cymen	9.96	1023	5,82	30,01	14,79	15,59	10,56
Limonene	10.13	1027	-	-	-	-	4,51
1,8-cineole	11.10	1036	-	-	-	-	34,22
γ-terpinène	11.34	1057	17,56	15,20	1,51	22,25	-
Linalol	12.94	1100	-	-	-	1,79	-
4-terpineol	15.85	1176	2,15	0,54	0,53	1,15	3,84
Myrtenal	16.79	1197	-	-	-	-	11,34
Nopol	16.30	1212	-	-	-	-	2,66
Careen (2)	17.50	1227	-	-	-	-	1,01
Carvone	18.82	1231	-	-	-	-	2,81
Thymolmethylether	19.03	1233	-	-	-	1,18	-
Carvacryl methyl oxide	19.96	1244	5,05	-	-	-	-
Geraniol	20.40	1245	-	-	-	-	-
Pulegone	20.42	1247	-	-	-	-	-
Thymol	21.50	1293	25,9	46,62	30,43	41,39	-
Carvacrol	21.87	1311	38,67	0.88	45,78	2,06	-
Myrtenyl acetate	23.20	1328	-	-	-	-	-
Caryophyllene	26.76	1417	-	-	-	1,3	-
Guaiol	42.54	1596	-	-	-	-	6.28
Cedrol	42.52	1607	-	-	-	-	16.13

[a] The compounds that present lower than 1 % were not showed
[b] Retention time (min)
[c] Retention indices on the HP 5MS column
[d] Chemotypes 1
[e] Chemotypes 2
[f] Chemotypes 3

The preliminary study showed that oregano essential oil (chemotype 3) gave the best mycelial inhibition results (100%) at low doses (0.15%), followed by thyme and oregano chemotype 2 at doses (0.25%). Several studies have shown that oregano essential oil completely inhibits the development of many pathogens such as *Eurotium herbariorum, Aspergillus wentii, Aspergilus tamarii, Botrytis cinerea, Alternaria alternata, common Penicillium, Stachybotrys chartarum, Penicilliumim plicatum* and *Cladosporium cladosporioides* [25–27]. Oregano essential oil has significant antifungal activity against *Aspergillus flavus* and *Aspergillus niger* at a concentration of 0.072 µL/ml [28]. Thus, Greek oregano essential oil at a dose of 4µL can also significantly inhibit the growth of *Verticillium dahlia, Sclerotina sclerotiorum, and Pythium spp,* isolated from infested tomato plants [29]. Thyme essential oil is also completely inhibited the growth of many plant pathogenic fungi, such as, *Penicillium digitatum,*

Galactomyces citri-aurantii, *Penicillium italicum*, *Alternaria citri* and *Botrytis cinerea* [25], [30–32]. These results are consistent with those obtained in our study, where oregano and thyme have good antifungal activity against *Cylindrocarpon spp* and *Verticillium spp* at low concentrations. Stević et al. (2014) indicated that many tested fungi were relatively uniformly susceptible to Oregano and Thyme oils. This represents a good basis for the formulation of products with potential efficiency in controlling fungal contamination [33]. The three Oregano oils gave different results in antifungal activity, so it was necessary to determine the chemical composition for each oils to know the major components, which can be the cause of a good antifungal activity, not to forget the role of minor components.

The differences observed in chemical composition of *O. compactum* chemotypes can be attributed to exogenous factors such as altitude where the plant grows, temperature, nature and soil composition, or even to endogenous factors such as genetic characteristics [17], [19]. Analysis of the chemical composition of the essential oils (thyme and three chemotypes of oregano) tested in our study reveals that they contain mainly aromatic mono-terpenes such as carvacrol, thymol and p-cymene. Numerous studies show that the activity of these oils is often attributed to these compounds (carvacrol and thymol) [34], [35]. In another study, the individual application of thymol and carvacrol showed a significant antifungal effect against the pathogens *Colletotrichum acutatum* and *Botryodiplodia theobromae*. These substances could be used as alternatives to chemical fungicides in pre- and post-harvest phases of many fruit and vegetable species [36]. Therefore, carvacrol and thymol may play an important role in the antifungal activity of oregano and thyme demonstrated in our study. However, in our study, *E. camaldulensis* essential oil was found to be relatively less active on mycelial growth compared to the other oils tested, this is clear when comparing inhibitory concentrations, equally, this oil was proven as a moderate antifungal agent against domestic moulds, wood rot fungi [37] and plant pathogenic fungi [38,39]. Antifungal activity of Eucalyptus oil in our experiment was low, despite being positive in other filamentous fungi [12,13]. 1,8-cineole is the major component of eucalyptus oil, and its antifungal activity against several pathogens has been proven by many researchers [40,41].

On the other hand, a higher concentration than 10µl/ml of Fenugreeks extract may be required to observe minimal mycelial growth inhibition of both fungi (Figure 3 and 4). Other results showed that fenugreek aerial parts are rich in phenols, alkaloids, flavonoids and tannins; and they possess high antifungal activities [32-34]. Whereas Fenugreek's absolute does not seem have an inhibitory effect.

Some investigators reported that the antifungal activity resulted from a direct effect of essential oil on fungal mycelium. This effect may be due to an attack on the phospholipid bilayer of the cell membrane, subsequent a breakdown of the enzymatic systems, followed by the modification of the genetic material of the fungi. As well as the formation of fatty acid hydroperoxides by the oxygenation of unsaturated fatty acids, which leads to the coagulation of the cytoplasm and the damage of lipids and proteins, in addition, the disruption of the protonic motive force of fungi [42].

The antifungal activity of essential oils could be influenced by the major components of these oils or due to a synergistic effect between the major and minor components. Synergistic and antagonistic effects of the compounds may also play an important role in the inhibition of fungi [48, 49]. In this respect, it is remarkable to note that the observed difference in the antifungal activity of the three chymotypes of oregano and thyme oil tested in our study may also be due to the synergistic effect between thymol and carvacrol, because the synergy between these two phenols has been observed in several studies [43], [44]. Therefore, the use of essential oils of oregano and thyme can help prevent fungal growth, with the advantage of having a positive impact on plant growth. The other advantage of using these oils is their non-toxicity for the explant and the fact that they do not interfere with the regeneration and differentiation process of plant tissues.

Conclusion

In this work, two fungal species, *Cylindrocarpon spp.* and *Verticillium spp*, were isolated from infected roots in tissue culture of *Calodendrum capense*. Six essential oils were tested on these fungi (three chemotypes of Oregano, Thyme, Eucalyptus and Fenugreek). The results confirm a good antifungal activity of three chemotypes of Oregano and Thyme essential oils, against both fungus *Cylindrocarpon spp.* and *Verticillium spp. in vitro*, and that the chemotype 3 has the lowest MIC (0.15 µl/ml). The evaluation of chemical compositions of collected Oregano samples made it possible to characterize three distinct chemical varieties: carvacrol/thymol/γ -terpinene chemotype, thymol/p-cymene/γ -terpinene chemotype and carvacrol/thymol/ p-cymene chemotype. In addition, Thyme oil includes thymol as the major component. Therefore, it is likely that these compounds (thymol and carvacrol) may constitute the main active compounds against the fungal strains *Cylindrocarpon spp.* and *Verticillium spp*. Further studies may be considered to test the efficiency of these compounds against these fungi. The findings of this study constitute preliminary evidence supporting the use of essential oils for contamination control in vegetative micropropagation of *Calodendrum*.

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(2020) ; http://www.jmaterenvironsci.com