Plant biology and pathology/Biologie et pathologie végétales

# The potency of lemon (*Citrus limon* L.) essential oil to control some fungal diseases of grapevine wood

## *Les huiles essentielles de citron (*Citrus limon *L.) pour lutter contre certaines maladies fongiques du bois de la vigne*

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ABSTRACT

This study aimed to evaluate the in vitro antifungal activity (AA) of the essential oil (EO) of lemon (*Citrus limon* L.) against three pathogenic fungi attacking grapevine wood. The composition of the EO was also studied. Ten volatile components were identified by gas chromatography mass spectrometry. The results showed that the EO consists of volatile components where monoterpene hydrocarbons are the most abundant ones. Four major components were identified, which represent 99.9% of the total EO (limonene, neral, β pinene, and γ terpinene). The AA of the EO was evaluated against three pathogenic fungi attacking grapevinewood (*Eutypa* sp., *Botryosphaeria dothidea*, and *Fomitiporia mediterranea*). The results showed that the EO exerts AA against all tested fungi and significantly inhibits their growth. *Eutypa* sp. is the most sensitive fungus. These results show, for the first time, a new use for the EO of lemon (*C. limon* L.) to control fungal diseases of grapevine wood.

RÉSUMÉ

Cette étude examine l'activité antifongique in vitro (AA) de l'huile essentielle (EO) de citron (*Citrus limon* L.) contre trois champignons pathogènes du bois de la vigne. La composition de l'EO a également été étudiée. Dix composants volatiles ont été identifiés par chromatographie en phase gazeuse spectrométrie de masse. Les résultats ont montré que l'EO est constituée de composants volatils où les hydrocarbures de monoterpènes sont majoritaires. Quatre composants principaux ont été identifiés, ce qui représente 99,9 % de l'EO totale (limonène, néral, β pinène et γ terpinène). L'AA de l'EO a été évaluée contre

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trois champignons pathogènes du bois de vigne (*Eutypa* sp., *Botryosphaeria dothidea* et *Fomitiporia mediterranea*). Les résultats ont montré que l'EO exerce une AA contre tous les champignons testés et inhibe de manière significative leur croissance. *Eutypa* sp. a été le plus sensible. Ces résultats montrent, pour la première fois, une nouvelle utilisation pour l'EO du citron pour lutter contre les maladies fongiques du bois de vigne.

## 1. Introduction

Fungi are the main infectious agents in plants, causing alterations during developmental stages, and are the main microorganisms responsible for losses in agriculture. The decline of the vine is due to fatal diseases such as esca, eutypiosis, and BDA (black dead arm) developed in vineyards [1,2]. These diseases are the most destructive ones of the woody tissues of grapevine and have been found on some hosts, causing decline and loss of productivity [3]. This kind of fungus colonizes wood tissue and causes dieback; the first disease, called esca, is a complex disease, including vascular symptoms and inter nal white rot in the trunk, which gradually changes the hard wood into a soft one [1]. *Eutypa* dieback, caused by the fungus Eutypa lata, and BDA, caused by the Botryosphaeria family, threatens the sustainability of vineyards, especially young ones. It is becoming a serious problem in most vine growing regions. These fungi produce toxic metabolites that are transported through vascular tissue to the foliage, causing necrosis of leaves, vascular symptoms, and internal brown rot in the trunk [4]. Since the ban of sodium arsenite, the only known way to fight the esca and the Black Dead Arm (BDA) in 2001, there is a worrying progression of these diseases in the vineyards around the world. The removal of sodium arsenite due to its toxicity on both environment and human health is causing concern to growers in that no satisfactory replacement control methods have existed up till today. The control methods available are prophylactic, and they involve the removal of cut wood and dead vineyard. Painting wounds with a drying product, following the ban, since 2007, of Escudo, fungicide to brush. Biocontrol, by treating size wounds with a preparation with *Trichoderma harzianum*, signifi cantly reduces the development of the pathogenic fungi Botryosphaeria, Phaeomoniella, and Phaeoacremonium. However, this last preparation showed its prophylactic efficacy in greenhouse only [5,6].

Fungi are generally controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment [7]. Wood diseases of the vine and many other trees are very complex, involving several fungi, and no effective control exists for the time being against this kind of pathogen. Natural products having components with various modes of action might provide an effective solution to some sanitary plant problems [8]. These products are now an endless source of interesting molecules for scientists and the industry. They have similar active ingredients that have specific properties giving them an intrinsic behavior [9]. Phyto compounds are expected to be far more advantageous than synthetic pesticides due to the sheer magnitude of their complexity, diversity and novelty of chemicals, reactions, and phenomena [10], as they are bio degradable in nature, non pollutant and possess no residual or phytotoxic properties [11].

Citrus essential oil (EO) has been identified in different parts of fruits as well as in leaves (particularly present in fruit flavedo), showing that limonene, β myrcene, α pinene, *p* cymene, β pinene, terpinolene, and other elements are the major aromatic compounds of many citrus species [12 15]. *Citrus limon* EO is used for many applications such as food, medicines, cosmetics and perfumes, detergents, aromatherapy, pathogen inhibition, and insect control [16], but, to our knowledge, no previous studies have been carried out on the use of *C. limon* EO against phytopathogenic fungi attacking grapevine wood trees or to control plant diseases.

This study aims to investigate, for the first time, the in vitro antifungal activity (AA) of *C. limon* EO against three phytopathogenic fungi (*Eutypa* sp., *Fomitiporia mediterranea* and *Botryosphaeria dothidea*) that attack grapevine wood trees. Also, this study determined the EO composition when extracted by steam distillation from the epicarp of *C. limon* fruits. The results showed that EO exerts a significant AA against the studied fungi attacking the wood of the grapevine and could be of use to control fungal diseases in agriculture.

## 2. Materials and methods

The present study also confirmed the AA of citrus oils already described in the literature, for example the antifungal efficacies of citrus oils against *Penicillium digitatum* and *P. italicum*, and found that *P. digitatum* was more sensitive to the inhibitory action.

### 2.1. Isolation of the EO

Fresh epicarps of lemon were collected from Blida (Northern Algeria) in April 2013. The EO was extracted by steam distillation of fresh plant material collected (100 g of epicarps). The bottles of oil were covered with aluminum paper to protect them from any negative effects of light and were stored in a refrigerator at a temperature of 4 °C.

### 2.2. Gas chromatography mass spectrometry (GC MS) analysis

The oils obtained were analyzed according the method previously used for lemon [17 19]. GC MS was performed using a PerkinElmer Clarus 600 mass spectrometer with a

silica capillary column of 50 m length and 0.22 mm inner diameter with 50 μm film thickness. Chromatograms were recorded with a temperature ramp with a 4 min step at 40 °C and a further increase up to 250 °C at a rate of 30 °C/min. Helium was used as the carrier gas at a rate of 1 ml/min. Oil samples (0.1 μl) were introduced directly into the source of the MS via a transfer line (280 °C) with a split ratio of 1:50. EO components were identified based on their retention indices (determined with a reference), calculated using Biot's law: $\alpha = [\alpha]\ lc$ where $[\alpha]$ is the specific rotatory power, $l$ is the length of the tank, and $c$ is the concentration of the solution. Individual components were identified by spectrometric analyses using two computer library MS searches. Visual mass spectra comparison data from the literature were used for confirmation, their relative retention index (RRI) was calculated in relation to the retention time of a series of alkanes (C7 C20) as reference chemicals.

### 2.3. Fungal material

The fungal material used to evaluate the efficiency of treatments based on the EO of lemon against three fungal strains was obtained from a personal collection: *F. mediterranea*, *B. dothidea*, and *Eutypa* sp., which were isolated from infected grapevine wood [2], identified using a combination of morphological and cultural characters and confirmed by molecular analysis (ITS and β tubulin primer). Cultures of each of the fungi were maintained on potato dextrose agar (PDA) and stored at 4 °C. AA was determined by using an in vitro volatility assay. The EO was dissolved in DMSO (dimethylsulfoxide) solution (97 and 3%, respectively) and four doses of this EO were prepared (0.25%, 0.50%, 0.75%, and 1%). Whatman paper discs of 8 mm diameter, previously sterilized by autoclaving, were first impregnated and saturated with 30 μl of each EO dilution, and then deposited on the lid of a Petri dish [16]. The control consisted of discs impregnated with the same volume of DMSO. All the Petri dishes were immediately closed and sealed with parafilm to prevent the evaporation of the oil. The plates were incubated at 25 °C. Mycelial radial growth was measured from the third day of incubation [20 22]. The inhibition percentage of mycelial growth was calculated as per the formula [17 19]: $(P_{lg} = (D_T - D)/D_T) \times 100)$, where $P_{lg}$ is percentage of growth inhibition, $D_T$ is the mean diameter of mycelial growth in the control, and $D$ is the mean diameter of mycelial growth in the treatment. The estimation of mycelial growth was carried out for 10 days from 3 days after the treatment with the EO. For a better measure of the growth diameter, digital pictures were taken of all plates, then treated with ImageTool software (3.1); three mea surements were taken for each diameter.

## 3. Results

### 3.1. EO extraction

EO was extracted from lemon epicarps (waste product) by the steam distillation method. The EO yield was 1.2%. Six components were identified in the EO by GC MS. The main components are presented in Table 1. The major constituents were limonene (61.69%), neral (21.66%), β pinene (10.23%), and $\gamma$ terpinene (6.42%) (Table 1). The identified components represent 99.9% of the total EO.

### 3.2. AA of EO

The AA recorded in this study, which represents the inhibition of radial growth on solid medium, revealed that the EO of *C. limon* possesses potential AA against *Eutypa* sp., *F. mediterranea*, and *B. dothidea* fungi. The effect of the EO dose (different concentrations) is summarized in Table 2. The tested doses inhibited the growth of fungus at all concentrations. At a concentration of 0.25%, the potency of the *C. limon* EO was greatest on *Eutypa* sp. (82% inhibition), followed by *B. dothidea* (48.1% inhibition) and *F. mediterranea* (33.1% inhibition). Statistical analysis of variance revealed a significant result. These results show that the EO significantly reduced the growth of all the tested fungi. Concentrations D4 (1%), D3 (0.75%) and D2 (0.50%) showed a greater inhibitory effect compared to D1 (0.25%). The optimal AA was obtained with concentration D4 (1%), whereas no inhibition was registered, even after 10 days with the control. Doses D4, D3 and D2 evolved from average toxicity at the beginning of treatment application to high toxicity up to 5 days, while dose D1 showed low toxicity at the beginning of its application and average toxicity at the end of the treatment (Table 2).

## 4. Discussion

The diseases caused by *F. mediterranea*, *B. dothidea* and *Eutypa* sp. result in significant losses in a variety of economically valuable agricultural crops. Infection due to these fungal pathogens has become more common. Over the last decades, concerns have been expressed about the increasing prevalence of pathogenic fungi that are resis tant, but no effective control exists for the time being against these kinds of fungi. For this reason, in the last decade, scientists have conducted an increased number of intensive studies on extracts and biologically active compounds isolated from natural plants [23,24]. Our results showed that the EO yield from *C. limon* fruit epicarp was 1.20%. This result is in agreement with the literature data for EO from *C. sinensis* [14]. Chemical analysis of the EO of *C. limon* led to the identification of several molecules. Its chemical composition is dominated by the presence of hydrocarbon monoterpenes. The main

**Table 1**
Composition of *C. limon* EO collected from the Blida region (Algeria).

| Pic | Compound | Molecular formula | Retention time (min) | % |
|---|---|---|---|---|
| 1 | Limonene | $C_{10}H_{16}O$ | 26.45 | 61.68 |
| 2 | Neral | $C_{10}H_{16}O$ | 44.32 | 21.66 |
| 3 | β-Pinene | $C_{10}H_{16}O$ | 24.04 | 10.23 |
| 4 | γ-Terpinene | $C_{10}H_{16}O$ | 27.86 | 6.42 |
| 5 | β-Myrcene | $C_{10}H_{16}O$ | Trace | – |
| 6 | Geraniol | $C_{10}H_{18}O$ | Trace | – |
| Total | | | | 99.9% |

**Table 2**
Antifungal activity (expressed as minimum inhibition concentration MIC, mm) of *C. limon* EO at different concentrations for different periods of time.

| Fungi | Period (days) | Treatment | | | | |
|---|---|---|---|---|---|---|
| | | D1 (0.25%) | D2 (0.50%) | D3 (0.75%) | D4 (1%) | Control |
| *B. dothidae* | 5 | 15.10 | 12.5 | 11.7 | 11.4 | 18.3 |
| | 10 | 13.2 | 9.65 | 9.07 | 8.04 | 13.73 |
| | 15 | 33 | 29.94 | 28 | 25.69 | 33.29 |
| *F. mediterrania* | 5 | 13.5 | 10 | 9 | 5 | 4.8 |
| | 10 | 14 | 9.47 | 8.44 | 7 | 17.64 |
| | 15 | 26.99 | 15.2 | 10.16 | 7.2 | 32.05 |
| *Eutypa* sp. | 5 | 7 | 7.1 | 7.6 | 5.4 | 6 |
| | 10 | 12.82 | 13.77 | 12.58 | 11.2 | 14.08 |
| | 15 | 23.2 | 20.68 | 17.2 | 13 | 24.5 |

representative of monoterpenes is limonene (Table 1). Studies of other citrus species such as *C. limonum*, *C. sphaerocarpa*, *C. sinensis*, and *C. reticulata* also showed limonene as the major component, about 70%, in the oils [12,13,17 19]. The other abundant monoterpenes in oil are β pinene, γ terpinene, and β myrcene (Table 1). The composition of many EOs has been described in the literature; it varies according to different factors, including the developmental stage of the plants, the organs harvested, the period, and the geographical area of harvesting [25].

The obtained antifungal results showed pronounced activity of the EO extracted from *C. limon*. These results are in agreement with the work of Sharma and Tripathi [26], who showed the effect of EO from *C. sinensis* (L.) Osbeck epicarp on the growth and morphogenesis of *Aspergillus niger*. The evaluation of the antifungal activity of oils revealed their inhibitory effect against all the fungal strains at all concentrations, but the results indicate that higher concentrations inhibit fungus more efficiently than when diluted. In the work of Sharma and Tripathi [14,26], mycelial growth was inhibited at 2.5 and 3.0 mg/ml of oil in PDA and agar medium, respectively. The EO of citrus significantly reduced the growth of *A. niger* in a dose response manner in earlier fungitoxic investigations on the EO of *C. sinensis* [14,26]. According to the statistical analysis, the oils exhibited different degrees of inhibition on the growth of the tested fungi; the species tested were the most sensitive to the action of the oil. Caccioni et al. [12] tested the antifungal efficacy of EO from *C. sinensis* against *P. digitatum* and *P. italicum*. They found that *P. digitatum* was more sensitive to the inhibitory action of the oils. *Citrus* species are known to possess AA; these observations indicate that the mode of AA of *C. sinensis* EO results from the attack of the cell wall by the oil, from the retraction of the cytoplasm in the hyphae, and, ultimately, from the death of the mycelium [26]. Generally, terpenoids and phenylpro panoids give EOs their antimicrobial properties [27]. The activity of these molecules depends both on the lipophilic character of their hydrocarbon skeleton and on the hydrophilic nature of their functional groups. The chemical structure of the constituents of EOs directly influences their activity [28]. The nature of the alkyl groups can influence this activity: alkenyl substituents are more active than alkyl substituents. In this study, the chemical results indicated that *C. limon* EOs are characterized by a relatively high content of limonene, which is known to possess important AA. Thus, limonene, which is substituted with an isopro pylene group at the 4 position, has a higher activity than its *p* cymene counterpart, substituted by an isopropyl group [29]. Interactions between the constituents of EOs may also affect their activity. For example, the effectiveness of the EO of thyme against *Staphylococcus aureus* and *Pseudomonas aeruginosa* is due to a synergy between its main constituents, carvacrol and thymol [30]. As the antimicrobial nature of EOs is apparently related to high terpene content, Klaric et al. [31] and Pinto et al. [9] proved that the higher the terpene level, the more EOs are effective, and they have a broad spectrum of activity against filamentous fungi and insects. Lahlou [32] reported that the activity of an EO is higher than that of its major component tested separately. Indeed, many authors have shown that not only phenols are responsible for the activity; all of the chemical composition should be taken into account [33]. We can conclude that the synergism between components does play an important role. As a result, plant secondary compounds possess several modes of action on fungal strains, but, in general, their action takes place in three phases: attack of the cell wall by the plant extract, resulting in increased permeability and loss of cellular constituents, acidification of the inside of the cell, blocking the production of cellular energy and synthesis of structural components, and, finally, destruction of genetic material leading to the death of the fungus [34].

According to the statistical analysis, the volatile oils exhibited different degrees of inhibition on the growth of the tested fungi. The obtained results showed that EO extracted from *C. limon* prevented the growth of the tested microorganisms with an average inhibition zone diameter increasing proportionally with the concentration of the tested samples, the most promising plants belonging to the Meliaceae, Rutaceae, Asteraceae, Annonaceae, Abiateae, and Canellaceae families. A wide variety of EOs are known to possess antimicrobial properties and, in many cases, this activity is due to the presence of active constituents, mainly attributed to isoprenes such as monoterpenes, sesquiterpenes, other hydrocarbons and phenol. In this study, the chemical results indicated that *C. limon* EO is characterized by a high proportion of limonene, which is reported to possess high activity towards microorganisms.

## 5. Conclusion

*C. limon* EO caused the greatest growth inhibition of *Eutypa* sp. (IM: 82%), followed by *B. dothidea* (IM: 48.1%)

and *F. mediterranea* (IM: 33.1%) at a concentration of 0.25%. This dose showed low toxicity at the beginning of its application, increasing to average toxicity at the end of treatment (after 15 days). All tested concentrations were found to be lethal under the test conditions. Based on the present study, it can be concluded that the EO of *C. limon* possesses fungitoxic activity, inhibiting the growth of the phytopathogenic fungi tested. It would be interesting to assess the morphological alterations and the modes of action of this oil on phytopathogenic fungi in later studies.

## Disclosure of interest

The authors declare that they have no competing interest.

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