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Evaluation of the antifungal activity of aromatic plants essential oils on *Phytophthora citrophthora*

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ABSTRACT

The objective of this study was to evaluate antifungal effect of essential oils (EO) from various Moroccan aromatic plants, namely: *Artemisia herba alba*, *Origanum compactum*, *Rosmarinus officinalis*, *Thymus vulgaris*, *Lavandula stoechas*, *Mentha viridis*, on *Phytophthora citrophthora*. Determination of oils antifungal activity was performed by measuring the diameter of mycelium. A growth model was established to evaluate fungal growth diameter as such: $1.834 * [\text{Oil concentration in } \mu\text{L}/20 \text{ mL PDA}]^2 - 6.166 * [\text{Oil concentration in } \mu\text{L}/20 \text{ mL}] + 4.99$. With correlation coefficient $r^2 = 0.999$. The EO of *O. compactum* was then used for further chemical examination by gaseous chromatography coupled with mass spectrometry. The results showed that the most significant antifungal effect and the highest yield were obtained with *O. compactum* oil. The inhibition of *Phytophthora* was proportional to the dose of *O. compactum* EO applied. Total inhibition was at 2 $\mu\text{L}/20 \text{ mL}$ PDA, which may be due to the high level of Carvacol. Chemical analysis of *Origanum compactum* EO, showed dominance of Carvacol (69%), followed in decreasing order by Cymene, Caryophyllene, Linalool and α -Pinene.

Key Words: Aromatic plants, Essential oils, Antifungal activity, *Phytophthora citrophthora*

INTRODUCTION

Phytophthora citrophthora induces substantial damage to orange trees by causing a number of diseases, including brown rot gummosis, rot root and rot fruits. This damage leads to a significant reduction in fruits yield. In many countries, as in Morocco, the cultures of *P. citrophthora* are the most isolated fungi [1].

Different synthetic fungicides such as *Metalaxyl* are often used to treat *P. citrophthora* plant disease however, these fungicides are expensive, toxic and are a significant source of environmental pollution [2, 3]. In addition, the fungi could develop resistance to fungicides leading to the development of more chemicals [4]. Therefore, a fungicide based on plant essential oils could be a better alternative to chemicals [2, 3].

Morocco with its bio climate and soil diversity has a native flora of > 7000 sp [5], which is rich in aromatic plant containing over 800 species that belong to 352 genres from 107 families [5,6].

The extracted oils are a mixture of different compounds, which have the potential to inhibit some fungi [7-13], via inhibition of spore germination [14], or through limitation of mycelium growth [2].

In our knowledge, no study has been done in Morocco, to investigate the effects of aromatic plant extracts on *P. citrophthora*. Thus, our objective was to evaluate the antifungal effects of essential oils from various Moroccan aromatic species (namely, *Artemisia herba alba*, *Origanum compactum*, *Rosmarinus officinalis*, *Thymus vulgaris*,

Lavandula stoechas and *Mentha viridis*) on *Phytophthora citrophthora*, the causing agent of gummosis disease in orange trees.

MATERIALS AND METHODS

Artemisia herba alba leaves, *Origanum compactum* leaves and inflorescences, *Rosmarinus officinalis* terminal inflorescences, *Thymus vulgaris* flowered stems, *Lavandula stoechas* terminal inflorescences, *Mentha viridis* leaves were collected in Meknes region, and were dried at shade and kept at dark.

In order to extract essential oils through steam stirring, about 100 grams of dry matter from each plant species were submitted to hydro distillation (Clevengertype). Yield of essential oils was determined and expressed as mL per 100 g of dry matter.

Biological tests

Antifungal activity of essential oils was investigated in vitro with diffusion method. *P. citrophthora* was isolated from orange trees cultivated in experimental station at INRA "Regional Agronomic Research Centre, Kenitra, Morocco".

PDA medium containing-plates were used to evaluate the inhibition effect of essential oils by measuring the diameter of the area covered by mycelium growth. Two distant (2 cm) wells were drilled on the PDA solid medium: one received a dose of 50 μ L of essential oils extracted from the six different plant dry matters investigated, and the second received the inoculum of *P. citrophthora*. Three replicates of each essential oil type were used.

The plates were incubated at 25°C and the fungal growth was evaluated via measurement of mycelium diameter at 24, 72 and 120 hours.

In the following part, only *Origanum* species was used to investigate the effect of oil concentration on *P. citrophthora* growth using suspension method. The doses were: 0.8 μ L, 1 μ L, 2 μ L, 4 μ L, 6 μ L, 8 μ L, each added to 20 ml of PDA in glass tubes that were agitated by vortex. The suspension obtained was tipped out on Petri plates. When the medium solidified, a fragment of fungi about 6 mm diameter was deposited on the plate. Each treatment was repeated three times. After 24 hours incubation, the fungi growth was measured at 48 hours intervals.

Two parameters were used:

- MIC (the minimal inhibitory concentration) corresponds to the minimum concentration of essential oil that inhibits fungi growth; thus, no growth was visible at eyes on the solid medium.
- MCF (the minimal fungicide concentration) is the minimum concentration of essential oil causing elimination of fungi at 99.99%.

Chemical analysis

1 μ L of essential oils from *O. compactum* diluted in Hexane was used to carry out chemical analysis by gaseous chromatography (*Hp 5980*) coupled with mass spectrometry (*Hp 5772*), in National Scientific and Technical Research Centre- Rabat- Morocco - (CNRST). Such equipment permits, first the chromatographic separation of each essential oil constituents, then the qualitative and quantitative determination of major compounds. At the end, the obtained specters were compared with results in data base about NBS 75 k reference or/and with specters already published.

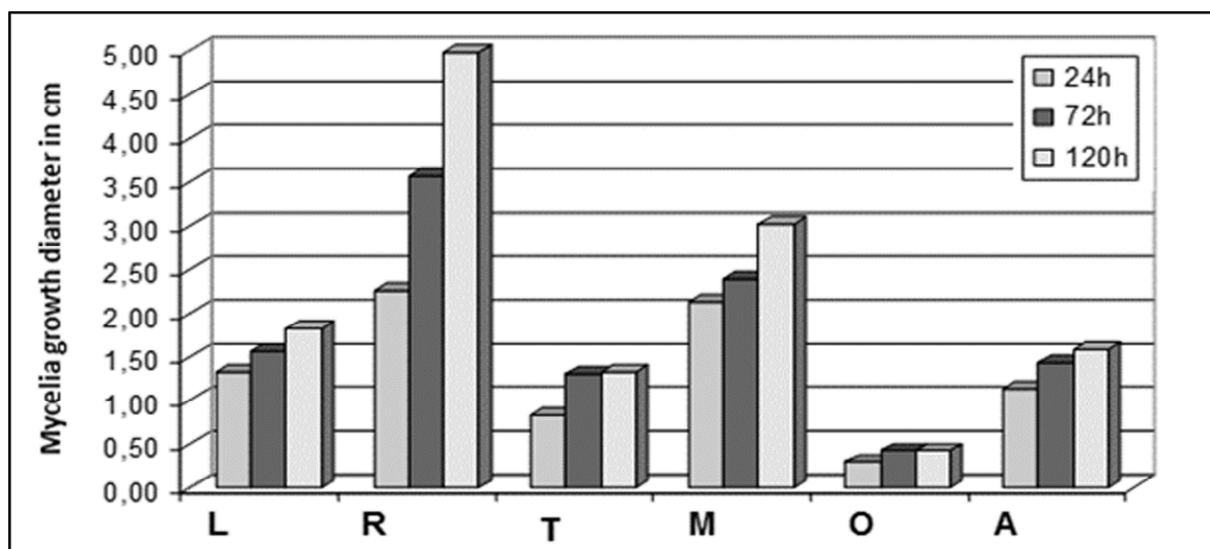
RESULTS AND DISCUSSION

Yield in essential oils

The yield of EOs recovered by hydro distillation from the six aromatic plants is given in ml per 100 g of dry plant. The highest yield was obtained from *O. Compactum* (3.44 %) and the lowest from *A. herba-alba* (0.62 %). As was reported [15, 16], *T. vulgaris*, *L. stoeckas*, *M. viridis* and *R. officinalis* gave (1.8 %, 1.65%, 1.56%, 1.36 %), respectively.

Antifungal activity

Biological tests on *P. citrophthora* using the diffusion method revealed significant difference between the six species (Fig. 1). Growth inhibition of *P. citrophthora* was the highest with *O. compactum*, which is in accord with previous work performed on *Candida albicans* [17]. *R. officinalis* and *M. viridis* only gave slight effects on growth of *P. citrophthora* mycelia. *Rosmarinus* sp. was reported to affect fungal spores germination of *Phytophthora* spp [18].



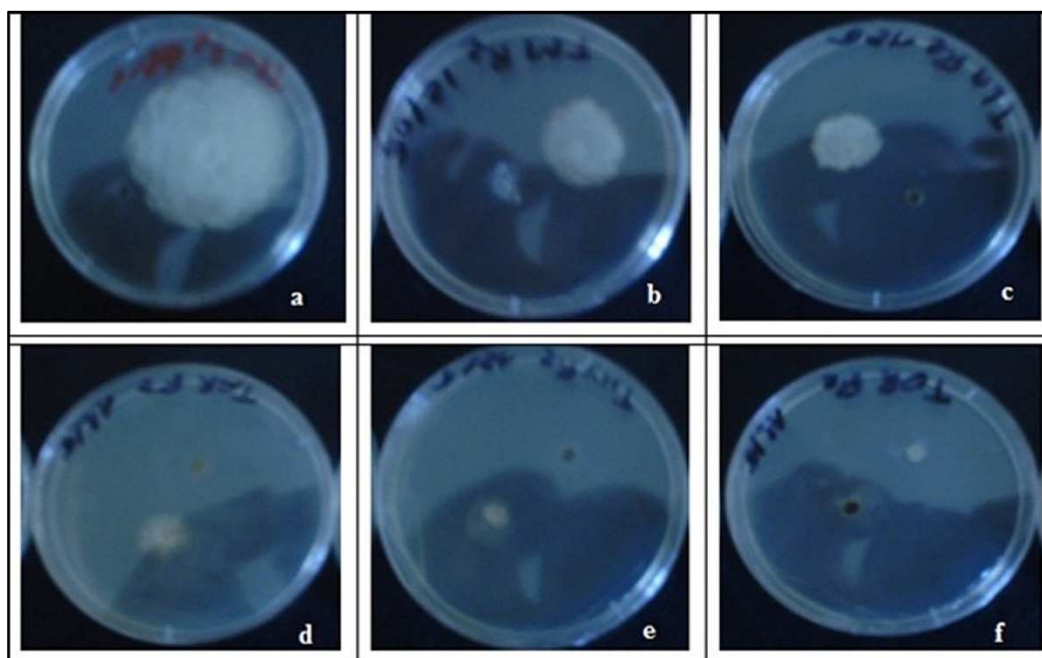
L: *L. stoechas*; R: *R. officinalis*; T: *T. vulgaris*; M: *M. viridis*; O: *O. compactum*; A: *A. herba alba*

Figure 1. Biological tests with essential oils from the six species tested on *P. citrophthora* using diffusion method

Table 1 shows that the mean of mycelium diameters was 0.43 cm with *O. compactum* whereas it was 4.97 cm, 3.03 cm with *R. officinalis* and *M. viridis*, respectively. This attests clearly that *O. compactum* possess the most efficient inhibitor of *P. citrophthora* (Photo 1: f).

Table 1. Growth in cm of *P. citrophthora* cultured on PDA plates after 120 hours incubation at 25°C. Means with the same superscripted letters did not show significant differences (at 5 % Scheffé test)

Species	Mean in cm
<i>A. Herba alba</i>	1.60 ^a
<i>L. stoechas</i> ²	1.83 ^a
<i>M. viridis</i>	3.03 ^b
<i>O. compactum</i>	0.43 ^c
<i>R. officinalis</i>	4.97 ^d
<i>T. vulgaris</i>	1.33 ^{ac}



a: *R. officinalis*; b: *M. viridis*; c: *L. stoeckas*; d: *A. herba alba*; e: *T. vulgaris*; f: *O. compactum*

Photo 1: *P. citrophthora* mycelia growth after 120 hours incubation at 25°C

As shown in photo 1, growth inhibition decreased from *O. compactum* (f) > *T. vulgaris* (e) > *A. herbaalba* (d) > *L. stoeckas* (c) > *M. virdis* (b) > *R. officinalis* (a). Similar results were found in previous work where *T. saturojoïdes* was more effective than *M. pulegium*, which was more effective than Rosmarinus [11].

Furthermore, the successful effects correlate with oil concentration [14] as well as with the type of fungal species. For example, with *M. pulegium*, 10 µL was necessary to inhibit growth of *Penicillium expansum* and *Alternaria alternata* [12] whereas 20 µL was needed for *Penicillium. spand* 2 µg/mL for mycosis agents [11, 14]. The work presented by [19] and [20] reported that pulegone and 1,8-cineole must be used in higher concentration to inhibit mycelia growth than when used as total essential oil. Thus, the activity of the oil probably results from the combination of all major compounds as well as from a synergic effect of the less dominant ones [10, 19].

Table 2. Effect of different *O. compactum* essential oil concentrations on the diameter of *P. citrophthora* growth in cm

Time	Concentration (µL / 20 mL PDA medium)							
	0 µL	0,8 µL	1 µL	2 µL	4 µL	6 µL	8 µL	10 µL
24 h	1	0	0	0	0	0	0	0
72 h	2	0.2	0	0	0	0	0	0
120 h	3	0.4	0.2	0	0	0	0	0
168 h	4	1	0.5	0	0	0	0	0

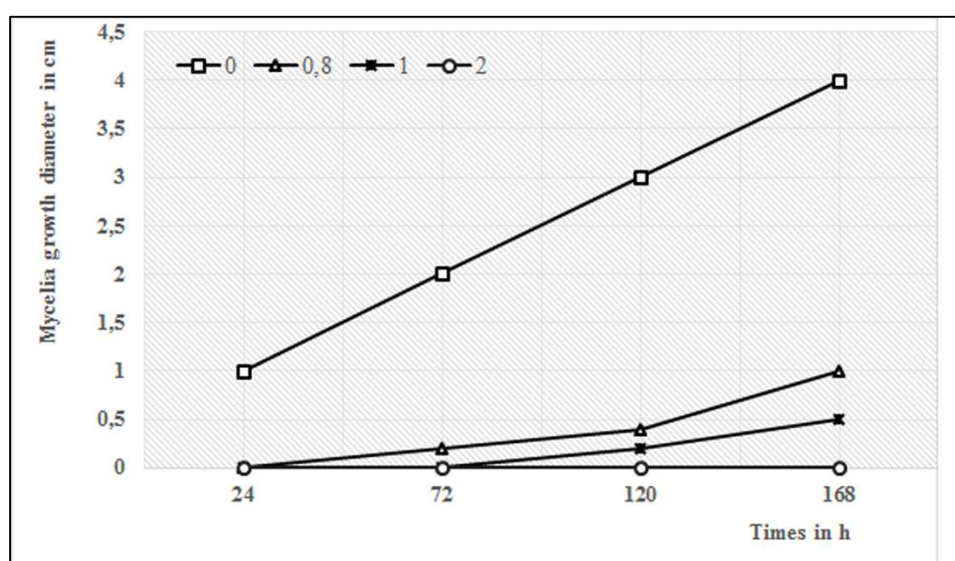


Figure 2. Observation of *P. citrophthora* mycelium diameter with varying concentration of oil from *O. compactum*

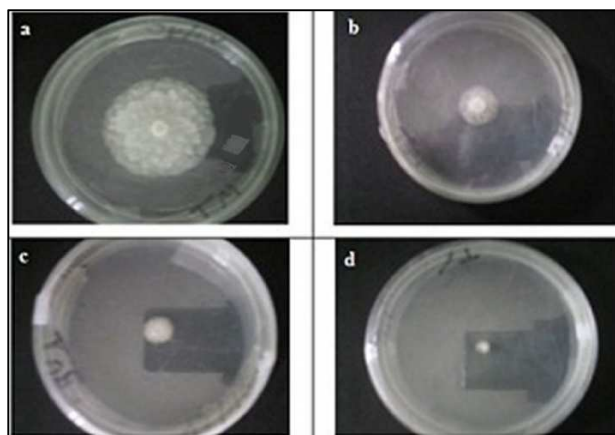
Growth inhibition of the fungi was proportional to increased doses of *O. compactum* EO. No growth was observed at 2 µL/20 mL PDA dose. At, 216 h incubation time, the diameter of the mycelium was 5 cm on the control plates, whereas, it was only 1.2 cm with 0.8 µL/20 mL PDA, 0.7 cm with 1 µL/20 mL PDA and 0 cm with 2 µL/20 mL PDA (Table 2, figure 2). *P. Citrophthora* growth correlated with time. A model was established by linear regression, to evaluate fungal diameter progression as follow:

$1.834 * [\text{Oil concentration in } \mu\text{L}/20 \text{ mL PDA}]^2 - 6.166 * [\text{Oil concentration in } \mu\text{L}/20 \text{ mL}] + 4.99$ with correlation coefficient, $r^2 = 0.999$.

We can conclude that concentration of essential oil is the determinant factor for the inhibition efficiency of the oil. Therefore, two intervals were proposed:

- Interval of minimum concentrations: from 0 µL to 2 µL.
- Interval of maximum concentration from 2 µL to 10 µL.

The MIC was 0.8 µl while MFC was 2 µL (Photo 2). It was reported that essential oils when combined with amphotericin B (AmB) provoked a strong drop of MIC compared to 80% of AmB alone, in the treatment of *Candida albicans* mycosis. This may mean that in the case of such combination with oils, a smaller dose of AmB will be needed, and this would reduce its side effects [21]. MIC obtained with oil from *T. saturojoïdes* and *M. pulegium* on agents responsible for mycoses are almost similar to those with imidazoles [11]. Then, if MIC is equal to MBC, it means that essential oil is bactericide [22].



a: Normal growth of *P. citrophthora* (control); b: Concentration (0.8 μ L); c: Concentration (1 μ L); d: Concentration (2 μ L)
Photo 2. Growth inhibition with an increasing concentration of oil from *Origanum compactum*

Chemical composition of Oregano essential oil

O. compactum EO composition was examined by CPG and mass spectrometer analysis (Fig.3).

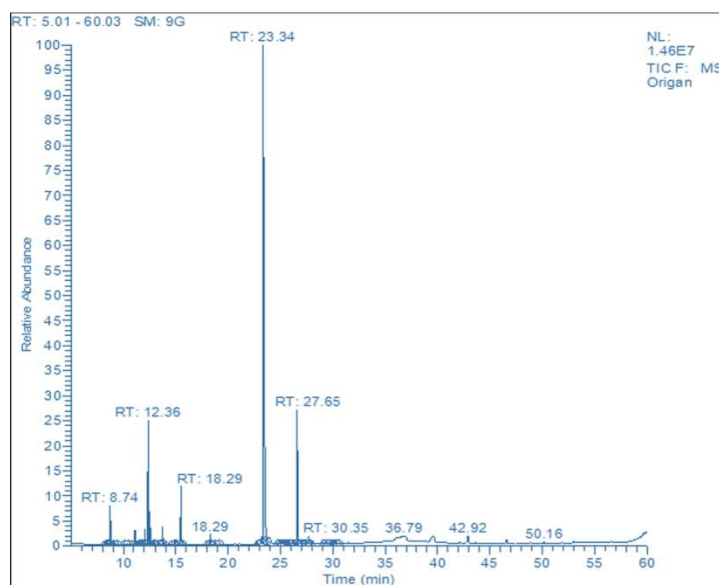


Figure 3. Chromatogram of Essential oil from *O. compactum* (GC/MS)

About thirty compounds were detected with the majors constituents are Carvacrol (69%), Caryophyllene (7.85%), p-Cymene (7.20%), Linalool (3.77%), α -Pinene (2.42%) and Dipentene (2.23%). Similar results were found in other reported work on Carvacrol and p-Cymene [23]. Whereas, it was found that oil of *O. vulgare* subsp. *Glandulosum* grown in Algeria was characterized by high Thymol content [24].

Climate, period of plant sampling, extraction method or time separating distillation from chromatographic analysis, especially with volatile or photodegradable products could affect the results.

For example, there was a difference in oil composition of *M. pulegium* grown in Morocco, Algeria and Tunisia [25-28]. In Morocco, Essential oil of *A. herba alba* from an east and a southern region are dominated by β -Thujone and Camphre [16], respectively.

Carvacrol was the most dominant element in *O. compactum* essential oils [23]. Carvacrol was also found in *T. vulgaris* oil [29] but its concentration was small than that of *O. compactum*, which explains the differences observed in their effects on the growth of *P. citrophthora*. Previous work affirmed that Carvacrol had bactericide and fungicide effects through its rupture of the pathogens membranes or alteration of their functions [30-32].

Both, Cetones (as Pulegone from *M. Pulegium*) were reported to be more active than Terpene oxides as 1,8-cineole (from *Eucalyptus camaldulensis*) on fungi such as *Penicillium expansum* and *Alternaria alternata* [9, 12].

In others work [33], it was reported that antifungal activity decreased with the chemical structure: Phenols > Alcohols > Aldehydes > Acetones > Ethers > Hydrocarbures, and within Aliphatic Aldehydes, Cinnamaldehyde was the most active. Also, for Phenols, the antifungal activity increase with steric molecular complication (phenol > Thymol > Isoeugenol > Eugenol) and the hydrophobic character of Phenolic or Aldehydes aromatic appeared to be necessary to give optimal antifungal propriety [33].

CONCLUSION

Aromatic plant essential oils used in this study have antifungal activities and constitute an important potential as a good alternative to synthetic pesticides that are expensive and a source of environmental pollution [2]. They can also serve as a mean for bio control against fungal rot in storage phase [12, 13]. Interestingly, some products are already formulated as botanic pesticides like Azadirachtin, Carvone, Pyrethroides and are successfully used in programs of integral defense against fungal and bacterial pathogens [2].

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