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Environmental factors influence on chemical polymorphism of the essential oils of *Lychnophora ericoides*

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Abstract

Lychnophora ericoides is a Brazilian medicinal plant used in folk medicine as an anti-inflammatory and analgesic agent. The essential oils from leaves of two populations with and without scent, collected at 2-month intervals during an 1-year period, were analysed by GC-MS. The results were submitted to principal component and cluster analysis which allowed two groups of essential oils to be distinguished with respect to sampling site and scent: cluster I (Vianópolis site, with specimens exhibiting an aromatic scent) containing a high percentage of α-bisabolol (44.7–76.4%) and α-cadinol (10.9–23.5%), and cluster II (Cristalina site, with specimens without scent) characterised by a high content of (E)-nerolidol (31.3–47.1%) and *ar*-dihydro-turmerone (4.8–15.4%). The canonical discriminant analysis showed that using the data set of the seven sampling months and (E)-nerolidol and α-bisabolol as predictable variables, it was possible to distinguish between the samples harvested according to Cerrado seasons, dry winter (May–September) and humid summer (November–March). In addition, canonical correlation analysis between the soil sampling sites and the populations revealed a significant relationship between oil components and edaphic factors. Oxygenated sesquiterpenes and potential acidity, Al saturation, cationic exchange capacity, silt, and sand load as the first canonical variate were fairly strongly related to samples collected in Vianópolis site. On the other hand, monoterpenes and sesquiterpene hydrocarbons were strongly related to chemical balance in soils (organic matter, P and base saturation), which is related to samples at the Cristalina site. The chemovariation observed appears to be environmentally determined.

Keywords: *Lychnophora ericoides*; Asteraceae; Essential oils; Chemical variability; Edaphic factors; Canonical correlation; Canonical discriminant analysis

1. Introduction

Lychnophora Mart. (Asteraceae) is an endemic genus from Brazil and comprises about 68 species known as "arnica da serra". Its species grow in restricted regions of the central Brazilian Cerrado (Coile and Jones, 1981; Robinson, 1992; Semir, 1993; Mansanares et al., 2002) at high altitudes in sandy sites, usually inside woodland and scrubs. *L. ericoides* Mart. is the most popular species, whose leaves and roots are largely commercialised in local markets as analgesic and anti-inflammatory agents (Cerqueira et al., 1987; Borsato et al., 2000; Kanashiro et al., 2004). Characteristic constituents are sesquiterpene lactones (Borella et al., 1998; Sakamoto et al., 2003) showing antiproliferative activity (Santos et al., 2005a), besides flavonoids with anti-inflammatory effects (Gobbo-Neto et al., 2005), triterpenes (Borella et al., 1998; Sargenti and Vichnewski, 2000), lignans and caffeoylquinic acids with analgesic activity (Borsato et al., 2000; Santos et al., 2005b).

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L. ericoides has been classified in the category of a threatened Brazilian species (Silva, 1994), due to its population decline. The latter is a consequence of its excessive exploitation and destruction of its habitats, as well as its low seed viability, which makes it difficult to propagate. Although cell culture has been recently reported (Santos et al., 2005a), cultivated plants have not yet been obtained (Paron, 2002). This plant shows an intense polymorphism (Semir, 1993), with some specimens lacking any scent, smaller leaves of green coloration and with erect-tortuous, ramified and delicate stems. Other types present pleasant and characteristic aromatic scents, larger leaves of silver green colour with a large amount of scales as well as erect and robust stems. Despite the different scents, both types are used in traditional medicine, although there is a preference for the latter.

As part of our ongoing work on the characterisation of essential oils of aromatic plants growing wild in the Brazilian Cerrado (Azevedo et al., 2001), we now report on the results obtained for the essential oil composition and variability of *L. ericoides*, which have not yet been described, nor other related *Lychnophora* species. For this purpose, two sampling sites were chosen, where each represented a different population with or without scent. The essential oils from leaves were collected at 2-month intervals during 1 year and analysed by GC-MS. To study the chemical variability, the chemical constituents were submitted to principal component, chemometric cluster and canonical discriminant analysis in order to detect the pattern distribution of samples and to identify which constituents can differentiate between these groups of individuals. In addition, the environmental factors affecting the essential oil variability were studied through the application of a canonical correlation analysis between the oil component data set and the edaphic-climatic data matrix with 21 variables for each sampling site.

2. Results and discussion

The essential oils of *L. ericoides* collected from two different sampling sites gave an average yield of 0.24%, s.d. = 0.16 (wt/wt). The annual mean yield of oils from the Cristalina site (0.35%, s.d. = 0.16) was higher than that of plants growing in the Vianópolis site (0.13%, s.d. = 0.06). The seasonal dynamics of the essential oil yield (Table 1) in both population types were similar to those of others Cerrado species, which had lower values during the dry season from May to September period (Silva et al., 2003). In total, 42 compounds were identified, accounting for 86–98% of the volatile constituents. The major components of the essential oils are presented in Table 1, arranged in order of elution. The main group of constituents in most of the samples was composed by oxygenated sesquiterpenes (27.4–91.3%). The Vianópolis population was characterised by the absence of *epi*-α-bisabolol (**15**), regardless of the sampling month, while α-bisabolol (**16**), which has important anti-inflammatory activity (Szoke et al., 2004), only occurs in this site and with a high mean content (61.53%, s.d. = 9.58). On the other hand, the Cristalina samples showed the highest content of (E)-nerolidol (**11**) (41.61%, s.d. = 4.88).

The results obtained from nearest neighbour complete linkage cluster analysis using Ward's technique (Fig. 1) and PCA revealed the existence of a high chemical variability within the essential oils of *L. ericoides*. Fig. 2 shows the relative position of the individuals in the discriminant space in relation to an axial system originated in the PCA. First PC accounts *ca.* 60% of the total variance and separates oxygenated sesquiterpenes (mean content, 83.37%, s.d. = 4.71) ($P < 0.001$) of Vianópolis sampling, with minimal differences among most individuals, from mainly sesquiterpene hydrocarbons of Cristalina samples (11.71%, s.d. = 2.26) ($P < 0.0001$). Therefore, two types of essential oils were found: cluster I (Vianópolis samples) with specimens exhibiting an aromatic scent and being characterised by a high percentage of α-bisabolol (**16**) (61.53%, s.d. = 10.56) ($P < 0.0001$) and α-cadinol (**14**) (17.34%, s.d. = 4.84) ($P < 0.0001$); cluster II (Cristalina population) with specimens without any scent and with (E)-nerolidol (**11**) (41.74%, s.d. = 9.81) ($P < 0.0001$), and *ar*-dihydroturmerone (**13**) (9.71%, s.d. = 3.91) ($P < 0.0001$) as principal constituents.

Canonical discriminant analysis has been performed to help predict clusters, based on the values of the determinate quantitative variables. Using the data set of the seven sampling months, it was possible to classify our proposed clusters using two predictor variables, (E)-nerolidol (**11**) and α-bisabolol (**16**). Fig. 3 gives a visual impression of how well the canonical functions are discriminated among the sampling months using the Cerrado seasons as *a priori* groupings. The first discriminant function ($F1$) accounts for *ca.* 99% of the total variability and separated the populations ($P < 0.0001$), while the second discriminant function ($F2$) distinguished the samples harvested according to dry winter (May–September) or humid summer (November–March) ($P < 0.023$). In addition, using the two discriminant functions, it is possible to accurately predict *ca.* 86% well classification in the original clusters by a cross-validation approach. This involves a number of slightly reduced modifications to the parent data set, estimating parameters from each of these modified data sets, and then calculating the precision of the predictions by each of the resulting models (Wold and Eriksson, 1995). The only one misclassification observed was the August sampling month from the Cristalina site that belongs to the dry season, as it was classified as belonging to the humid season. This misclassification could be caused by the elevated levels of **11** during the August month (characteristic of humid season).

All these findings may be correlated with factors other than the genetic determination, as terpenoid biosynthetic pathway could change during plant development, or under herbivory pressure (Sturgeon, 1979; Langenheim, 1994) or because of differences in environmental conditions (Figuei-

Table 1
Main essential oil constituents from *L. ericoides* leaves collected during 1 year from two sampling sites in Brazilian Cerrados

	Constituents	R_I	Site	July	September	November	January	March	May	August
1	Tricyclene	932	Vianópolis	2.46[a] Ba	2.11 Bab	1.83 Bb	1.12 Bb	0.79 Bb	2.19 Bab	1.18 Bab
			Cristalina	7.04 Aa	2.36 Aab	2.15 Ab	2.44 Ab	2.41 Ab	3.87 Aab	2.94 Aab
2	*o*-Cymene	1021	Vianópolis	2.9 Ba	2.29 Ba	1.92 Ba	1.27 Ba	1.22 Ba	2.47 Ba	2.47 Ba
			Cristalina	7.25 Aa	1.17 Ab	0.78 Ac	1.96 Ac	2.06 Abc	3.4 Abc	3.18 Ab
3	Limonene	1027	Vianópolis	0.95 Ba	0.8 Bb	0.74 Bb	0.55 Bb	0.31 Bb	0.76 Bb	0.56 Bb
			Cristalina	4.5 Aa	1.29 Ab	1.2 Ab	1.69 Ab	1.66 Ab	2.22 Ab	1.93 Ab
4	Linalool	1098	Vianópolis	0.88 Ba	0.72 Ba	0.82 Ba	0.66 Ba	0.54 Ba	0.7 Ba	0.78 Ba
			Cristalina	3.73 Aa	1.48 Aa	1.91 Aa	2.48 Aa	2.02 Aa	3.06 Aa	4.75 Aa
5	Terpinen-4-ol	1175	Vianópolis	2.43 Ba	1.54 Bb	1.6 Bab	1.79 Bab	1.1 Bab	1.75 Bab	1.63 Bab
			Cristalina	7.07 Aa	2.9 Ab	3.65 Aab	4.45 Aab	3.28 Aab	4.46 Aab	5.58 Aab
6	α-Terpineol	1189	Vianópolis	1.35 Ba	0.58 Bab	0.67 Bb	0.68 Bb	0.52 Bb	0.92 Bab	0.76 Bab
			Cristalina	1.84 Aa	0.71 Aab	0.8 Ab	1.12 Ab	0.82 Ab	1.14 Aab	1.35 Aab
7	(E)-Caryophyllene	1418	Vianópolis	0.07 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba
			Cristalina	2.79 Aa	3.31 Aa	4.42 Aa	2.36 Aa	2.27 Aa	2.41 Aa	1.64 Aa
8	*ar*-Curcumene	1480	Vianópolis	1.48 Aa	0.65 Aa	0.95 Aa	0.74 Aa	1.06 Aa	1.44 Aa	0.9 Aa
			Cristalina	1.67 Aa	0.8 Aa	0.72 Aa	0.79 Aa	1.11 Aa	1.15 Aa	1.17 Aa
9	Cameroonan-7α-ol	1507	Vianópolis	0.03 Aa	0.04 Aa	0.0 Aa	0.0 Aa	0.0 Aa	0.0 Aa	0.0 Aa
			Cristalina	3.91 Aa	3.42 Aa	2.57 Aa	3.19 Aa	2.18 Aa	3.11 Aa	2.07 Aa
10	Silphiperfolan-7β-ol	1515	Vianópolis	3.16 Aa	2.1 Aa	1.96 Aa	1.3 Aa	2.09 Aa	1.96 Aa	1.47 Aa
			Cristalina	0.84 Aa	0.83 Aa	0.57 Aa	0.85 Aa	0.86 Aa	1.02 Aa	1.06 Aa
11	(E)-Nerolidol	1564	Vianópolis	0.23 Ba	0.19 Ba	0.01 Ba	0.0 Ba	0.07 Ba	0.14 Ba	0.07 Ba
			Cristalina	31.31 Aa	38.83 Aa	44.6 Aa	43.7 Aa	47.08 Aa	41.1 Aa	43.97 Aa
12	*ar*-Turmerol	1576	Vianópolis	1.33 Aa	1.49 Aa	0.62 Aa	0.47 Aa	0.75 Aa	0.93 Aa	0.93 Aa
			Cristalina	0.0 Ba	0.09 Ba	0.11 Ba	0.11 Ba	0.11 Ba	0.0 Ba	0.09 Ba
13	*ar*-Dihydro-turmerone	1596	Vianópolis	0.71 Ba	0.55 Ba	0.45 Ba	0.04 Ba	0.15 Ba	0.24 Ba	0.49 Ba
			Cristalina	4.77 Aa	15.44 Aab	12.05 Abc	10.8 Ac	9.83 Abc	7.39 Ab	7.28 Aab
14	α-Cadinol	1646	Vianópolis	23.5 Aa	19.94 Aa	13.4 Aab	10.89 Ac	19.59 Abc	16.32 Aab	17.71 Aa
			Cristalina	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba
15	*epi*-α-Bisabolol	1682	Vianópolis	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba
			Cristalina	2.45 Aa	0.28 Ab	0.0 Ab	0.36 Ab	0.13 Ab	0.08 Ab	0.0 Ab
16	α-Bisabolol	1685	Vianópolis	44.65 Ad	53.01 Acd	68.6 Aab	76.44 Aa	64.82 Ab	60.68 Abc	62.52 Ab
			Cristalina	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba	0.0 Ba
Oil yield (%)			Vianópolis	0.09 Ba	0.14 Ba	0.17 Ba	0.13 Ba	0.04 Ba	0.31 Ba	0.1 Ba
			Cristalina	0.23 Ab	0.3 Aa	0.69 Aab	0.25 Ab	0.35 Ab	0.32 Ab	0.24 Ab

[a] Percentage data. Means followed by the same capital letter in the columns and small letter in the rows did not share significant differences at 5% probability by Tukey test.

redo et al., 1997; Robles and Garzino, 2000). In fact, the Canonical Correlation Analysis between populations and soils (Table 2) revealed that α-cadinol (**14**) and α-bisabolol (**16**) from the first set, and potential acidity, Al saturation, cationic exchange capacity, silt, and sand from the second set, load fairly strong onto the first canonical variate and are related to the Vianópolis sampling site. In addition, (E)-nerolidol (**11**) presents a strong relationship to chemical balance in soils (organic matter, P and base saturation), which is related to the sampling at the Cristalina site. Although Hannover (1992) provides evidence that terpene chemotypes are strongly controlled by genetic factors, he also reported instances of environmental variation in terpene expression under extreme habitat conditions. Influence of environmental factors in the chemical composition of essential oils have also been reported in the Asteraceae (Haider et al., 2004), and are well known for Lamiaceae (Ložiene and Venskutonis, 2005; Karousou et al., 2005).

Concerning the morphological polymorphism, our personal repeated field work in sampling sites did not reveal any morphological variability within each population. In this study, the existence of chemotypic differentiation cannot be concluded, because the plants grew on different environments at each sampling site. However, these results may indicate that the Vianópolis and Cristalina populations represent two separate ecotypes. The Corumbá River Basin geographically separates the Vianópolis population from

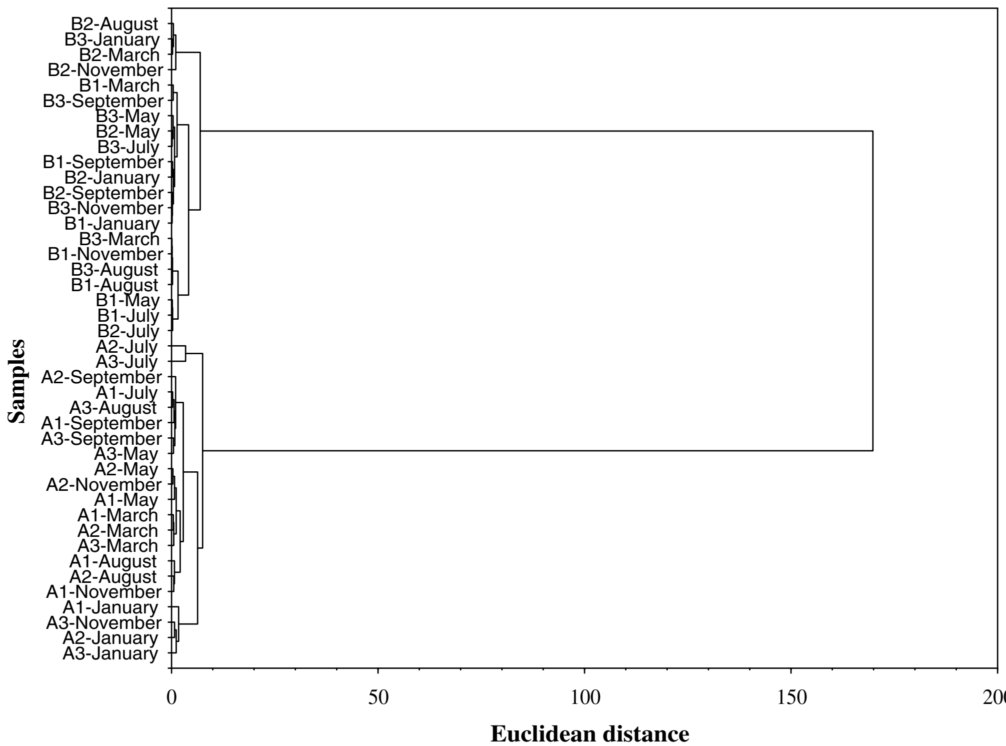

Fig. 1. Dendrogram representing chemical composition similarity relationships among 42 samples of *L. ericoides* to which cluster it belongs: I, Vianópolis site (samples A); II, Cristalina site (samples B).

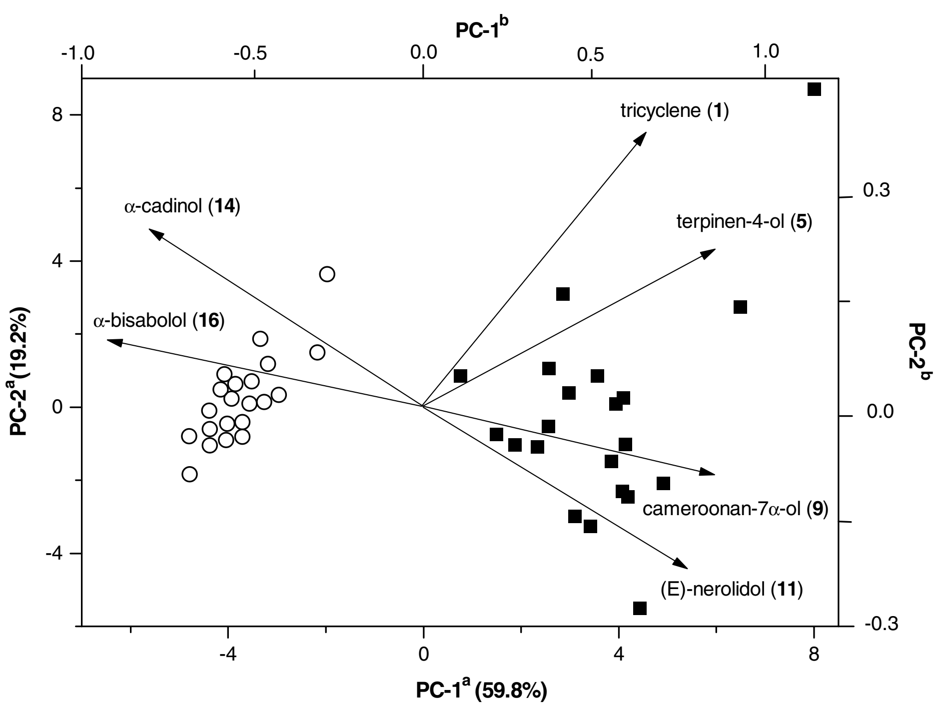

Fig. 2. Scatterplot of *L. ericoides* samples for the two principal components extracted in PCA to which cluster it belongs: I, Vianópolis site (○); II, Cristalina site (■). [a]Axes refer to scores from the samples. [b]Axes refer to scores from discriminant oil components which are represented as vectors from the origin.

the Cristalina site, and this species does not occur in lower altitude areas, corresponding to the depression formed by the river and its tributaries. At least in part, this spatial barrier could contribute to an ecological isolation, a pre-requisite for speciation and chemovariation. Zucchi et al. (2005) analysed the genetic structure by RAPD markers

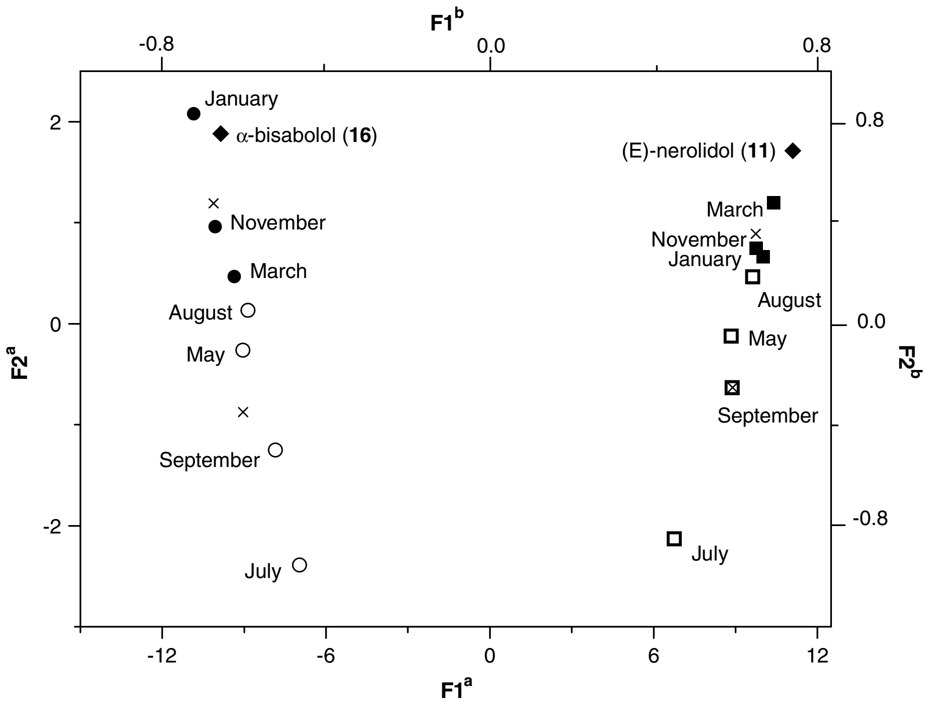

Fig. 3. Canonical discriminant ordination of *L. ericoides* sampling months from Vianópolis (circle symbols) and Cristalina sites (square symbols) according to Cerrado seasons: dry winter (unshaded symbols) and humid summer (shaded symbols). Crosses represent the group centroids on the canonical variates. [a]Axes refer to scores from the sampling months. [b]Axes refer to scores from predictor variables, (◆): (E)-nerolidol (**11**) and α-bisabolol (**16**).

Table 2
Canonical correlation structure (loadings) of the oil components and edaphic-climatic factors with their canonical variates

	Discriminant oil components (set 1)	Canonical variate V1	Edaphic-climatic factors (set 2)	Canonical variate W1
1	Tricyclene	−0.4582	% Sand	0.9994
2	*o*-Cymene	−0.2259	% Silt	0.9994
4	Linalool	−0.6882	% Clay	−0.9994
5	Terpinen-4-ol	−0.6921	% Organic matter	−0.9994
9	Cameroonan-7α-ol	−0.9055	P (μg/ml)	−0.9994
11	(E)-nerolidol	−0.9471	H+Al (mE/dl)	0.9994
13	*ar*-Dihydro-turmerone	−0.8547	Al (mE/dl)	0.9994
14	α-Cadinol	0.9289	CEC (mE/dl)	0.9994
15	α-Bisabolol	0.9788	% Base saturation	−0.9994
			% Al saturation	0.9994
			Precipitation (mm)	0.0427
			Temperature (°C)	−0.0358
Eingenvalues				0.9952
Canonical correlation				0.9976
Wilks' lambda				0.0005
Bartlett's *Chi*-square				234.18
Degrees of freedom				108
P-value				0.0001
Cumulative variance (%):				
Of discriminant oil component data				60.62
Of discriminant oil component-edaphoclimatic relation				82.85

of natural *Eugenia dysenterica* populations in the same areas, and observed a spatial pattern of genetic variability among populations. The authors attributed the differentiation to a stochastic process, where the gene flow depends on geographic distribution, compatible with the isolation by distance model. Here, it might be speculated that the phenotypic plasticity of *L. ericoides*, which ultimately results in qualitative difference of scent is only one factor contributing to differences in the essential oil profiles of natural populations growing in different sampling sites.

Genetic adaptation to the specific environment of the growing site is an other factor to be taken into account.

In spite of the correlation obtained for the two populations of *L. ericoides* with edaphic factors and the high variance explained by environmental data set (Table 2), there is an outstanding percentage of variability in the composition oils that should be the subject of subsequent genetic studies.

In summary, the essential oil from *L. ericoides* grown on the Brazilian Cerrado revealed a high polymorphism, which could be influenced by edaphic factors. The knowledge of the chemical phenotypic plasticity in the essential oil among natural populations of *L. ericoides* could contribute to germplasm conservation of this species in *ex situ* and *in situ* conditions.

3. Experimental

3.1. Plant material

Leaves of *L. ericoides* were collected at the mature vegetative stage in July, September and November 2001, and in January, March, May and August 2002 from their natural habitats and were identified by Dr. Vera Lúcia G. Klein of Departamento de Biologia Geral, Universidade Federal de Goiás, Goiás State, Brazil; 15 randomised individual plants at the same age representing the local population were collected as homogenous samples from each accession of the two localities: (A) Vianópolis site, with a population exhibiting aromatic scent (accessions A1: 16° 40′ 47″ S/48° 12′ 52″ W/917m; A2: 16° 40′ 45″ S/48° 12′ 52″ W/896m; A3: 16° 40′ 45″ S/48° 12′ 48″ W/897m). (B) Cristalina site, with a population without scent (accessions B1: 16° 49′ 26″ S/47° 41′ 43″ W/1140m; B2: 16° 49′ 28″ S/47° 41′ 43″ W/1140m; B3: 16° 49′ 29″ S/47° 41′ 43″ W/1140m). In geographical terms, the Cristalina site presents a coarse sand soil texture, while the Vianópolis site has sandy loam. Site elevation's, mean annual rainfall, mean temperature and mean annual relative humidity are, however, similar. Voucher specimens of each accession have been deposited in the Herbarium of the Universidade Federal de Goiás (UFG), Goiás State, Brazil (code numbers from 27033 to 27038).

The leaf samples were dried in an oven, with circulating air, at temperature of 30 °C for 7 days until constant wt. Then, the total plant material from each population was chopped and submitted to hydrodistillation (3 h) using a modified Clevenger apparatus. The oil yields (%) were based on the dry wt of plant samples. All experiments were conducted in triplicate. Soil samples were also collected at 20 cm depth in all localities. The pH was determined in a 1:1 soil/water volume ratio. Ca, Mg and Al were extracted with 1 M KCl, and P was extracted with Mehlich's solution (Silva, 1999). Organic matter, cationic exchange capacity (CEC), potential acidity (H + Al), base saturation, Al saturation and texture of the soils were determined by applying the usual methods (Silva, 1999). The climatic data were obtained from Núcleo de Meteorologia e Recursos Hídricos do Estado de Goiás-SIMEGO, Goiás State, Brazil.

3.2. Essential oil analysis

The oil sample analyse was performed on a GC-MS Shimadzu QP5050A employing the following conditions: a column CBP-5 (Shimadzu) fused silica capillary column (30 m long × 0.25 mm i.d. × 0.25 μm film thickness composed of 5% phenylmethylpolysiloxane) connected to a quadrupole detector operating in the EI mode at 70 eV with a scan mass range of 40–400 *m*/*z* at a sampling rate of 1.0 scan s^{-1}; carrier gas: He (1 ml min^{-1}); injector and interface temperatures were 220 °C and 240 °C, respectively, with a split ratio of 1:20. Injection volume was 0.2 μl (20% in CH_2Cl_2) in the split mode and the oven temperature was raised from 60 °C to 246 °C, with an increase of 3 °C min^{-1}, then 10 °C min^{-1} to 270 °C, holding the final temperature for 5 min.

Individual components were identified by comparing their retention indexes made through co-injection with a C_8–C_{32} *n*-alkanes series (van Den Dool and Kratz, 1963), mass spectra with those of the literature (Adams, 2001) and a computerised MS-data base using NIST libraries. Major compounds were also identified by ^{13}C NMR spectroscopic analysis using a Varian Gemini spectrometer operating at 75 MHz with C_6D_6 as internal reference. The identification was performed by analysis of the ^{13}C NMR spectrum of the total oil, by comparing the signals obtained with those in the literature (Kubeczka and Formácek, 2002).

3.3. Statistical analysis

Principal component analysis (PCA) was applied to examine the interrelationships between populations and its chemical constituents using Système Portable d'Analyse des Données Numériques-SPAD.N software package, version 2.5 (Centre International de Statistique et d'Informatique Appliquées, France, 1994). Cluster analysis was also applied to the study of similarity of samples on the basis of constituent distribution. Nearest neighbour complete linkage technique by Benzécri algorithm (Benzécri, 1980) was used as an index of similarity and hierarchical clustering was performed according to the Ward's variance minimising method (Ward, 1963).

Canonical discriminant analysis using SAS CANDISC procedure (Statistical Analysis System/SAS Institute Inc., Cary, NC, 1996) was used to differentiate the seven sampling harvested months according to Cerrado seasons. The predictive ability of canonical discriminant functions was evaluated by cross-validation leaving one group approach as implemented in SAS statistical package.

Oil variability and edaphic factors relationships were obtained by a canonical correlation analysis implemented using the SAS CANCORR procedure. The predictive ability was evaluated by canonical redundancy analysis with a standardised variance coefficient. Average multiple comparisons were established by the Tukey test with percentage data $(\%+0.5)^{1/2}$ transformed. *P*-values less than 0.05 were considered to be significant.

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