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Interspecific hybridization of *Eucalyptus* as a potential tool to improve the bioactivity of essential oils against permethrin-resistant head lice from Argentina

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Abstract

The essential oils extracted from *Eucalyptus grandis*, *Eucalyptus camaldulensis*, *Eucalyptus tereticornis*, and the hybrids *E. grandis* × *E. camaldulensis*, and *E. grandis* × *E. tereticornis* were analyzed by GC–MS, and evaluated for their fumigant and repellent effects on permethrin-resistant head lice. Fumigant activity of both hybrids was higher than that for pure species. *E. grandis* × *E. tereticornis* and *E. grandis* × *E. camaldulensis* showed KT_{50} values of 12.99 and 13.63 min, respectively. *E. grandis*, *E. camaldulensis*, and *E. tereticornis* showed KT_{50} values of 25.57, 35.01, and 31.31, respectively. A simple regression analysis revealed a significant correlation between KT_{50} data and % of 1,8-cineole in these essential oils. Repellency varied from 47.80 ± 16% to 80.69 ± 6% for the five *Eucalyptus* essential oils tested. Interspecific hybridization improves the pediculicidal activity of *Eucalyptus* essential oils.

Keywords: Head lice; *Eucalyptus*; Interspecific hybridization; Repellents; Fumigants

1. Introduction

Pediculus humanus capitis De Geer, commonly known as the human head louse, is a cosmopolitan parasite found worldwide, both in developed and developing countries. Each year, an estimated 5 million people are newly infested with head lice and these parasites mostly affect children 6–12 years old (Gratz, 1997). Their presence is unpleasant and may cause itching, loss of sleep, and secondary skin infections due to scratching of irritated scalp (Gratz, 1997; Durden, 2005). For decades, control has been mainly based on the continued or repeated chemical application of insecticides such as DDT, lindane, malathion, carbaryl, permethrin and *d*-phenothrin. Repeated use of this insecticide has led to development of resistance to one or more of these products in several countries, including Argentina (Picollo et al., 1998; Burgess, 2004). During the last two decades, pediculosis has increased worldwide as a result of product failures through resistance, improper application, formulation changes and misdiagnosis (Burgess, 2004; Kim et al., 2004).

Recently, there has been a great interest in the use of plant extracts as new control alternatives to synthetic insecticides. Essential oils (EOs) seem to be good candidates because many have lesser mammalian toxicity and lesser persistence in the environment than synthetic insecticides (Isman, 1999). Some of these natural products are effective against a wide variety of insects pest (Isman, 1999; Lee et al., 2003), including head lice (Mumcuoglu et al., 1996; Burgess, 2004; Priestley et al., 2006; Toloza et al., 2006a,b).

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The majority of essential oils are obtained by steam-distillation of plant tissues from either wild or cultivated plants, and consist of mixtures of hydrocarbons (terpenes, and sesquiterpenes) and oxygenated compounds (alcohols, esters, ethers, aldehydes, ketones, lactones, phenols and phenol ethers) (Guenther, 1972). These oils are often responsible for a plant's distinctive scent. An estimated 3000 essential oils are known, of which 300 are of commercial importance (FAO, 1995).

Among plants containing essential oils, are *Eucalyptus* species belonging to the Myrtaceae family. Several hundreds species of *Eucalyptus* contain volatile oils, though probably fewer than 20 of these have ever been exploited commercially for essential oil production. The oils are classified in the trade into three broad types according to their composition and main end-use: medicinal, perfumery and industrial (FAO, 1995).

Interspecific hybrids of *Eucalyptus* have been used in forestry for decades (Griffin et al., 1988). The main hybrid areas are planted on a large scale are in Brazil, Congo, China, Indonesia and South Africa. However, there are also small plantations in other countries in Asia (e.g. Phillippines, Vietnam, Thailand and Malaysia) and South America (e.g. Argentina, Chile, Paraguay and Uruguay) (Dungey and Nikles, 2000). Apart from growth, the main traits which have been identified for improvement by hybridization include propagation, coppicing, frost, drought and salt resistance, wood density, resistance to pests and pulp yield (Potts and Dungey, 2004). On the other hand, *Eucalyptus* hybridization has been used as a technique to improve the quality and quantity of essential oils (Farah et al., 2002).

In Argentina, the importance of *Eucalyptus* species for reforestation and as a source of timber or pulp was recognized in the middle of the 1950s (Marcó and Harrand, 1999). In the last decade, the used of hybridization methods to intercross some species of *Eucalyptus* has received some attention. Only 2% of the *Eucalyptus* products obtained in Argentina were employed for medicinal purposes (FAO, 1995). This paper describes a laboratory study aimed at assessing the fumigant and repellent activity of essential oils of *Eucalyptus grandis*, *Eucalyptus camaldulensis*, *Eucalyptus tereticornis*, and the hybrids *E. grandis* × *E. camaldulensis* and *E. grandis* × *E. tereticornis* against permethrin-resistant head lice. In addition, yield and chemical composition analyses of the evaluated essential oils are reported.

2. Methodology

2.1. Plant materials

The species were purchased from a forest tree nursery, Paul Forestal SRL (INASE Register Number J/5 188), Argentina, with quality and origin certificates. They were planted in an experimental plot site in the Centro de Investigaciones de Plagas e Insecticidas (CITEFA-CONICET) (34°33′42″S, 58°30′39″W), located in Villa Martelli, Buenos Aires, Argentina. After 18 months of growth (March 2006), fresh leaves of *E. grandis*, *E. camaldulensis*, *E. tereticornis*, and the hybrids *E. grandis* × *E. camaldulensis* and *E. grandis* × *E. tereticornis* were collected.

2.2. Extraction and essential oil yields

Fresh leaves of each *Eucalyptus* species were extracted for 70 min using the hydrodistillation method in a modified Clevenger-type apparatus, at a laboratory scale. Moisture in the oil was removed by anhydrous sodium sulfate (Merck, Buenos Aires, Argentina). The oils obtained were stored at 4 °C prior to bioassays. Essential oil yields were expressed as oil weight/100 g fresh plant material (% w/w).

2.3. Essential oil analysis

The chemical compositions of *Eucalyptus* essential oils were determined by gas chromatography coupled to mass spectrometry using a GCMS-QM 5050 *A* (Shimadzu, Japan) instrument. Gas chromatography conditions were as follows: injection of a 0.4 μl sample of an hexane solution of *Eucalyptus* essential oil (1 mg/ml); capillary column HP-1 (Crosslinked Methyl Silicone Gum) (50 m × 0.32 mm × 0.52 μm); helium as carrier gas (1.3 ml/min). Analytical conditions: injector and interface temperatures of 250 and 280 °C, respectively, split ratio of 13:1, initial isothermic temperature of 50 °C during 10 min, programmed temperature of 50 °C to 68 °C (1 °C/min), programmed temperature of 68 °C to 75 °C (0.5 °C/min), programmed temperature of 75 °C to 250 °C (20 °C/min), final isothermic temperature of 280 °C during 10 min, electron impact 70 eV.

Compounds identified in the samples were confirmed by comparing the GC retention times with standard compounds where possible, with comparison of mass spectra with available NIST or Wiley mass spectral libraries. Quantification of essential oil components (expressed in relative % on total area of chromatogram) was carried out by peak area normalization measurements.

2.4. Head lice

Head lice were collected from 530 infested children 6–12 years old, using a fine toothed antilouse comb. Lice were obtained from two elementary schools (Guardia de Honor and República de Turquía) located in different parts of Buenos Aires city. In a previous study, a topical application method had demonstrated high levels of resistance to permethrin in these two schools, with resistance ratios of 665.2 and 373.0, respectively (Vassena et al., 2003). Head lice were collected and transported to our laboratory as previously reported (Picollo et al., 1998; 2000). The protocol for louse collection was approved by the ad hoc committee of the Centro de Investigaciones de Plagas e

Table 1
Yield of essential oil (w/w), expressed as means of four replicates and standard deviation

Eucalyptus	Yield (% w/w)	Standard deviation
E. grandis × *E. tereticornis*	0.89	0.02
E. grandis	0.36	0.02
E. tereticornis	0.60	0.03
E. grandis × *E. camaldulensis*	0.54	0.04
E. camaldulensis	0.39	0.01

Insecticidas (Research Center of Pests and Insecticides, Buenos Aires, Argentina), and archived in our laboratory.

2.5. Test for fumigant and repellent activity

The method of Toloza et al. (2006a,b) was employed to evaluate the fumigant and repellent activity of five *Eucalyptus* essential oils against head lice. Fumigant tests were conducted in an enclosed chamber that allowed a build up of vapor. A drop of 60 μl of pure essential oil was introduced to the chamber. Controls consisted of the same experimental unit without addition of any substance. Batches of 15 adult lice were exposed to a test substance and knockdown was recorded every 5 min for 1 h. Knockdown consisted of the inability of the insects to walk on a filter paper. Three replicates were made for each tested oil. During each study, the assembled units were kept at 28 ± 1 °C and 60 ± 5% RH.

Repellency testing was conducted on a filter paper divided into an inner circular zone and an outer ring. The outer zone was impregnated with 0.5 ml of a test solution (50 mg/ml) dissolve in acetone, while the inner zone was impregnated with acetone only (control). In a preliminary test, both zones were treated with acetone only and it was found that 86.6 ± 6% of released insects moved to the outer zone. Groups of 15–20 adult head lice were placed on the inner zone and repellency was evaluated as the % of lice that avoided the treated zone. The number of lice found in each zone was recorded every 5 min and each experiment was completed within 1 h. There were three replicates for each tested essential oil. A repellency index (RI) for each essential oil was calculated as the percentage of insects repelled from the treated zone, $RI = [(N_c - N_t)/N_c] \times 100$, where N_c was the average

Table 2
Chemical composition of essential oils from five species of genus *Eucalyptus*, expressed as relative percentage on total area in the chromatogram

Plant species as source of essential oils					
	E. tereticornis	*E. grandis* × *E. tereticornis*	*E.grandis*[b]	*E. grandis* × *E. camaldulensis*	*E. camaldulensis*
Oil constituents [a]					
α-Thujene	1.99	–	–	–	1.7
α-Pinene	1.35	22.8	52.71	30.65	<1
Camphene	–	–	–	<1	–
β-Pinene	–	3.29	–	1.1	–
Sabinene	1.26	–	–	–	1.05
Myrcene	1.54	–	–	–	1.21
α-Phellandrene	9.44	–	–	–	6.45
Linalool acetate	–	–	–	1.43	–
α-Terpinene	<1	–	–	–	<1
p-Cymene	14.51	1.92	9.7	2.77	17.93
β-Phellandrene	22.64	–	–	–	16.34
1,8-Cineole	18.59	63.04	18.38	49.65	19.13
γ-Terpinene	2.25	–	5	–	1.87
α-Terpinolene	–	–	–	–	<1
Linalool	<1	–	–	–	3.08
p-Menth-2-en-1-ol	<1	–	–	–	<1
Fenchol	–	<1	–	<1	–
Cryptone	4.14	–	–	–	5.71
α-Campholene aldehyde	–	<1	1.19	<1	–
Trans-pinocarveol	–	1.22	1.93	2.75	–
Borneol	–	1	2.95	1.42	–
Terpinen-4-ol	5.83	<1	1.04	<1	6.73
α-Terpineol	1.59	2.55	5.67	4.05	–
4-Isopropilbenzaldehyde	1.32	–	–	–	2.6
Phellandral	<1	–	–	–	1.45
γ-Elemene	<1	–	–	–	–
Spathulenol	6.83	<1	–	<1	7.32
Caryophyllene oxide	1.69	–	–	–	–
Globulol	–	<1	–	–	–
Unidentified compounds	2.01	1.56	1.43	2.66	2.96

[a] Main constituents of essential oils, determined by CG–MS. Unidentified compounds: less than 90% similarity index.
[b] Data from Lucia et al., 2007.

number of lice in the control zone, and N_t was the number of lice found in the treated zone.

2.6. Statistical analysis

Probit analysis was conducted on fumigant data to estimate knockdown of 50% of exposed individuals (KT_{50}), using statistical software POLO-PC (LeOra Software, 1987). Values were considered to be significantly different if the 95% confidence limits (CL) did not overlap. The relationships between 50% knockdown time of the essential oils and their main components α-pinene and 1,8-cineole were estimated by either simple or multiple linear regression analysis (Statistica 99, StatSoft, Tulsa, OK). Analysis of variance (ANOVA) was performed on repellency data, and means were separated using the least significant difference (LSD) test (Statistica 99, StatSoft, Tulsa, OK).

3. Results and discussion

3.1. Chemical constituents

The average essential oil yields based on fresh weight for each plant extracted are shown in Table 1. The hybrid *E. grandis* × *E. tereticornis* showed the greatest yield (0.89%, w/w). The qualitative and quantitative chemical compositions of the essential oils are presented in Table 2. These results revealed that the principal constituents of *E. grandis* were α-pinene (49.52%) and 1,8-cineole (15.72%). In contrast, the main constituents of *E. tereticornis* and *E. camaldulensis* were *p*-cymene (19.7%) and 1,8-cineole (20.49%); and *p*-cymene (25.77%), 1,8-cineole (23.95%), respectively. Oils of the hybrids mainly constituted of 1,8-cineole and α-pinene (63.04% and 22.8% for *E. grandis* × *E. tereticornis*, and 49.65% and 30.65% for *E. grandis* × *E. camaldulensis*, respectively). Farah et al. (2002) reported a similar content of 1,8-cineole (47.69%), but a lower percentage of α-pinene (14.11%) in an interspecific hybrid of *E. grandis* × *E. camaldulensis*. Additionally, these authors showed that the interspecific hybridization of eucalyptus grown in Morocco produced essential oils with high content of 1,8-cineole. However, in that study, the objective was not to improve the insecticidal activity of eucalyptus essential oils but rather its pharmaceutical quality.

3.2. Fumigant activity

In the present work, there were significant differences in vapor effects against head lice among the hybrids and species (Table 3). Fumigant activity of both hybrids was higher than that for pure species. The higher fumigant activity of hybrids could be based on higher 1,8-cineole concentrations as determined by GC–MS analysis. To test this hypothesis, a regression analysis was made. A simple regression analysis revealed a significant correlation between KT_{50} data and % of 1,8-cineole in the essential oils ($KT_{50} = 33.515 \pm 4.87–0.2645 \pm 0.09\%$ 1,8-cineole; $r^2 = 0.68$; $F = 8.56$; df = 1; $P < 0.05$) (Fig. 1a). Thus, the higher the concentration of 1,8-cineole, the more effective it was as a fumigant. In contrast, KT_{50} values were independent of α-pinene concentration ($KT_{50} = 23.258 \pm 7.54 + 0.10 \pm 0.15\%$ α-pinene; $r^2 = 0.09$; $F = 0.43$; df = 1; $P < 0.548$). When a multiple linear regression analysis was made, the resulting model was $KT_{50} = 42.2 \pm 2.8–0.226 \pm 0.06$ (% α-pinene) − 0.389 ± 0.07 (% 1,8-cineole); $r^2 = 0.96$; $F = 26.01$; df = 2; $P < 0.05$), showing a significant and better relationship when both predictors where included (Fig. 1b).

Our results demonstrate the importance of 1,8-cineole to the fumigant activity of Eucalyptus essential oils. In a previous study, Toloza et al. (2006a,b) analyzed the fumigant activity of 21 monoterpenoids against head lice, and reported that the KT_{50} value of 1,8-cineole was 3.84-fold shorter than that of α-pinene. Additionally, Lahlou et al. (2000) studied the activity of 15 terpenoids isolated from plants of Morocco against head lice, and found that 1,8-cineole was also two to three times more effective than α-pinene. These results are also in accord with those of Papachristos et al. (2004) in the fumigant analysis of monoterpenoids against stored product insects.

Repellent activity of essential oils and synthetic components against body and head lice has previously been

Table 3
Fumigant and repellent activity of *Eucalyptus* essential oils against head lice

Substance	Fumigant activity				Repellent action
	Slope ± SE	KT_{50} min (CL)	χ^2	df	(RI ± SE)
E. camaldulensis	7.98 ± 0.99	35.01 (32.15 − 37.76)	13.51	12	73.16 ± 11
E. grandis	11.17 ± 1.57	25.57 (23.97 − 27.24)	2.52	13	74.73 ± 9
E. tereticornis	14.63 ± 2.25	31.31 (29.47 − 33.09)	5.72	15	53.13 ± 11
E. grandis × *E. camaldulensis*	11.04 ± 1.80	13.63 (12.50 − 14.68)	1.73	7	80.69 ± 6
E. grandis × *E. tereticornis*	15.62 ± 3.46	12.99 (11.98 − 14.05)	2.18	7	47.80 ± 16
Piperonal[a]	N/S	N/S	N/S	N/S	72.26 ± 11.16
DDVP[b]	3.93 ± 0.68	40.15 (34.63 − 48.86)	7.27	N/S	N/S

N/S, not studied. Piperonal was only employed as the repellency positive control, while DDVP was only employed as the knockdown positive control.
[a] Positive control for repellent activity. Data from Toloza et al., 2006a,b.
[b] Positive control for fumigant activity. Data from Toloza et al., 2006a,b.

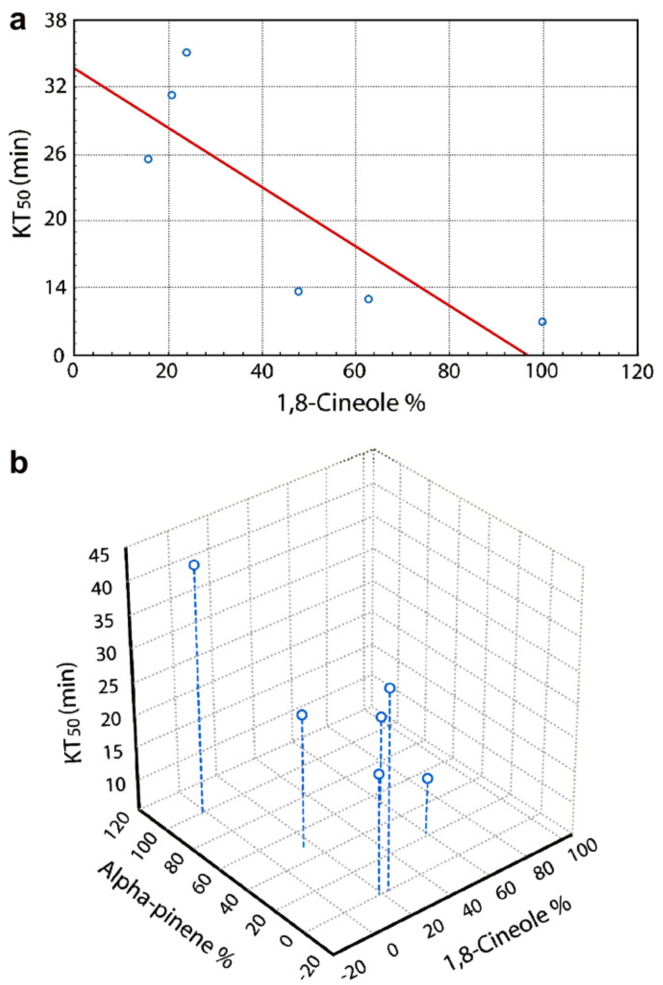

Fig. 1. Relationship between fumigant activity (KT_{50}) from each essential oil and their corresponding concentration of the main constituents (1,8-cineole and α-pinene). (a) Simple regression of fumigant toxicity (KT_{50}) and % of 1,8-cineole in tested oils. (b) Multiple linear regression among % of 1,8-cineole, % of α-pinene and KT_{50} (min).

reported (Burgess, 1993; Mumcuoglu et al., 1996; Toloza et al., 2006a,b). The impetus for studying repellent alternatives is to deter lice from climbing onto a new host (Burgess, 2004). The problem of lice resistance to existing pediculicides makes the development of effective lice repellents more attractive (Mumcuoglu et al., 1996).

3.3. Repellent activity

For the five *Eucalyptus* essential oils tested, repellency varied from 47.80 ± 16% to 80.69 ± 6% of (Table 3). Although the oil from the hybrid *E. grandis* × *E. camaldulensis* was the most repellent, it was not significantly different from the other tested oils ($F = 1.71$; df = 4; $P < 0.283$). These results suggested that 1,8-cineole or α-pinene concentration are not related to the repellent activity of *Eucalyptus* essential oils. The RI values of the *Eucalyptus* EOs obtained herein are in accord with the RI value obtained previously in our laboratory with different populations of head lice using a well known repellent commercial product known as piperonal (1,3-benzodioxol-5-carboxaldehyde) (Toloza et al., 2006a,b).

It is appropriate to point out that subtle differences in oil composition can result in major differences in repellency. For instance, *E. grandis* and *E. camaldulensis* presented very different terpenoid compositions but comparable RI values. A similar pattern was previously found in a study on several insects, like the house fly, red flour beetle and Southern corn rootworm (Rice and Coats, 1994). In that report, when very subtle differences of monoterpenoid composition were found, similar RI values were obtained.

It is important to note that essential oil insecticidal activity is species dependent and that the chemical composition of the EO (i.e. monoterpenoids) can influence their insecticidal properties (Rice and Coats, 1994; Isman, 1999; Enan, 2001). Moreover, factors such as shape, degree of saturation and type of functional groups can influence the insecticidal activity of the whole oil. These can affect penetration into the insect cuticle, movement and interactions of the constituents inside the insect and their biodegradation. Thus, differences in effect at the site of action may account for the differential response found in the repellent and fumigant bioassays reported here. It has been suggested that contact repellents act upon specialized chemoreceptors which are not normally sensitive to vapors (Dethier, 1956). On one hand, vapors might enter through tracheae to haemolymph and then bind to some putative target site. These oils posses a neurotoxic mode of action and some mechanisms for this have been proposed. For example, some authors have proposed that these oils are competitive inhibitors of acetyl-cholinesterase (AChE) as well as cholinesterase (Grundy and Still, 1985; Ryan and Byrne, 1988), while others suggest interference with the neuromodulator octopamine (Enan, 2001; Kostyukovsky et al., 2002) or with GABA-gated chloride channels (Priestley et al., 2003). On the other hand, human lice are so closely attached to their host that they do not require much in the way of host seeking senses. It seems that rather than attraction to favorable stimuli, the human louse reacts by klino-tactic or klino-kinetic avoidance of unfavorable zones. Thus, it is suggested that repellency in this ectoparasite could be due to anti-gustatory effects or chemical irritancy that deters insects from crawling into close contact with the repellent material (Busvine, 1971).

As discussed by Isman (2006), there are three main barriers to commercialization for botanical insecticides: sustainability of the botanical resource, standardization of chemically complex extracts, and regulatory approval. One, to produce a botanical insecticide on a commercial scale, the source plant biomass should be obtained on a commercial scale and preferably not on a seasonal basis. The *Eucalyptus* EOs obtained in the present study showed relative high yield values (i.e. allowing commercial scale) and were not affected by seasonal bias due to standardized cultivation and harvesting methods. Moreover, the seedings of origin certificate guarantees a regular harvesting and chemically homogenous system. Two, for a botanical insecticide to provide a reliable level of efficacy to the user, there must be some degree of chemical standardization, based on the putative active ingredient(s). The hybrids of this study showed less number of ingredients than pure species, allowing a better chemical identification and manipulation of the individual compounds. Three, regulatory approval remains the most formidable barrier to the commercialization of new botanical insecticides. Certain botanical insecticides could be exempt from regulatory scrutiny in some countries based on their presumed safety.

4. Conclusions

Our study shows that *Eucalyptus* essential oils have pediculicidal and repellent potential for development as products for head lice control. Production of *Eucalyptus* essential oils is feasible owing to their low cost, availability and accessibility. Interspecific hybridization appears to be an interesting biotechnological tool to improve essential oils with more favorable properties for pest control. As some oil constituents applied to the human head may have a high rate of evaporation, essential oils or their components should be formulated to produce slow release of these. However, acute and chronic toxicological testing for safety should be conducted before a product is released into the market.

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