Inter. J. Acad. Stud. 3(6):(2017) 104-128
Journal home page: www.ijacademicstudies.com

International Journal of Academic Studies

Effects of *Phoracantha recurva* Newman, 1840 (Coleoptera: Cerambycidae) attacks on the content and chemical composition of essential oils of *Eucalyptus grandis* leaves and its clone 977 and evaluation of their potential bioactivity against xylophagous organisms (fungi and insects)

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ARTICLE INFO

Article history:
Received 18th May 2017
Received in revised form 16th June 2017
Accepted 27st June 2017

Key words:

Phoracantha recurva,
Eucalyptus grandis,
Clone 977
Essential oil
Fungicide
Termiticide

ABSTRACT

Clonal eucalyptus are highly valued in Morocco and in the Mediterranean basin for the production of industrial wood, but nothing is known about their ability to resist xylophagous plants. This study aims to investigate the chemical defenses response of eucalyptus trees to artificially attacks by young larvae of *Phoracantha recurva* by assessing changes in essential oil production and composition of leaves from *Eucalyptus grandis* and its clone 977. Fungicidal and termicidal essential oil activities were evaluated to value their biocidal properties. In the Northwest region of Morocco, six of vigorous eucalyptus-trees were selected for each wood species studied. In contrast to a control tree without infection, three from each species were artificially infested in three part of the trunk by hatched larvae of *P. recurva.* After two months, the most important cambial reaction was observed with *E. grandis* compared to clone 977. For both species, the primary reaction is less important compared to the reaction of deep tissues. The essential oils recovered by hydrodistillation show that the infested trees have the highest average yield. Arian parties are 1.51% and 2.36% respectively for *E. grandis* and its clone 977, after infestation, we can observe a decrease of the production of essential in the leaves, respectively 1% and 1.5% for *E. grandis* and its clone 977. The analysis of the chemical composition by GC-MS method of essential oils shows that the leaves of infested trees of *E. grandis* and its clone 977, have specific constituents resulting from infestation such as Endo-fenchol, trans-carveol, Nerol and Spathulenol. Equally, this analysis revealed that the attacked trees hemi-synthesized more components compared to their controls. However, the evaluation of fungicidal and termiticidal activities showed that the essentials oil of the clone 977 is more active compared to the parental species *E. grandis*.

1. Introduction

The need for timber and wood industry in Morocco is growing despite the decline in the area and the vulnerability of natural forests (Goujon, 1961). Climate changes observed in recent times are another handicap that does not promote sustainability and multifunctionality of Moroccan forests. For reducing pressure on natural forests and meet energy needs of rural and urban population in firewood, plantations of various species of eucalyptus have been massively put up in Morocco. This exotic species, fast growing, has opened new perspectives in industrial production pulpwood for pulp (MCEF, 1998). An ambitious program of genetic improvement of introduced eucalyptus species has been developed since 1987 by the Department of Forestry. Several clones deemed interesting, to increase wood productivity, have emerged and have been widely planted in Morocco the last twenty years (MCEF, 1998) due to their rapid growth and their ability to survive into a large type of terrain.

However, these species planted outside their natural range, are threatened by fungal action (chlorosis) or specific insects. *Phoracantha recurva* Newman, 1840 (Coleoptera: Cerambycidae) is the main factor who threatens eucalyptus in Morocco and throughout the Mediterranean region. One of the most practical measures that could prevent further spread of this xylophagous pest, clearance and cutting of all attacked and dead trees then removing them from the forests. Moreover, there is no satisfactory method for controlling longicorn beetle. Regarding the weather, the adoption of appropriate and adaptive management practices and the use of vigorous trees are now widely recommended. At the same time, the search for biologically active molecules is highly desirable when we study the trees defense mechanism for developing new biological control strategies. This approach has been developed in some northern species (Morewood *et al.*, 2003). This new process will serve as a platform for strategies against other wood-eating pests.

The plant defense mechanisms against pathogens and herbivores are various and more complex (War *et al.*, 2012). Each host plant has one or more strategies enabling it to face the aggression of the parasite. These strategies are based on the development of biochemical and molecular parameters that determine the type of resistance (Zouiten, 2002). Phenolic compounds are group of secondary metabolites widespread in the plant kingdom, and they are often involved

in plant defense against insect, fungal or bacterial attack (Freeman & Beattie, 2008; Carmona *et al.*, 2011). They also have a significant effect in repelling plants regarding many insects (Havlickova et *al.*, 1998) and other harmful agents (Hedin et *al.*, 1983; Hedin et *al.*, 1991; Hartley & Lawton, 1991; Zouiten, 2002; Akhtar & Isman, 2004).

As yet, there is no information regarding eucalyptus clones strategy to prevent *Phoracantha* sp. attack and the biochemical factors involved in this resistance. The present work is the exploration of metabolic pathways activated after artificial infestations of two eucalyptus species of great economic value to the country. Indeed, the defense reactions of the clone 977 and its parental species *E. grandis* were examined. The analysis focused on the change in the chemical composition of the leaves by extraction and analysis of their essential oils (before and after infestation) then on the evaluation of their biocidal activities against wood decay fungi and termites.

2. Materials and Methods

Rootstock

Three trees of *Eucalyptus grandis* from Sidi yahya Gharb forest (N: 34 20 541; W: 006 18 695) and three of his clone 977 from Sidi Amira forest (N: 34 02 719; W: 006 40 520) were infested with hatchings larvae of *P. recurva*. Each tree received sixty larvae on three grooves made spiral in the longitudinal direction of the shaft (r/r cambium). Outbreaks occurred in June 2012 period corresponding to a sharp reproduction of the pest. After a period of two months, perforated larval galleries has been noted and characterized on each tree and the induced reaction at the cambium was measured in length and width. Its mean area (S = length x width) and standard error (SE) has been calculated and compared among the two study species by using non-parametric Mann-Whitney U test. We also selected three trees from each species as control.

2.1. Extraction of essential oils (EO)

Samples of eucalyptus leaves from infested trees and their control (not attacked trees) were collected for each species (*E. grandis*, 977 clone). The extraction of essential oil was performed by steam distillation in a Clevenger-type apparatus (Clevenger, 1928). Three independent distillations were performed by boiling a half hour of 200 g of fresh plant material with a liter of water in 2 liters flask surmounted by a column of 60 cm in length

connected to a condenser. The yield of essential oil was determined by reference to the dry matter evaluated from three samples of 30 g dried 48 hours in an oven at 60°C. The essential oil was stored at 4°C in the dark in the presence of anhydrous sodium sulfate. It was diluted in methanol (1% v/v) before perform the analyses.

Chromatographic analysis

Chromatographic analysis were performed on a gas chromatograph electronically controlled Hewlett Packard type pressure (HP 6890 series) equipped with a capillary column HP-5 (30 mx 0.25 mm) with a film thickness of 0.25µm, with an FID detector powered by a gas mixture of H2 / air and a split-splitless injector set at 250 °C. The injection mode is split (leak report: 1/50, flow: 66 ml min^{-1}). The gas used was nitrogen with a flow of 1.7 mL min^{-1}. The column temperature is programmed from 50 to 200 °C at 4 °C min^{-1} for 5 min. A computer system type "HP ChemStation" managing the operation of the device and to track the evolution of chromatographic analyzes controls the unit. Identification of components was performed based on their indices Kováts (IK) and the gas chromatography coupled to mass spectrometry (GC-MS). The latter was performed on a gas chromatograph Hewlett-Packard type (HP6890 series) coupled to a mass spectrometer (HP 5973 series). Fragmentation was done by electron impact in a field of 70 eV. The column used was a capillary column HP-5MS (30 m x 0.25 mm), the film thickness is 0.25µm. The column temperature was programmed from 50 to 200 °C at 4 °C.min^{-1} for 5 min. The carrier gas was helium with a flow rate set at 1.5 mL min^{-1}. The injection mode was split (leak report: 1/70, flow 112 ml min-1). The device was connected to a computer system running a NIST9 mass spectral library.

The identification of the components was based on the comparison of their mass spectra (GC / MS) spectra with the respective library (NIS 98) and on the basis of calculating indices Koválts (Jalili & Sereshti, 2007).

The percentage of identified components was estimated from the peak areas for each compound without any correction for essential oils of the leaves of *Eucalyptus grandis* and its clone 977.

2.2. Antifungal Test

Four wood-fungal species *Gloeophyllum trabeum* (Persoon ex Fries) Murril, *Poria placenta* (Fries) Cooke senu J. Eriksson, *Coniophora puteana*

(Schumarcher ex Fries) Karsten, and *Trametes versicolor* (Linneaeus) Quelet which cause brown and white rots wood, were tested. These four fungal strains were originated from the collection of the Microbiology Laboratory of Forest Research Centre, Rabat, Morocco. These species were chosen because of the considerable damage, which they cause to timber and derived products. The strains were grown on nutrient media PDA (potato dextrose agar) for seven days at 25°C and in the dark.

The evaluation of the antifungal activity of essential oils was carried out according to the direct contact method reported by Remmal *et al*., (1993) and Satrani *et al*., (2001). The technique consist to add essential oils at different concentrations in the middle of still liquid culture at a temperature of 56°C before placing the mycelium and then determine the minimum inhibitory concentrations (MIC) of essential oils have been determined.

Due to the immiscibility of essential oils with water and therefore to the culture medium, an emulsification was conducted with an agar solution 0.2%. It provides, in the culture medium, a homogeneous distribution of essential oils to maximize the contact mycelial agent/essential oil. Dilutions are prepared 1 / 10th, 1 / 25th, 1 / 50th, 1 / 100th, 1 / 200th, 1 / 300th and 1 / 500th, 1 / 600th, 1 / 700th, 1 / 800th, 1 / 900th and 1 / 1000th in the agar solution. The tubes were agitated to disperse the essential oil in the culture medium before pouring into Petri dishes. Controls containing the culture medium and the agar solution at 0.2% alone were also prepared to ensure the virulence of the strains.

Contamination was carried out by deposit of fragments of 1 cm in diameter, taken from the periphery of a mycelial mat and from a culture of 7 days in malt extract. Incubation is at 25 °C for 7 days for fungi. For each essential oil and each fungus test, the bioassa was carried out in trireplicate. The analysis focused on the essential oils of *Eucalyptus grandis* leaves and its clone 977 before and after infestation (Control).

MIC is situated between the concentration corresponding to the mycelial growth and the concentration corresponding to an absence of growth. The MIC determination allows the evaluation of the antifungal activity of EO tested.

Antitermite test

The anti-termite activity of essential oils was performed according to the non-choice test. This study makes it possible to test the preventive efficacy of treatment oils with

regard to termites. Indeed, it makes it possible to judge the value of the treatment product studied and its ability to protect preventively the wood. The latter is applied by a surface treatment (Chang & Cheng, 2002).

Antitermite test involves impregnation of a cellulose support with different concentrations of essential oils and they were place in the presence of termites. The insecticide potential of EO extracted from control and infested trees was evaluated at two concentrations: 50% and 100% (v/v) on termites (*Reticulitermes flavipes*). 20µL of each test solution was applied to 1cm² (1x1 cm) a cellulose support (Whatman 1001185). These treated cellulose were dried for 4 hours (20 °C and 65% RH) and placed in the middle of a petri dish containing moistened Fontainebleau sand 20g (1 part water to 4 parts sand) with 50 workers. We performed a control group with the cellulose support treated with deionized water to ensure the virulence of the termites, and a second set called 'diet' containing no cellulose support is used to evaluate the survival of termites without source of food. The analysis focused on the essential oils of *Eucalyptus grandis* leaves and its clone 977 before and after infestation (Control). Essential oils have been tested pure. Stock solutions are diluted again to 50% in ethanol.

For each test solutions, control lots and diet lots, trials were conducted in trireplicate. All petri dishes were placed in a culture chamber (27 °C and 75 ± 5% RH) for 8 days. Every 2 days a visual inspection was used to evaluate the consumption of cellulose support and the workers mortality.

During the period of the test, regular observations every 2 days until the end of the test, a visual examination of the degradation of the papers is carried out. The papers are scanned for comparison with the initial surface (before testing). The survival rate of the workers was calculated.

The results were subjected to parametric statistical treatment by the Kruskal-Wallis test at the 0.05 (5%), which will decide on the validity of the termite test.

3. Results and Discussions

1. Monitoring reactions after infestation

Subject to attacks *P. recurca* larvae, *Eucalyptus*, like many other species, implements defense mechanisms to circumscribe the contested area to avoid any risk of exposure to various microorganisms unfavorable to survival of the species. With eucalyptus, this defense mechanism is

reflected by anatomical and chemical changes in the phloem and sapwood up after a period of 2 to 8 weeks. The observations made during in vivo study show that the shape of the exudate surface varies between the two species tested (*E. grandis* and its clone 977). Wood cells accumulate polyphenolic compounds irregularly scattered in the tissue.

The most important cambial reaction was observed with *E. grandis* (S = 779,22 cm²; SE = 195,98 cm²) compared to clone 977 (S = 238,41 cm²; SE = 57,59 cm²) (U = 13,00 ; P = 0,002). For both species, the external reaction is less important compared to the reaction of deep tissues. Indeed, no trace of galleries on the bark or dryness of the superficial tissues was observed with clone 977. The larvae have penetrated directly to deep tissues after the cambium in clone 977. However traces of larvae can be observed from the external tissues on the bark only in *E. grandis*.

The phenolic compounds have an undeniable role in plant-parasite interaction. Furthermore, it is known that phenolic compounds enter into the composition of the lignin and suberin, ensuring the rigidity of the plant cell wall including the reinforcement constitutes a protective barrier against drying, penetration of microorganisms or the attack of phytophagous insects (Ride *et al.*, 1983; Macheix *et al.*, 1990). Few data exist with regard to the biochemical mechanisms involved in the interaction couple *Phorcantha* sp. / *Eucalyptus*, especially the phenolic compounds in the attraction or repulsion of process (Zouiten *et al.*, 1998; Zouiten *et al.*, 2000).

Yield essential oils (EO) of *E. grandis* and its clone 977

Without infestation, the clone 977 shows higher content of essential oil than its parental species *E. grandis*. Average yields of essential oils in aerial parts of *E. grandis* and its clone are respectively 1,5% and 2.4%. After infestation, we can observe a decrease of the production of essential in the leaves of wood species, respectively 1% and 1,5% for *E. grandis* and its clone 977 (Figure 1).

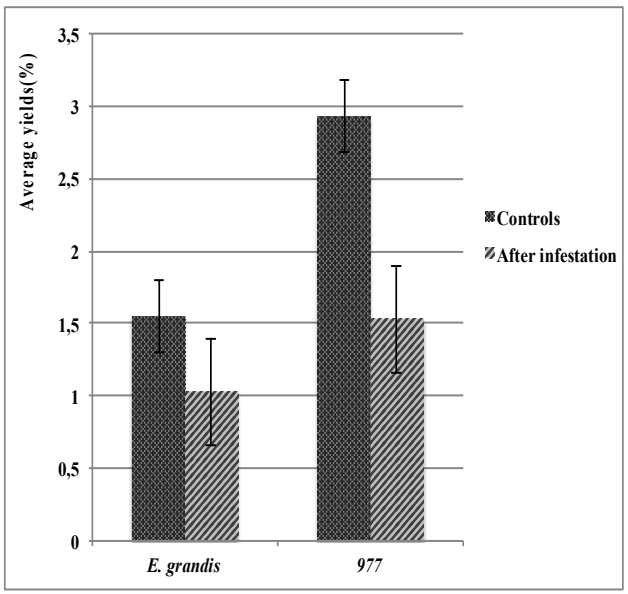

Figure 1. Average yields of essential oils from *E grandis* and clone 977 before (controls) and after infestation by the larvae of *Phoracantha recurva*.

Several studies have shown that the average yields of EO of *E. grandis* leaves is less than 1% (Ahmadouch 1984; Zrira *et al.*, 1992; Zrira & Benjilali., 1996; Farah *et al.*, 1999, Farah *et al.*,2002). On the other hand, the yields ratio obtained are lower than those reported by Ogunwande *et al.*, (2003), Yu Chang Su *et al.*, (2006) and Soyingbe *et al.*, (2013) who observed values that vary from 1,78% to 4.7%.

The difference of essential oil content observed between these wood species without infestation can be attributed to several factors, mainly the species geographical origin. However, the infestation had as much effect on the parental species *E. grandis* and its clone 977. The decrease of essential oil yield observed in the leaves after *Phoracantha* attacks could be explained by the disruption of biosynthesis that has been directed towards the production of constituents that will fight against this pest.

We can hypothesize that the more sensitive the eucalyptus tree, it is called to change its metabolism to defend itself than to improve its yield. In some plant, it has been shown that the mixture of volatile compounds emitted can vary qualitatively or quantitatively depending on the type of damage (Connor *et al.*, 2007) or the herbivore species involved (Turlings *et al.*, 1998).

This study has shown that activation of defense mechanisms is not restricted to the organs involved in the attack but can also extend to non-infected and healthy organs such as leaves.

Chemical composition of essential oils

The chemical composition of essential oils was determined by GC and GC / MS. The results obtained are summarized in Table 1.

Table 1. Chemical composition of essential oils of *Eucalyptus grandis* and clone 977 after attack of *Phoracantha recurva* and their control.

		E. Grandis		*E. 977*	
KI	**Compounds.**	**NA**	**A**	**NA**	**A**
932	α-pinene	1.47	17.53	55.81	6.91
944	Camphene	0.05	1.32	0.57	-
974	β-pinene	-	-	0.29	-
988	Dehydro-1,8- Cineole	0.04	-	-	0.2
1020	p-cymene	15.21	43.32	19.86	11.2
1025	Limonene	-	1.93	-	-
1026	1,8cineole	79.44	-	9.3	**15.6**
1053	γ-terpinene	1.13	0.77	2.69	-
1083	Fenchone	-	-	0.17	-
1087	Terpinolene	-	-	0.14	1.36
1095	Linalol	-	0.12	0.12	-
1102	Isopenthyle isovalerate	-	0,3	-	-
1114	Endo-fenchol	0.05	2.07	0.77	**4.24**
1122	α-campholenal	-	1.07	1.31	**11.8**
1139	Cis-pinene hydrate	-	3.53	2.47	**16.65**
1141	Camphor	0.1	-	-	-
1154	β pinene oxide	-	0.23	0.13	1.01
1162	δ−terpineol	-	0.66	0.23	1.12
1163	Borneol	-	-	0.05	0.63
1174	Terpinene-4-ol	0.16	-	1.26	**10.84**
1179	p-cymene-8-ol	0.27	-	0.25	4.58
1186	α-terpineol	-	-	-	1.17

1190	Myrtenal	2.03	6.26	2.69	-
1215	trans-carveol	-	0.32	-	0.63
1226	Cis-carveol	-	-	-	0.95
1227	Nerol	-	0.13	0.03	0.16
1239	Carvone	-	-	-	0.22
1281	α-terpinen-7-al	0.02	0.27	-	0.68
1343	α- terpinyl actate	0.02	0.4	-	1.05
1354	Eugenol	-	0.48	-	-
1371	Longicyclene	-	-	0.06	0.28
1414	caryophyllane 4,8-α,-epoxy	-	0.79	0.15	0.96
1425	caryophyllane 4,8-β,-epoxy	-	1.59	-	-
1448	ε−isoeugenol	-	9.71	-	-
1452	α-humulene	-	-	-	1.22
1457	Allo-Aromadendrene	-	0.15	-	-
1488	β−selinene	-	0.41	-	-
1490	NI	-	-	-	0.66
1495	NI	-	-	0.06	0.19
1513	γ-cadinene	-	-	0.18	1.57
1521	trans-calamene	-	-	-	0.88
1559	β-Germacrene	-	-	-	-
1565	isoeugenol Z-acetate	-	0.2	-	-
1570	Caryophyllenyl alcool	-	1.58	-	-
1577	Spathulenol	-	0.23	-	0.45
1587	Cis-β-Elemenone	-	0.66	-	-
1590	Globulol	-	-	-	0.5
1592	NI	-	2.98	-	0.43
1620	10-epi-γ-eudesmol	-	0.81	-	-
1638	Epi-α-cadinol	-	-	-	0.62
1640	Epi-α-muurolol	-	-	-	0.2
1652	α-eudesmol	-	-	-	0.31
	Total	**99.99**	**99.82**	**98.59**	**99.27**

NA : not attacked**; A :** attacked**; IK:** Kovats index**; -:** traces**; %:** Percentage

The results of GC/MS analysis of essential oils from the leaves of trees attacked and not attacked of *E. grandis* and its clone 977 (Table 1) resulted in the identification of 35 total constituents for both species. The number of molecules in the essential oils from attacked trees is higher (29 and 33 constituents respectively for *E. grandis* and its clone 977) than that of control trees (13 and 23 constituents respectively for *E. grandis* and its clone 977). These results show that after *P recurva* attack, trees have synthesized more components compared to controls to deal with attacks. Without attack, from a qualitative point of view, γ-terpinene, terpinene-4-ol, p-cymene, p-cymene-8-ol, 1,8 cineole and α-pinene represent 70% of the essential oil composition of *E. grandis* and its clone 977 with their controls.

After the attack, we can observe a decrease of the proportion of some constituents or appearance of new molecules. In the case of the clone 977, there is an increase of α-campholenal proportion (1.31% to 11.80%); terpinen-4-ol (1.26% to 10.84%); p-cymene-8-ol (0.25% to 4.58%); γ-cadinene (0.18% to 1.57%). Meanwhile there is a decrease in the proportion of α-pinene from 55.81% to 6.91%; of p-cymene 19.86% to 11.20%. Attack of *P. recurva* larvae promoted the biosynthesis of 12 new compounds in essential oils leaves that are mainly α-terpineol; trans-carveol; α-humulene; spathulenol; globulol; epi-α-α-muurolol and eudesmol.

Concerning *E. grandis*, we noted after the attack, an increase in the proportion some constituents such as α-pinene (1.47% to 17.53%); camphene (0.05% to 1.32%); p-cymene from (15.21% to 43.32%); endo-fenchol (0.05% to 2.07%); myrtenal (2.03% to 6.25%); a decrease of γ-terpinene (1.13% to 0.77%). As for the clone 977, there is the appearance of 22 new compounds including limonene; linalool; α-campholenal; hydrate cis-pinene; β-pinene oxide; δ-terpineol; trans-carveol; nerol; eugenol and 10-epi-γ-eudesmol.

The various compositions of essential oils from the leaves of *E. grandis* have been reported by different authors. According to Farah *et al.*, (2002) who studied composition essential oil from leaves of *E. grandis* from Mechraâ of El Kettane, located in the western Moroccan Maâmora forest they showed high content of α-pinene (14.64%), p-cymene (23.20%), 1.8 -cineole (21.00%) and α-terpineol (4.94%). However, the chemical composition of essential oils samples of 2414 (*E. grandis* x *E.*

camaldulensis) and 949 Eucalyptus clones (*E. grandis* x *NI*) shows the presence of 1,8-cineole as a main component with varying rates from 45% to 69% (Farah *et al.*, 2001).

The work done by Dagne *et al.*, (2000) showed that α and β-pinene are the main components of essential oils *E. grandis* from Ethiopia and the essential oil of *E. grandis* from Spain is rich in α-pinene and 1.8-cineole (Mora Martinez *et al.*, 2002). Estanislaus *et al.*, (2001) identified γ - terpinene, p-cymene, and β-pinene as the main components. By cons, studies by Soyingbe, (2013) of University of South Africa have shown that the main constituents are m-xylene (33.04%), ethylbenzene (11.59%), the eucalyptol (1,8-cineole) (15.50%), p-xylene (9.61%) and limonene (3.48%).

On the other hand, Lucia *et al.*, (2007) work on essential oils from the leaves of *E. grandis* grown in Buenos Aires is rich in β-pinene. However, in South Africa, the essential oil of. *E. grandis* is rich in α-pinene (29.69%), p-cymene (19.89%), 1,8-cineole (12.80%), α-terpineol (6.48%), borneol (3, 48%) and D-limonene (3.14%) (Soyingbe, 2013) further studies also showed that α-pinene, γ-terpinene, limonene, α-terpineol, and spathulenol globulol were the most commonly found in the constituents essential oils sheets of *E. grandis* (Boland *et al.*, 1991; Zrira *et al.*, 1992; Zrira 1992; Zrira & Benjilali 1996; Farah *et al.*, 1999; Farah *et al.*, 2002). These same compounds were also detected in oil sheets *E. grandis* grown in Nigeria (Ogunwande *et al.*, 2003). The absence of 1,8-cineole and β-pinene in the essential oils of the leaves of the same species grown in Nigeria and Uruguay could be attributed to several factors, including weather conditions (Ogunwande *et al.*, 2003). In the face of adversity, species change their chemical profiles. This could confer a direct or indirect plant resistance to attack (Werner & Roth, 1983; Sookar *et al.*, 2003; Witzell & Martin, 2008). Indeed, the observed differences of the chemical composition of different origins of *E. grandis* could be explained by several factors such as climatic conditions, the nature of the soils, the effects of biotic and abiotic stress without ignoring also the attacks of the pests.

This study showed that the activation of defense mechanisms is not restricted only to the bodies involved in the attack, but they can also be extended to non-infested and healthy organs like leaves. In this context, we have seen a change in the composition of

volatile organic compounds known for their role in the defense against herbivores.

Antifungal activity of essential oil

The results of this study showed that essential oils exhibit inhibitory activity against all tested fungi (Table 2) with different activity threshold. Essential oils from control trees are less active against all the studied strains compared to those from the attacked trees (clone 977 and *E. grandis*).

Table 2. Antifungal activity of essential oils from leaves of *E grandis* and clone 977 after attack by *Phoracantha recurva* and their control.

	Concentration of essential oil from control								**Concentration of essential oil from attacked trees**							
	1/100	1/250	1/500	1/1000	1/2000	1/3000	1/5000	T	1/100	1/250	1/500	1/1000	1/2000	1/3000	1/5000	T
							E. grandis									
***C.** versicolor*	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	+
***G.** trabeum*	-	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+
***P.** placenta*	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+
***C.** puteana*	-	-	-	+	+	+	+	+	-	-	-	-	+	+	+	+
							Clone 977									
***C.** versicolor*	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+
***G.** trabeum*	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	+
***P.** placenta*	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	+
***C.** puteana*	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	+

-: Inhibition**; +:** Growth**; T:** control (culture medium without essential oil).

The four tested fungi have shown great sensitivity in contact with the EO from *Eucalyptus grandis* and clone 977 obtained after attack. They showed the same minimum inhibitory concentration (MIC) of 1/5000 v/v. against *C. versicolor* and *G. trabeum*, which showed less sensitivity to EO from control trees of *E. grandis* clone and *977,* respectively with the inhibitory concentration of 1/500 and 1/1000. Hawever they were more resistant to essential oils from attacked *E. grandis* (inhibitory concentration of 1/2000). *P placenta* and *C. puteana* were more sensitive to EO from

leaves of attacked *E. grandis* and their growth was stopped at the low concentration of 1/1000 v/v and slightly more resistant to EO from control trees of the same species with the concentration of inhibition of 1/500.

Several studies (Erler *et al.*, 2006; Tang, 2007; Cheng *et al.*, 2009) have focused on the study of the degree of antimicrobial activity of major compounds of the essential oils they sorted in descending order next: phenols> alcohols> aldehydes> ketones> ethers> hydrocarbons.

The fact that EO from clone 977 is the most active, this may be due to its chemical profile, which is more complex after the attack (32 compounds) than essential oil without attack (23 compounds). The attack of *P recurva* is at the origin of a modification of the EO composition, which impact its bioactivity against white and brown rot. In fact, *Eucalyptus* sp. essential oil is composed primarily by 1,8-cineole which is a terpene oxide. Previous work (El Arch *et al.*, 2003) showed that the terpenic oxides are active against microbial agents. Chebli *et al.*, (2003) and Vilela *et al.*, (2009) showed that, at higher concentrations, these compounds cause inhibition of mycelial growth. Thus the essential oil bioactivity is the result of a synergistic effect of different molecules (Ouraini, 2007; Chebli *et al.*, 2003).

In addition, the terpene alcohols are particularly active against the microbial cells as soluble in aqueous media and they cause serious damage in the cell walls of microorganisms (Griffin *et al.*, 1999; Dorman *et al.*, 2000; Carson *et al.*, 2002; Hammer *et al.*, 2003). Indeed alcohols have microbicidal activity rather than microbiostatic (Cox *et al.*, 2001; Inouye *et al.*, 2001; Hammer *et al.*, 2003).

Antitermite activity of essential oil

Mortality rates obtained after a period of 7 days are shown in Table 3 and Figure 2. The results obtained with the batch control allow observing a low mortality rate (3.30%), reflecting the virulence of termites and these data validates the test (survival rates above 50% at the end of the test). In this lot, termite activity is intense; the untreated cellulose support was totally consumed by the workers.

Table 3. Mortality rate of pure essential oils after 7 days (end date of the test screening).

	Day 1	**Day 3**	**Day 5**	**Day 7**
Control Virulence	1.92	2.30	2.82	3.33
Control Diet	2.30	3.45	3.88	4
Control Ethanol	1.50	1.72	1.90	2.82
E. grandis attacked (100%)	0	8.07	23.56	41.33
E. grandis not attacked (100%)	0	4.56	9.41	35.33
E. grandis attacked (50%)	0	7.30	10.31	22.0
E. grandis not attacked (50%)	0	3.69	8.02	16.00
977 attacked (100%)	22	25	40.24	81.33
977 not attacked (100%)	18.63	23.42	38.94	36
977 attacked (50%)	17.04	23.19	30.67	56.66
977 not attacked (50%)	15	17.94	19	22.66

In contact with the cellulose support treated with essential (100%) derived from the attacked and not attacked clone 977, we observed a mortality rate of 22% from attacked clone 977 and 18,63% from not attacked clone 977 after 24 hours of contact with termites and the support remained intact. At the end of the test, the cellulose support remained intact for both essential oils (from attacked and not attacked clone 977). In the latter 98% of the individuals died with black spots and no trace of activity is visible on the sand. This showed high toxicity. These essential oils are toxic to termites. This toxicity gives clone 977 significant power insecticide.

With the essential oils from non attacked *E. grandis* at 100%, workers consume paper and their behavior is normal during the first day (mobile, burrowing in the sand ...). The following days were observed an increase of the mortality which remained significantly lower compared to EO *E. grandis* attacked. This essential oil has a moderate insecticidal effect against the termites.

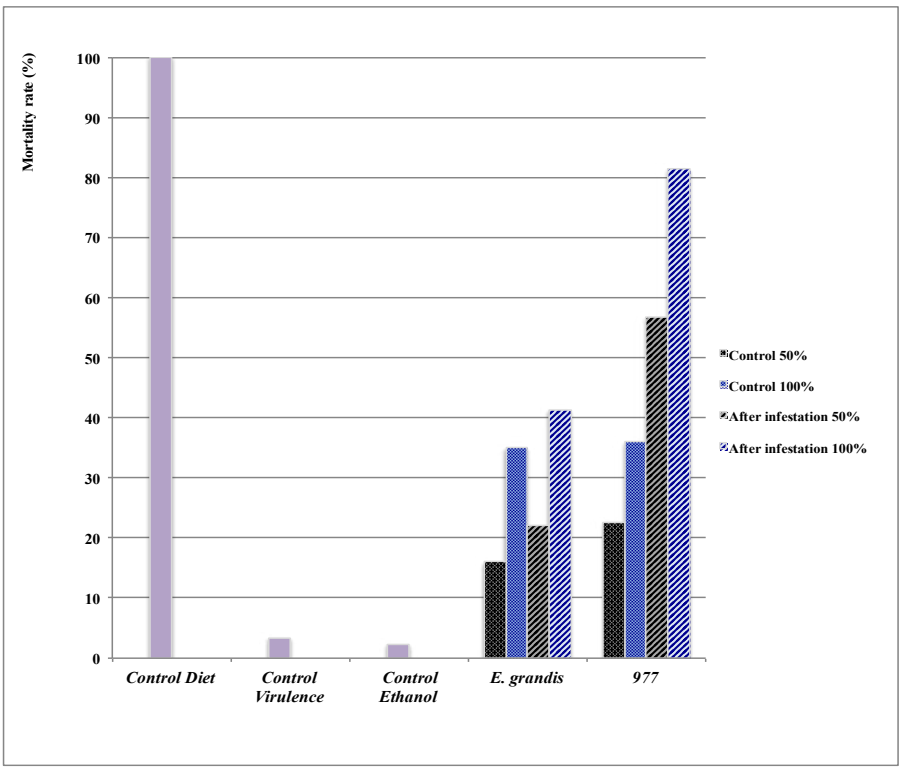

Figure 2. Termite mortality rate contacted with pure essential oils of trees after *Phoracantha recurva* attack and their witnesses

Tests screening of Essential Oils diluted to 50%

We felt good idea to test the highly effective essential oils at the concentration of 20 µl/cm²) at lower concentrations (10 µl/cm²) to determine the activity threshold.

The results of the control confirm the validity of previous tests, as the mortality rate in these groups after 7 days were: 2.59% for white light, 2.45% for the ethanol group and 100% for the Diet group (Table 3).

In concentrations of 10 µl/cm² mortality rates are important in the first 24 hours for clone 977 (80% mortality before attack and 85% after attack), which means that the effectiveness threshold is below 50% for this species. However, the termiticide efficacy of *E. grandis* oils is moderate, but this activity increases with the attack caused, trees by *P. recurva* and concentration.

Statistics:

The results were subjected to parametric statistical treatment by the Kruskal-Wallis test at the 0.05 (5%), which will decide on the validity of the termite test. The choice of

the latter was justified by the low number of tested parameters (less than 30) and which are quantitative variables with non-Gaussian distribution.

The Kruskal-Wallis test at the 5% threshold showed that mortality after attack kills 50% and 100% after attack are significant.

Table 4. Results of the parameters studied at the 0.05 by Kruskal-Wallis test.

		Mortality before at 50	**Mortality after at 50**	**Mortality before at 100**	**Mortality after at 100**
Average of 977		22.66	56.66	36	81.33
Median		22	56	40	76
Standard deviation		3.05	7.02	12.49	12.85
percentiles	25	20	50	22	72
	50	22	56	40	76
Average of *E .grandis*		16	22	35.33	41.33
Median		18	20	42	40
Standard deviation		9.16	13.11	13.31	10.06
percentiles	25	6	10	20	32
	50	18	20	42	40
Asymptotic significance		0.27	0.05	0.82	0.05

The results of this experiment show the great termiticide bioactivity of essential oils tested. The insecticidal effectiveness of attacked trees is greater than that of non-attacked trees in pure form (100%) and 50%.

It should also be noted that the termiticide activity of essential oils before and after the attacks the clone 977 is stronger than those of *E. grandis*. This demonstrated that parental species is less active than his clone.

The analysis of the test results screening of termite, has identified several important points. The tests have shown that activation of the defense mechanisms is not limited only to the bodies involved in the attack. It can also extend to non-infested and healthy organs like leaves. In this context, a change in the composition of volatile organic compounds, known for their role in the defense against herbivores, was observed.

It is also important to note that the mortality of workers is not due to the

ingestion of the product, but rather is due to inhalation of volatile compounds present in the medium. These products would act on the nervous system of insects and cause sudden death termites. This explanation is most consistent with the data of the literature which report that the inhalation toxicity is the main mechanism of action of essential oils on adult insects. (Smythe *et al.*, 1970; Taylor *et al.*, 2006).

4. Conclusion

Many phytochemical studies have shown that essential oils from plants often have toxic activity for animal cells (Bakkali *et al.*, 2008), insecticides (Stammati *et al.*, 1999) or antifungals (Hammer *et al.*, 2003). There is an insecticidal effect that varies depending on the dose and duration of exposure (Bouchikhi *et al.*, 2010). The toxic to insects and fungi are those that cause high mortality in populations at low concentration (ketoh *et al.*, 2004).

The present results show a variability of responses either in terms of anatomical changes in tissues but also in the inhibitory activity with respect to the fungi tested. The latter is mainly due to the chemical composition of essential oils. The qualitative and quantitative analysis of these oils has identified new components after attack and then confirms a variety of constituents.

This study highlighted a variety of defense mechanisms. Some are more effective than others as the case of clone 977. The latter has a strong fungicidal activity and termiticide has low concentrations. This clone could be a very good natural product against wood-destroying fungi and wood-eating insects.

From this work, chemical formulations essential oil of clone 977 could be developed and be recommended against these harmful agents. Nevertheless, it should conduct research into understanding these defense mechanisms by addressing benefit relationship to the host / insect and attractiveness that can serve as a basis in genetic improvement programs.

Further investigations must be undertaken on the use of these oils. Thus, in vivo tests on samples of wood that gasoline will be done to confirm its effectiveness against wood destroying fungi and insects to develop a biological control method based on natural substances.

Research on essential oils for wood processing give another dimension valuation of aromatic plants and are the cause of strengthening and development of research

in this area that would take into account environmental factors and protection of human health.

Acknowledgements

The authors gratefully acknowledge the financial support provided by the PRAD 11-02 (Project financed by bilateral cooperation between Morocco and France).

I thank the whole team that works in CIRAD (Guyana and Montpellier), in the Forestry Research Center (Rabat) and the team of the laboratory of Ecology and Environment (Casablanca), and all the people who helped me during the trips made and assured us intellectual support or support moral or material. I thank everyone who contributed in any way to the realization of this work (Marie-France, Christine, Nicolas and Alexis from CIRAD, Abderrahmane Belhouari, Salwa Elantry., Saad Hanane, Hassan Dahmani and Khalid Micha from FRC).

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