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Full Length Research Paper

Seasonal variation of fixed and volatile oil percentage of four *Eucalyptus* spp. related to lamina anatomy

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This experiment was conducted during the four seasons: Spring, summer, autumn and winter of two successive annual cycles; 2008/2009 and 2009/2010 (starting from May 2008). Four *Eucalyptus* species were under investigation; *Eucalyptus camaldulensis* Dehnh., *Eucalyptus cinerea* F. Muell. ex Bentham, *Eucalyptus citriodora* Hook. and *Eucalyptus globulus* Labill. Seasonal variations in the amount of fixed and volatile oils in *Eucalyptus* spp. matured leaves were investigated. It was determined that the amount of total lipids and essential oils significantly varied by the seasons (P < 0.01). The amount of total lipids in *Eucalyptus* spp. reached its peak mostly in spring. But the amounts of essential oils in different species were determined to be higher in summer, autumn and spring seasons, than in winter. Furthermore, the amount of total lipids and essential oils was higher in *E. camaldulensis* and *E. cinerea* than in other species. The anatomical investigation in the four studied *Eucalyptus* species, in relation to lipids percentage indicated that, the best lipids percentage amounts in this study were exhibited in *E. cinerea* and *E. camaldulensis,* for spring and winter; and were in agreement with these species highest lamina thickness. In general, fluctuation in lipids percentage is more correlated to the internal structure of lamina (duct average diameter, ducts total numbers, and open ducts numbers) in the same season; whereas, among seasons, it is thought that metabolism contributed more greatly. Cuticle thickness is true correspondence to seasonal environmental fluctuation, since it increases in all species, by shifting up from spring to summer then decrease to winter. Essential oils secretion which coincided with lipids percentage may be due to environmental stress influence over metabolism rather than structural adaptation.

Key words: *Eucalyptus*, seasons, fixed and volatile oils, lamina, anatomy, glands.

INTRODUCTION

Eucalypti species are mostly evergreen trees of immense size; around 800 species belong to the Magnoliopsidous family, Myrtaceae and native mainly to Australia and some neighboring islands (Bailey, 1958; Cronquist, 1981; Heywood, 1993; Brooker, 2000).

They are grown for their ornamental values, as windbreaks, for timber and fuel, and for oil, distilled from leaves, which is secondary compounds with pleasant aroma used as fragrance components in soap, detergents and toiletries, and also have pharmacological properties (Santos et al., 2008). *Eucalyptus* oil is being extracted in many countries like China, India, South Africa, Portugal, Brazil and Tasmania on commercial scale. In 1992, the world *Eucalyptus* oil production was estimated to be 4000 tons of which 60 to 70% was consumed in medicinal market (Bhatti et al., 2007). *Eucalyptus* leaves oil amounting to less than 20% is enough in the commercial point of view, and only 10% of these accounts for the entire world production of essential oil (Peter, 2000).

Essential oils are complex mixtures which comprised primarily of the lower classes of terpenes (Julia, 1992; Langenheim, 1994). The secondary compounds are probably co-evolution result from interaction of plants

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Table 1. The effect of different seasons on the total lipids content (%) of the four studied *Eucalyptus* spp. during the two successive annual cycles, 2008/2009 to 2009/2010.

Season	Species			
	E. camaldulensis	***E. cinerea***	***E. citriodora***	***E. globulus***
Spring	18.0^{b}	18.75^{a}	16.0^{a}	13.0^{a}
Summer	13.2^{c}	13.5^{d}	11.0^{c}	11.75^{c}
Autumn	13.0^{c}	13.75^{c}	10.75^{d}	11.25^{d}
Winter	19.0^{a}	15.75^{b}	14.75^{b}	12.75^{b}
LSD at 0.01	0.431	0.183	0.236	0.174

Values with different superscript letters within the same column are significantly different ($P < 0.01$).

versus pathogens, herbivores, pollinators (Croteau et al., 2000). Although monoterpenes predominate in most essential oils, many also contain sesquiterpenes. Many sesquiterpenes play defensive roles in plant-insect and plant-fungal interactions (Samuelsson, 1999).

Oil concentration is limited by gland capacity and density, which in turn is linked to leaf area reduction and increment in its thickness; thicker leaves were more likely to be smaller in area than thinner leaves, suggesting the expansion of leaves in one plane to a degree mutually exclusive of expansion in the other. Aside glands, probable sink of hydrocarbon monoterpenes in the leaves may be the leaf cuticle through which significant portion of monoterpenes is volatilized (King et al., 2006).

Thicker epidermis and presence of two or more layers of palisade cells in leaves are the xeromorphic features adapting to water limited environments like drought (Iftikhar et al., 2009). Mediterranean climate is characterized by the incidence of two stress periods; summer drought and winter cold (Nahal, 1981). Seasonal variations in the composition and concentration of sequestered oils have been observed (Silvestre et al., 1997). There are several reports that the concentration of terpenoids in aromatic plants varies during the day and seasons (Hendriks et al., 1997).

The aim of this study is to investigate the effect of seasonal fluctuations over the year on oil yield, fixed and volatile, of perennial evergreen tree (*Eucalyptus*), related to varying anatomical features in order to suggest best species (among studied ones) and season for harvesting oil from leaves.

MATERIALS AND METHODS

This experiment was conducted during four seasons; spring, summer, autumn and winter of two successive annual cycles; 2008/2009 and 2009/2010, at the laboratories of Agricultural Botany and Agricultural Biochemistry Departments, Faculty of Agriculture, Cairo University.

Collection of samples

The used plant materials were four *Eucalyptus* species (Quattrocchi, 2000); red gum (*Eucalyptus camaldulensis* Dehnh.), Argyle apple (*Eucalyptus cinerea* F. Muell. ex Bentham), Lemon-scented gum (*Eucalyptus citriodora* Hook.) and blue gum (*Eucalyptus globulus* Labill.). The leaf materials were gotten from the gardens of the Faculty of Agriculture, Cairo University. Fully expanded mature leaves were cut at the mid-date of each season (starting from May 2008) and gathered early in the morning. The leaves were pre-cooled (4°C) and wrapped in Kraft paper in bunches, and then transported under dry conditions to the laboratories within two hours.

Seasonal effects on essential oils and lipids content (percentage) of different *Eucalyptus* species were investigated, and then correlated with lamina anatomical correspondence to affected by climatic fluctuation over the seasons.

Extraction and determination of total lipids

Lipids were extracted by a modified method described by Xu et al. (1998). The cells (ca. 5 g) were extracted twice with a mixture of distilled water (H_2O), chloroform and methanol (8:10:20, v/v/v) and sonicated for 10 min using a microtop of Microson Ultrasonic cell disrupter. Then, the sonicated cells were filtered through GF/C Whatman glass microfiber (47 mm). Chloroform (10 ml) and distilled water (10 ml) were added sequentially to the filtrate and sonicated again for 10 min. The resultant solution was filtered under vacuum through Whatman glass filter microfiber. The filtrate was washed by 30 ml of 5% NaCl solution, and then, the chloroform layer was separated and dried over anhydrous sodium sulfate. The solvent was removed through evaporation at 40°C under reduced pressure. Then, the total lipids were weighed and stored at -20°C until analysis.

Essential oil extraction

Plant samples (100 g) were hydro-distilled in Clevenger-type apparatus (Council of Europe, 1997). The essential oil samples were stored in the dark at 4°C. The amount of oil (Table 1) obtained from plant material was calculated as:

Oil (% v/w) = Observed volume of oil (ml)/Weight of sample (g) × 100

Anatomical studies

Leaves were randomly chosen as specimens for anatomical study over the four successive seasons of the year. The micro technique procedures given by Nassar and El-Sahhar (1998) were applied. Specimens were killed and fixed for at least 48 h in formalin aceto alcohol (FAA) solution. Then, materials were washed in 50% ethyl

Table 2. The effect of different seasons on essential oil content (%) of the four studied *Eucalyptus* spp. during the two successive annual cycles, 2008/2009 to 2009/2010.

Season	Species			
	E. camaldulensis	***E. cinerea***	***E. citriodora***	***E. globulus***
Spring	1.78c	1.35c	0.60c	0.10c
Summer	2.5a	1.95a	1.8a	0.66a
Autumn	1.92b	1.50b	1.05b	0.23b
Winter	0.98d	1.0d	0.25d	0.05d
LSD at 0.01	0.033	0.086	0.033	0.028

Values with different superscript letters within the same column are significantly different ($P < 0.01$).

alcohol and dehydrated in normal butyl alcohol series before being embedded in paraffin wax (mp 52 to 54°C). Transverse sections, which were cut on a rotary microtome to a thickness of 20 , were stained with safranin/light green before mounting in Canada balsam. Slides were examined; measurements of different tissues were recorded using light microscope with micrometer eye piece and micrometer stage, and then photomicrographed using Microscope Olympus AX70 made in Japan. The following anatomical characters were measured: Cuticle thickness (μ), lamina thickness (μ), opens ducts number (in 40 x field), ducts total number [in 10x (eye lens) X 4x (object lens) field] and duct diameter (μ).

Statistical analysis

Data were subjected to an analysis of variance. Combined analysis was used for the two annual seasonal cycles. Means were compared by calculating the least significant difference (LSD) values at alpha 1% according to Snedecor and Cochran (1982).

RESULTS AND DISCUSSION

Chemical studies

The amount of total lipids obtained from various *Eucalyptus* spp. during different seasons was investigated, and it was observed that values were between 10.75 to 19.0%. It scored the minimum level in autumn season and increased significantly, till it reached the maximum at spring season in all studied species, except for *E. camaldulensis* which had maximum value in winter (Table 1). Species *E. camaldulensis* and *E. cinerea* reflect best lipids amount (19 and 18.75%, respectively). The variations of total lipid contents were affected by the species, age, water temperature, nutritional condition and seasonal variation, as confirmed by Folch et al. (1957), Gill and Weatherley (1984), Lathi (1987) and Christiansen et al. (1989). Recent studies have shown that lipids and fatty acids composition was influenced by seasonal variations (Yılmaz et al., 1995). The same trend shows that, the variations of the levels of lipids are the results of irregular seasonal variations and water temperature was determined by Dutta et al. (1985), Agren et al. (1987) and Yılmaz et al. (1995).

From Table 2, it can be seen that the content of essential oils in different seasons varied from 0.05% during winter to 2.5% during summer season. Best essential oils percentages were estimated in *E. camaldulensis* and *E. cinerea* (2.5 and 1.95% respectively) in the summer season. Data from Table 2 indicated that, the level of these components reached its maximum in all studied plant species during the summer season and had the lowest value during winter, and this may be due to the physical and chemical stress on plant especially during summer drought. This stress led to plant secretion to different defense components called secondary metabolites, as protecting agents, especially terpenoid compounds (essential oils). These results are in agreement with the results obtained by Samuelsson (1999). As to the significance estimates, more consistent trend in percentages variation of *Eucalyptus* spp. during different seasons are present for essential oils than for lipids, and this further confirm the formers are due mainly to the effect of environmental condition on plant synthesis. Wildy et al. (2000) investigated four promising *Eucalyptus* species reported 0.01 to 13.0% for oil production, from Western Australia at six locations. Also, Zafar et al. (2003) reported similar results; that is, 0.58 to 1.47%, regarding oil potential for the different *Eucalyptus* species. These variations might be attributed to different agro-climatic regions and soil composition in the districts of Punjab.

Anatomical studies

Aiming to explain the seasonal differences affecting lipids content (%) of the four studied species, microscopical counts and measurements of certain characters were recorded to investigate the internal differences in lamina of these species. The most important criteria in this study were to compare fluctuations in glands (number and size) over seasons, in random specimen’s descent by half meter from the top, in addition to measure the cuticle and lamina thickness as correspondence to heat/cold stress.

Microphotographs of transverse sections illustrating four studied *Eucalyptus* spp. (Plate 1) showed that

Plate 1. Transverse sections in leaves of the four studied *Eucalyptus* spp. (X = 9).

mesophyll of adult vertically positioned lamina is often isobilateral, with cells containing tannin abundant in all unlignified tissues and bicollateral vascular bundles. Epidermis coated by wax lamina characterized by the presence of secretory cavities, which are found below the epidermis on either sides of the lamina. Cavities are schizolysigenous in origin; lined with distinct epithelium when young which soon is obliterated by compression. Cavities are responsible for secreting oily substances.

Data presented in Table 3 and Plate 1 for the leaf specimens subjected to histological investigation cleared that as typical correspondence to seasonal environmental fluctuation, cuticle thickness increase in all species, by shifting from spring to summer, then decrease to winter,

Table 3. Different tissue counts and measurements (μ) in lamina of the four studied *Eucalyptus* spp. during the two successive annual seasonal cycles, 2008/2009 to 2009/2010 (average of 5 readings).

Season	Measurement (μ)	Species			
		E. camaldulensis	***E. cinerea***	***E. citriodora***	***E. globulus***
Spring	Average cuticle thickness	18.33	10.83	9.17	9.17
	Average lamina thickness	466.67	323.33	423.33	213.33
	Open ducts number	7	6	1	2
	Ducts total number	9	6	3	3
	Duct average diameter	135	152.5	125	85
Summer	Average cuticle thickness	25	13.33	15	10.83
	Average lamina thickness	456.67	290	400	290
	Open ducts number	5	3	4	3
	Ducts total number	7	5	4	3
	Duct average diameter	157.5	172.5	115	150
Autumn	Average cuticle thickness	16.67	9.17	15	10
	Average lamina thickness	430	290	400	333.33
	Open ducts number	5	5	4	3
	Ducts total number	6	6	4	3
	Duct average diameter	147.5	172.5	105	117.5
Winter	Average cuticle thickness	13.33	8.33	15	6.67
	Average lamina thickness	463.33	350	486.67	290
	Open ducts number	3	3	3	4
	Ducts total number	5	5	5	5
	Duct average diameter	187.5	140	125	110

except for *E. citriodora* which also increase in summer (from 9.17 μ) but score the same thickness throughout the rest two subsequent seasons (15 μ).

Lamina thickness varied over the seasons (by shifting, in samples, from spring to winter) for various studied species by what could be named 3 types; decreasing-sustaining-increasing type as in *E. cinerea* and *E. citriodora*; increasing-increasing-decreasing type as in *E. globulus* and finally, decreasing-decreasing-increasing type as in *E. camaldulensis*. In harmony with estimated lipid amount percentage, *E. cinerea* and *E. camaldulensis* exhibited their highest lamina thickness in spring and winter.

In a trial to investigate the association between lipids percentage and the most direct contributed anatomical features to it, average diameter and number of glands were studied. In the same season, referring to Table 3, Plate 1 and Figure 1, it could be realized that, average diameter, total number of ducts, and also, open ducts number have positive relation with lipids percentage, for all species understudied. Over seasons, for each individual species, it could be determined that, open ducts number (also total number) showed limited range. Relation over seasons could not be distinguished as clear as in the same season, due to inconsistent expression of these features. It could be interpreted that, physiological balance between anabolism and catabolism activities, due to seasonal growth rate, has the principal role in determining the amount of stored lipids (within cavities) over seasons. Also, it could be noticed that, mostly, duct average diameter increases by removing from spring to summer samples, then decreased over autumn to winter. As coincide to this relation, *E. citriodora* decrease in summer and increase in winter, whereas, duct average diameter of *E. camaldulensis* increase in both summer and winter samples.

The anatomical investigation in the four studied *Eucalyptus* species, in relation to lipid percentage indicated that, fluctuation in lipids percentage is more correlated to internal structure of lamina (ducts average diameter, ducts total numbers and open ducts numbers) in same season, whereas between seasons it was thought that, metabolism contributed more greatly. Best lipid percentage amounts in *E. cinerea* and *E. camaldulensis* in spring and winter, and were in agreement with these species highest lamina thickness. Cuticle thickness is true correspondence to seasonal environmental fluctuation, since it increases in all species, by shifting from spring to summer, and then decrease to winter.

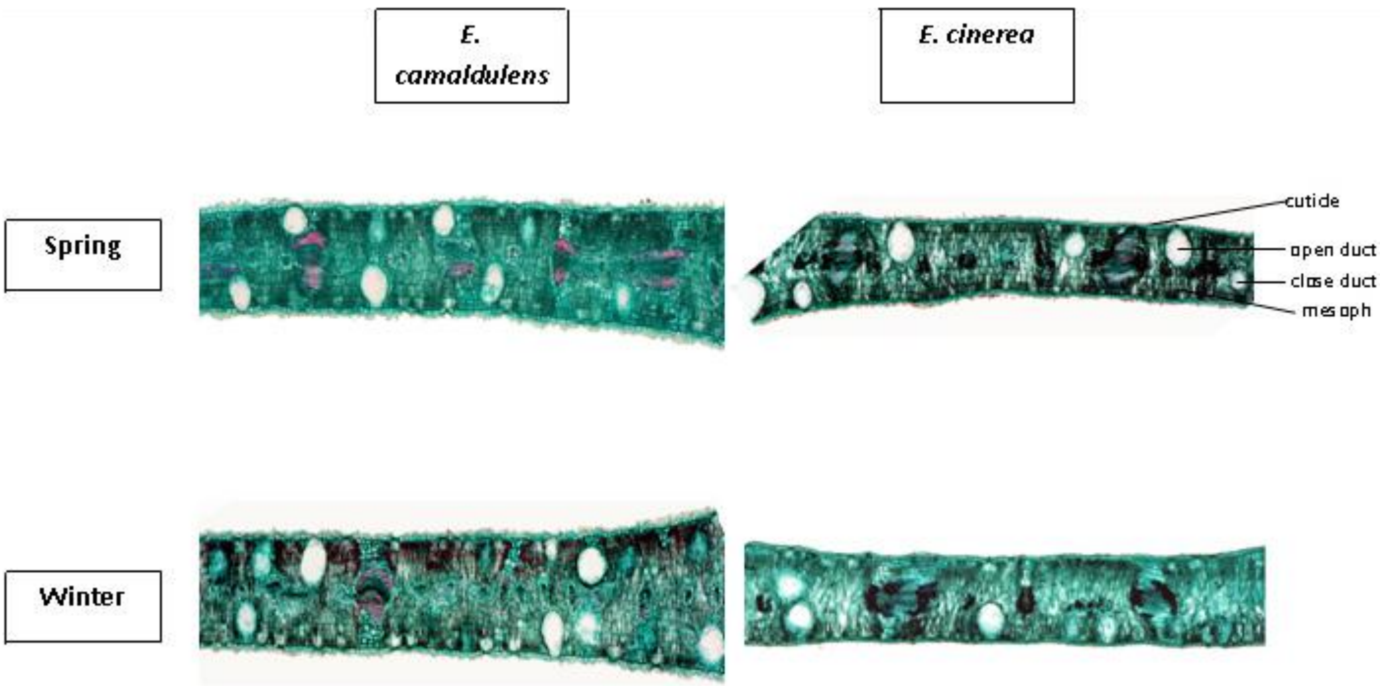

Figure 1. Magnified portion of transverse sections in lamina of the most higher studied *Eucalyptus* spp. in lipids amount for optimal season (X = 28).

As regards essential oils, it was clear that they coincides lipids percentage since their highest amount was estimated in summer, and then autumn, whereas lowest amounts were in winter, and spring, for all studied species. This may confirm that, essential oils secretion is due to environmental stress influence over the metabolism rather than structural adaptation.

The previously mentioned structure of *Eucalyptus* lamina is in agreement with those given by Metcalfe and Chalk (1950) as well as by Bailey (1958), King et al. (2006), Santos et al. (2008) and Iftikhar et al. (2009).

Conclusion

These studies confirmed that, *Eucalyptus* spp. Is a potential source of oil (volatile and fixed) around the year, with regard to significant fluctuation in oils yield due to subsequent seasonal climatic conditions. Putting economical factor into consideration, *E. camaldulensis* and *E. cinerea* showed best amount of total lipids and essential oils than other studied species. For purposes mostly industrial, yielding these two species in spring and winter was best (biggest lipid percentages (%)), whereas, for other usages, mostly medical, extracting rich essential oil yield is more preferable in summer and autumn.

The anatomical investigation indicated that, fluctuation in lipids percentage was more correlated to internal structure of lamina (ducts average diameter, ducts total numbers, and open ducts numbers) in same season, whereas between seasons it was thought that, metabolism contributed more greatly. Cuticle thickness is a true correspondence to seasonal environmental stress; same applies to essential oils secretion, which increased from spring to summer, and then decrease in winter.

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