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ANTIFUNGAL ACTIVITY OF THE LEAF ESSENTIAL OIL OF CITRUS AGAINST ALTERNARIA ALTERNATA IN VIVO

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ABSTRACT

The essential oils were extracted by hydro-distillation from the leaves of citrus by using hydrodistillation. The largest yields were recorded with *Citrus limon* L (1.02%) and *Citrus sinensis* (0.96%).GC/SM of the essential oil of *Citrus* reveled limonene (7.18 –36.10%), ß- pinene (4.35 - 30.0%) and linalool (0.21 –63.03%) as the principal major compounds. Essential oils of citrus exhibit a strong inhibiting effect on the development of *Alternaria alternata* on leaves and potato. Low concentrations generate the lowest severity indexes of disease for all citrus species. According to the results, the essential oils of citrus could be used as potential antifungal agents for the control of *Alternaria alternata in vivo*.

KEYWORDS: Citrus, Essential Oils, *Alternaria Alternata*, Potato, *In Vivo* Antifungal Activity

INTRODUCTION

The phytopathogenic fungi are known to cause several plant diseases and yield losses for many economically important crops (Fletcher et al., 2006; AbdelKader et al, 2012.). In agriculture, each year there are about loss of efficiency of about 20% mainly due to fungal diseases (Bajwa et al., 2004).

The genus Alternaria includes saprophytes and plant pathogens (Thomma, 2003). It has over 100 species ubiquitous extremely common in soils, vegetation, air or food (Calmes, 2011). Due to their growth, even at low temperature, Alternaria spp are pathogens postharvest familiar, responsible for food spoilage during refrigerated transport and storage (Ostry, 2008). The economic losses are mainly related to the reduction in quality due to a decrease in the nutritional value (Kosiak et al., 2004).

Algeria is the second largest potato producer in the Arab world and in Africa after Egypt with a production of 4.219.476.00 ton in 2012 (FAOSTAT 2014). *Alternaria alternata* is one of prevalent pathogens causing brown leaf spot on potato worldwide (Thomma, 2003).

The disease brown spots on leaves is known as one of the most destructive and communes of potatoes cultivated in regions with frequent precipitation and high relative humidity conditions. The disease can occur on a wide range of climatic conditions (Pitt and Hocking, 2009).

Studies were conducted *in vitro* and *in vivo* to evaluate the antifungal activity of essential oils of citrus and their active compounds. They reported their inhibitors powers against some phytopathogenic fungi such as Alternaria, Fusarium,

Penicillium and Aspergillus (Sharma and Tripathi 2008; Viuda-Martos et *al*., 2008; Cosic et *al*, 2010;. Gumus et al. 2010; Philips et *a*l, 2012; Hamdani et al, 2015; Hamdani et allem, 2015). This study aims to evaluate the antifungal activity of essential oils of citrus in vivo against *Alternaria alternata.*

MATERIALS AND METHODS

Plant Materials

Fresh leaves of *Citrus sinensis*, *Citrus aurantium* and *Citrus reticulate* were collected from the adult trees of the orchards of Chlef, Algeria during Mars (2011).

Fungal Strain Used

Alternaria alternate was providing from the Culture Collection of the laboratory of the pathology of the plants, Institute of agronomic sciences, university of Chlef. The fungal strain cultures were maintained on a Potato Dextrose Agar (PDA) at 22 ± 2° C.

Extraction of Essential Oil

Fresh leaves of *Citrus sinensis*, *Citrus aurantium* and *Citrus reticulate* were hydrodistilled in a Clevenger-type apparatus for 3h to obtain essential oil. 300g of leaves were in boiling water during distillation. The obtained essential oils were dried over anhydrous sodium sulphate and kept at 4 °C for other experiments. Yield of leaf essential oil was 1.02 %.

Analyze GC / MS

The composition of the oil was analyzed using Hewlett–Packard 6890 gas chromatograph and Agilent Technologies HP 5973N mass spectrometry equipped with a HP5MS capillary column (30 m x 0,25 mm, thickness of film 0,25 µ m). Oven temperature was initially held 60 ℃ during 2 min, then increased 260° C at a rate of 5 °C/min and held for 10 min. Carrier gas was helium at a flow rate of 1 (ml /min). Components were identified by comparing their mass spectra with Wiley and NIST library data and standards of the main components. Quantification of components was obtained by integrating the peak area of the chromatogram (Hamdani et al, 2015) .

Antifungal Activity on Potato Leaf

The evaluation of the antifungal potential of the essential oil *in vivo* was determined by direct contact, the essential oil is added directly to the culture medium to allow good adhesion of the oil with all of the leaf area and reduce the risk of evaporation of the essential oil (Feng et al., 2011).

After disinfecting a surface followed by several rinses with distilled water, leaflets were placed in Petri dishes 9 cm in diameter on water agar 4% to ensure saturating humidity throughout the test.. Inoculation of the leaflets was made using 20 µl of a spore suspension (10^5spores/ml) divided into six points. The dilution of essential oil was made in ethanol (10 µl/ml ethanol) to prepare solutions at different concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg/ml).

The essential oil was tested using three treatments application namely preventive, curative and at the same time. For preventive tests, the essential oil is used 24 hours before inoculation of the leaflets by the spore suspension and it is used 24 h after inoculation for curative tests. for the test at the same time, the essential oil is supplied 5 min after inoculation with the spore suspension. Controls without essential oils for each test were prepared in the same as the treatment. .

The Petri dishes are incubated in the dark for 48 hours and after placed in the light under ambient laboratory conditions (El abdellaoui et *al.*, 2005). Each test is repeated three times. Scoring the disease severity is calculated by reference to leaf area and estimated from the rating scale Notteghem et al. (1980). The severity index is calculated using the formula:

$IS= (\sum X_i n_i/9Nt)*100$

IS:Disease severity index.

Xi:Disease severity.

ni:number of leafs with the severity of the disease.

Nt: Total number of leafs

Antifungal Activity on Wound-Inoculated Potato Tuber

Experiments were conducted with commercial potato tuber (*Solanum tuberosum*) var Spunta of Chlef, Algeria. Fresh potato tubers were harvested at the mature stage and sorted based on size and absence of physical injuries or disease infection. Surface potato tubers were sterilized and prepared for inoculation by inflicting a deep rind wound as described by (Entsar and Badawy, 2012). The tubers were randomly distributed into groups of ten tubers and three replicates were used for each treatment. The first treatments (same time) tubers were dipped in the essential oil solution of concentration 1 mg / ml diluted in ethanol for five minutes followed by a dipping in the inoculums for 3 minutes.

Preventative treatments were done by an initial applying of *Citrus* oil by spraying (1000 **µg**/ml) to injured potato tuber for five minutes, 24 hours before soaking in the inoculum for 3 minutes (Plooy et al., 2009). Control treatments consisted of spraying the wounded potato tuber with sterile water only, before or after inoculation as relevant. Potato tubers inoculated were incubated in an incubator at 20 ± 2 ° C. After fifteen days of incubation, they were revealed to be stored under ambient laboratory conditions for two months. After storage, the number of wounds that showed disease symptoms was recorded and disease incidence (%) was calculated as follows:

$$\text{Disease incidence } DI(\%) = \left(\frac{\text{number of infected wounds}}{\text{total wounds per replicate}}\right) x100$$

Statistical Analysis

Mean values and standard deviations were calculated for all tests and were subjected to statistical analysis using Statistica.06, Statistical package (Statsoft, 1995). Differences between means were tested using test LSD of Fisher

Analysis GC / MS of the Essential Oil of Citrus

Essential oils are extracted from the sheets of *Citrus sinensis*, *Citrus aurantium* and *Citrus reticulate* by hydro distillation. The yields obtained are 0.96, 0. 51 and 0.73% respectively for *Citrus sinensis*, *Citrus aurantium* and *Citrus reticulata*. The qualitative and quantitative analysis of the chemical profiles of essential oils is indicated in Table 1. The table in cludes the time of retention and percentage of the 51components identified, accounting for 92.47 to 98.82% of all the components. Using a capillary column HP5MS, chromatograms of three essential oils of citrus tested were obtained and represented in figure 1.The outputs obtained from the essential oils are: The results obtained proved that there are

qualitative similarities between the four essential oils, although the quantities of some corresponding compounds are different. 10 componds were recorded jointly among the 51 identified, where limonene (7.18 –36.10%), ß- pinene (4.35 - 30.0%) and linalool (0.21 –63.03%) represented the principal major compounds. The greatest volatile fraction returns of monoterpenes hydrocarbons for *Citrus sinensis*, *Citrus reticulata* are 73.64 and, 63.75 respectively. The oxygenated monoterpenes explained approximately 71.85% of the identified components of the essential oil of *Citrus aurantium* .The high percentage of monoterpenes in essential oils of citrus has been reported by several studies (Baaliouamer 1987; Settanni et al, 2012.).GC/SM of the essential oil of *Citrus reticulata* revealed the presence of strong concentrations in γ Terpinene (26.62%), limonene (22.52%) and ß-pinene (4.35%) representing 53.49% of the total surface of the peak(Table 1). The essential oil of *Citrus sinensis* is characterized byhigh concentrations in ß-pinene (30%), limonene(9.37%) and the presence of two is omerssesquiterpenes (Z) and (E) ß - Elemene with apreponderance of the isomer (E) ß – Elemene (8.97%). For the essential oil of *Citrus aurantium*, linalool (63.03%)

Antifungal Activity on Potato Leaf

The virulence of Alternaria alternata was confirmed by the presence of typical spots on the leafs. The results of the effect of different concentrations of essential oils of *Citrus sinensis, Citrus aurantium* and *Citrus reticulata are shown in Figure* 1.It shows that the essential oil of *Citrus reticulata* exposes a strong antifungal power. The same inhibitory potency was observed *in vitro* (Hamdani et allem, 2015)The concentrations of 0.0625, 0.125 and 0.25 mg / ml generate lowest severity index of the disease.

The concentrations of 0.5 and 1 mg / ml involve the appearance of the signs of phytotoxicity and leafs have translucid leaf surfaces of yellow-brown to dark brown. This condition increases the mycelial growth of *Alternaria alternata*.

Feng and Zheng (2007) note that the development of *Alternaria alternata* was inhibited by high concentrations of essential oil of *Cinnamomum aromaticumin vivo*on tomato. Tian et al. (2011) obtain a reduction in the development of *Alternaria alternata* 83.3% on cherry tomatoes treated with the essential oil of *Anethum graveolens* L.

The necrotic spot wasreducing in preventive treatment. Reducing of necrotic spots in preventive treatment may be attributed to the contact time of the essential oil (24 h prior to infection) with the leaf which promotes the increased resistance of plant against *Alternaria alternata*

Several studies mention the contribution of essential oils in increasing plant resistance against external aggression and provide an important defense strategy for plants, in particular against parasitic insects herbivores and phytopathogenic fungi(Langenheim, 1994; Dayan et al., 2009). They act as molecular signals and illustrate the evolutionary links with their functional roles Theis ET Lerdau, 2003).

Antifungal Activity on Wound-Inoculated Potato Tuber

The results (Figure 3) illustrate the inhibitory effect of essential oils applied in preventive treatment compared with the control. It records disease indexes in the order of 0% for essential oils of *Citrus sinensis, Citrus aurantium* and *Citrus reticulata.*, this inhibitory effect disappears when the essential oils are used in processing simultaneously. Changes in the shape and firmness accompanied by the appearance of mycelial growth at areas of injury were observed. Plooy et *al*., (2009) by testing both preventive and curative treatment, they notice that the application of the essential oil *Lippia scaberrima* preventively reduced significantly the percentage of inoculated citrus fruit with *Penicillium digitatum*

compared

CONCLUSIONS

Essential oils of citrus exhibit a strong inhibiting effect on the development of *Alternaria alternata* on leaves and potato. Low concentrations generate the lowest severity indexes of disease for all citrus species. The results obtained suggest the use of essential oils in food preservation. Effectively, considerable interest has developed on the conservation of food by the employ of essential oils to pronounce the effect on slowing growth and limiting the production of mycotoxins (Smith et al., 2005; Omidbeygi et al., 2007)

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APPENDICES

Table 1: Chemical Composition of Essential Oils of *Citrus sinensis, Citrus aurantium* and *Citrus reticulata*

Composants	KI	RT(Min)	Citrus Reticulata (% Area)	Citrus Sinensis (% Area)	Citrus Aurantium (% Area)
3 hexen- 1-ol	866	4.71	-	-	0.91
α-Thujene	931	5.81	2.00	1.49	0.02
α -Pinene	939	5.99	4.36	2.04	0.38
camphene	953	6.37	0.10	-	0.03
β - Pinene	979	7.12	4.35	30.0	5.25
β - Myrcene	991	7.46	1.19	4.52	1.40
α Phellandrene	1008	7.86	0.10	0.68	-
3 Carene	1012	8.02	-	6.41	0.16
(+)-4-Carene	1035	8.20	0.38	2.77	-
D-Limonene	1036	8.71	22.52	9.37	7.18
β Ocimene	1055	8.74	-	0.74	-
1,3,6-octatriene,3,7-dimethyl-	1070	9.11	0.94	8.59	1.13
γ Terpinene	1080	9.41	26.62	4.42	-
Cis sabinene hydrate	1094	9.77	-	0.21	-
Terpinolene	1096	10.25	1.16	2.53	-
Linalool	1128	10.71	0.21	1.24	63.03
Z Alloocimene	1152	11.38	0.03	0.08	-
Citronellal	1159	12.06	-	0.90	-
Terpinen-4-ol	1177	12.94	0.15	2.14	0.43
α Terpineol	1189	13.34	0.10	0.07	1.00
Citronellol	1226	14.36	-	1.05	-
Citral	1254	14.70	-	-	0.46
1,6-octadien-3-ol,3,7-dimethyl	1280	14.87	0.11		5.79
2,6-octadienal,3,7-dimethyl	1301	15.45	-	0.22	0.15
Thymol	1360	16.97	0.30	-	-
σ-Elemene	1370	17.23	-	-	0.05
Cis-2,6-Dimethyl-2,6octadiene	1382	17.53	-	0.45	-
Benzoic acid 2-amino-	1392	17.79	0.09	-	-

methyl					
2,6-octadien-1-ol,3,7dimethyl	1394	17.84	-	0.22	0.99
Copaene	1408	18.20	-	-	-
(Z)*β* Elemene	1393	18.45	-	0.44	-
(E)*β* - Elemene	1398	18.71	-	8.97	0.08
*C*aryophylene	1417	19.41	-	3.48	2.62
Methyl N-methylanthranilate	1418	19.74	34.02	-	-
Aromadendrene	1439	19.93	-	-	0.05
(E)*β* Famesene	1458	20.20		0.65	0.12
α -humulene	1474	20.29	-	1.33	0.39
β -Selinene	1485	21.13	-	0.47	-
γ Elemene	1498	21.35	-	-	0.26
α -Farnesene	1509	21.49	-	0.61	-
γ -cadinene	1513	21.80	-	0.56	0.01
δ -Cadinene	1514	21.95	-	0.22	0.08
Nerolidol	1534	22.95	-	-	0. 37
*C*aryophylene oxide	1606	23.50	0.09	0.23	0.04
α Cadinol	1654	25.26	-	-	0.04
β Sinensal	1706	26.04	-	0.72	-
α Sinensal	1750	27.27	-	0.30	-
β Farnesol	2150	33.89	-	-	-
Phytol	2178	34.36	-	-	0.05
	Monoterpènes hydrocarbonés (%)		63.75	73.64	15.55
	monoterpènes oxygénés (%)		34.98	6,05	71.85
	Sesquiterpène hydrocarboné (%)		-	15.95	3.66
	Sesquiterpènes oxygénés (%)		0.09	1.25	0.45
	autres (%)		-	-	0.96
	Total identifié (%)		98.82	96.89	92.47

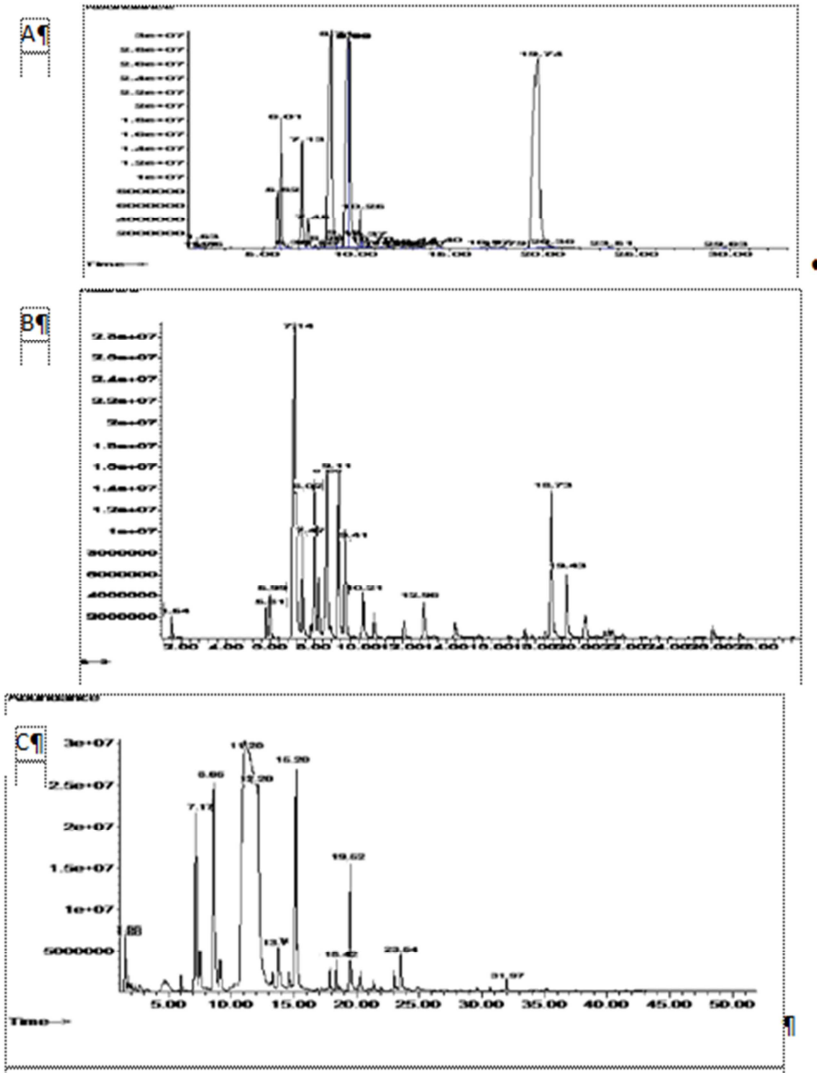
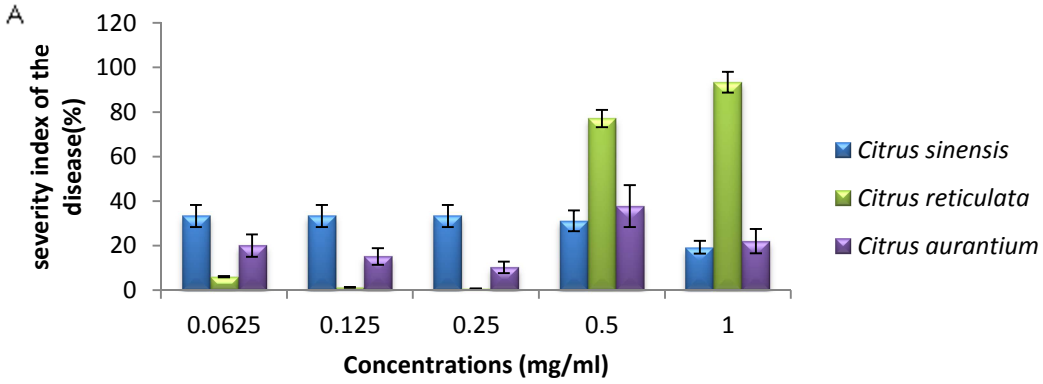


Figure 1: Chromatogram of Essential Oils of Citrus Leaves A: *Citrus reticulata* Blanco B: *Citrus sinensis Osbeck;* C*: Citrus aurantium* amara



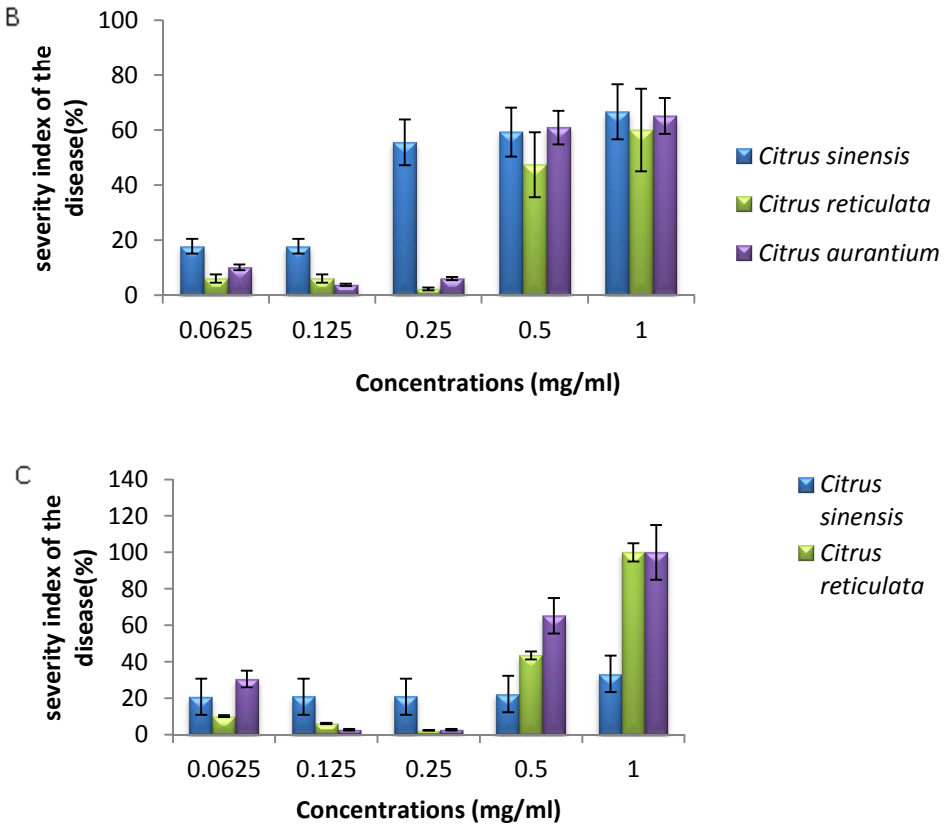


Figure 2: Effect of Concentrations of the Essential Oils of Citrus on the Index of Disease Severity (Test on Leafls) to: Curative, B: Preventive and C: The Same Time. Data Are Means ± Standard Deviations (Error Bars)

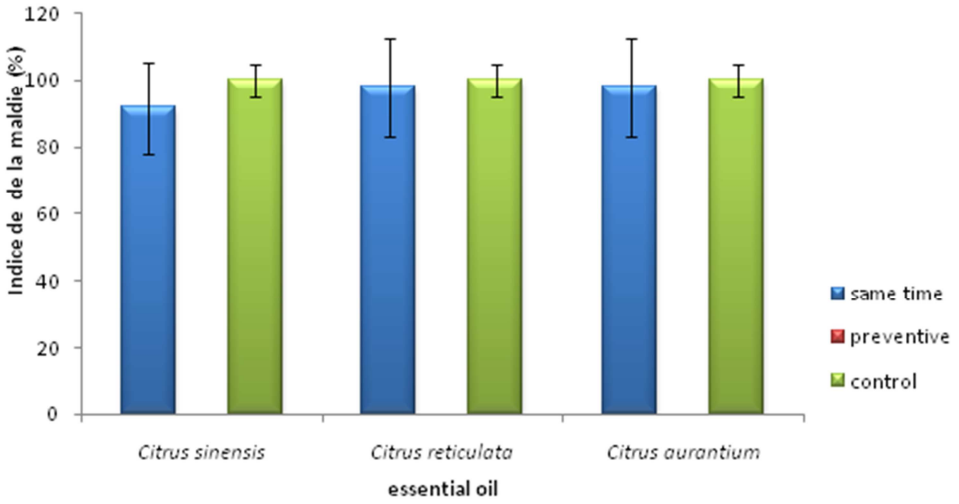


Figure 3: Effect of Applying Essential Oils of Citrus on the Index of the Disease (On Potato Tuber). Data Are Means ± Standard Deviations (Error Bars)