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Chromosome banding and essential oils composition of Brazilian accessions of *Lippia alba* (Verbenaceae)

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Abstract: The essential oil components and a karyotypic analysis of five *Lippia alba* (Verbenaceae) accessions from Brazil were performed with the objective of investigating the variation among different populations. The chemistry analysis allowed the grouping of the accessions in two main chemotypes: neral chemotype (LaCat, LaJF and LaRJ) and linalool chemotype (LaGua and LaVC). However, large karyotypic differences, verified by different chromosome banding techniques, were not detected. The results presented the same chromosome number for all accessions ($2n = 30$) with 10 metacentric chromosomes and 5 submetacentric. The chromosome banding showed great blocks of constitutive heterochromatin (C-bands) around the centromeric region, which was rich in AT bases (DAPI+), while the CMA bands were observed only in terminal regions of six chromosomes. Through Ag-NOR techniques, only two active pairs of NORs were detected on the three pairs of secondary constrictions (the NOR activity is discussed). This work relates the pattern of heterochromatin for *Lippia alba* for the first time.

Key words: *Lippia alba*; Ag-NOR; chemotypes; chromosome banding; fluorochromes; Verbenaceae

Introduction

Lippia alba (Miller) N.E. Brown (Verbenaceae) is an aromatic shrub widely distributed in South America, that grows in sandy soils on the margin of rivers, lakes and ponds (Moldenk 1965). This species is used in folk Brazilian medicine as sedative, carminative, spasmolitic, emanogoge, sudorific, antidysenteric, antireumatic, antihypertensive and expectorant (Corręa 1992; Di Stasi & Huruma-Lima 2002; Di Stasi et al. 2002; Lorenzi & Matos 2002; Zétola et al. 2002; Zoghbi et al. 1998). This species shows a great phenotypical plasticity (Montanari et al. 2004) and a great variation in essential oil constituents, being divided into different chemotypes (Matos 1996).

Cytological studies have been made with this species and the chromosome number $2n = 30$, firstly described by Bose & Choudhury (1960). Brandão et al. (2005), observed 15 bivalents and confirmed the somatic number of 30 chromosomes. Molecular cytogenetic data was recently obtained and showed three pairs of chromosomes presenting 45S rDNA sites and two chromosome pairs presenting 5S rDNA sites (Brandão et al. 2007).

The C-banding, Ag-NOR and fluorescent staining methods performed with counter-staining reagents are useful to mark species-distinctive regions of chromosomes. They can also be applied to cytotaxonomy and chromosome evolution, including the comparison among individuals of the same species distributed among different populations (Kokubugata & Kondo 1996). Considering the medical importance, the existence of various chemotypes and the large phenotypic plasticity, it is necessary to obtain more detailed information about different populations. With the objective of obtaining a better characterization of this species, four chromosome banding techniques were applied in addition to the essential oil characterization of five different Brazilian accessions.

Material and methods

Five accessions of *L. alba* obtained from five different Brazilian regions (Table 1) were collected and subsequently propagated in a greenhouse with the same environmental conditions. The Voucher specimens (Table 1) were deposited in the CESJ Herbarium, located in the Universidade Federal de Juiz de Fora – UFJF. For each chemotype identification, the essential oils from fresh leaves were obtained through hydrodistillation by a Clevenger-type apparatus during 2 h. The GC-MS analyses were performed on a Shimadzu gas chromatograph equipped with Supleco DB-5 column using helium as a carrier gas and 200 °C of injection temperature. The detector was operated at 250 °C and the column oven program was 50 °C to 200 °C at 4 °C/min. Identification of the constituents was based on the comparison of their retention times and mass spectra with Shimadzu library data of the GC–MS and literature data (Adams 2001).

Root tips were pre-treated with 8-hydroxyquinoline (3 mM) during 7–8 h at 4 °C and fixed in a 3:1 methanol:

Table 1. Origin of five different accessions of *Lippia alba*.

Accessions	City	State	Herbarium register number
LaCat	Cataguses	Minas Gerais	48821
LaGua	Guarani	Minas Gerais	48819
LaJF	Juiz de Fora	Minas Gerais	47724
LaRJ	Rio de Janeiro	Rio de Janeiro	48820
LaVC	Vitória da Conquista	Bahia	48818

Table 2. Percentage of different constituents of essential oil of *Lippia alba* for the five different accessions.

Compounds	Accessions				
	LaCat	LaGua	LaJF	LaRJ	LaVC
7-octen-4-ol	3.65	–	–	–	–
B-myrcene	14.52	–	–	2.36	3.37
Limonene	–	–	–	3.70	2.68
Eucaliptol	–	–	–	–	3.22
Linalool	–	46.82	–	–	33.42
Neral	32.87	19.47	37.05	37.19	19.10
Geraniol	–	4.99	–	4.88	5.04
Geranial	41.97	24.96	54.19	51.86	25.03
Cariophyllene	4.66	3.76	2.99	–	3.96
Germacrene	2.33	–	5.77	–	–

acetic acid solution at least 24 h before the preparation of slides. Root tips were macerated in an enzymatic solution [2% cellulase (Sigma) plus 20% pectinase (Sigma) diluted in 0.001M citric acid-sodium citrate pH 4.8 buffer] at 37 °C (3 h) and the slides were prepared according to Carvalho & Saraiva (1993, 1997). For meiotic analysis, the anthers were collected and fixed in a 3:1 methanol: acetic acid solution for at least 24 h and the slides were prepared according to Viccini et al. (2005).

For heterochromatin studies, the C-banding procedure was performed according to Schwarzacher et al. (1980) with minor modifications. Aged slides were hydrolysed in acetic acid 45% (60 °C) for 12 min and then immersed in 5% Ba $(OH)_2$ at 28 °C for 10 min. After that, they were incubated in 2× SSC at 60 °C for 80 min and the slides were stained with a 10% Giemsa solution for 30 min. The fluorochrome staining was performed according to Schweizer (1976). Aged slides were doubled-stained with 0.5 mg/mL of chromomycin A_3 (CMA) (90 min) and 2µg/mL of diamidino-2-phenyl-indole (DAPI) (30 min) and mounted in 1:1 (v/v) McIlvaine's pH 7.0 buffer-glycerol. Slides were aged for 3 days before analysis and the chromosomes were observed using an epifluorescence microscope (Olympus BX 60) with appropriate filter sets. The NOR visualization method was performed according to Howell and Black (1980) and applied in both mitotic and meiotic chromosomes.

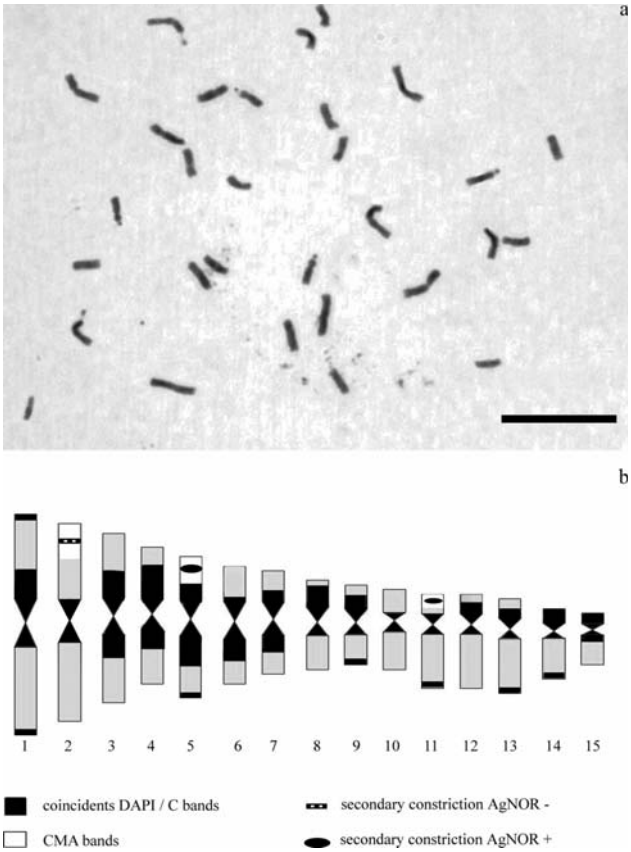

Fig. 1. *Lippia alba*. a – Metaphase stained with Giemsa; b – Idiogram with heterochromatin map. Scale barr 5µm

Results and discussion

The chemical analysis of essential oils showed a qualitative and quantitative difference among the accessions of *L. alba*. The major components identified in the essential oils analysis are listed in Table 2. Through this analysis, it was possible to classify the five accessions in two principal chemotypes. The accessions LaCat, LaJF and LaRJ were classified as a neral chemotype, while the others (LaGua and LaVC) were classified as linalool chemotypes.

The karyotypes of all *L. alba* accessions were very similar, consisting of fifteen pairs of chromosomes, of

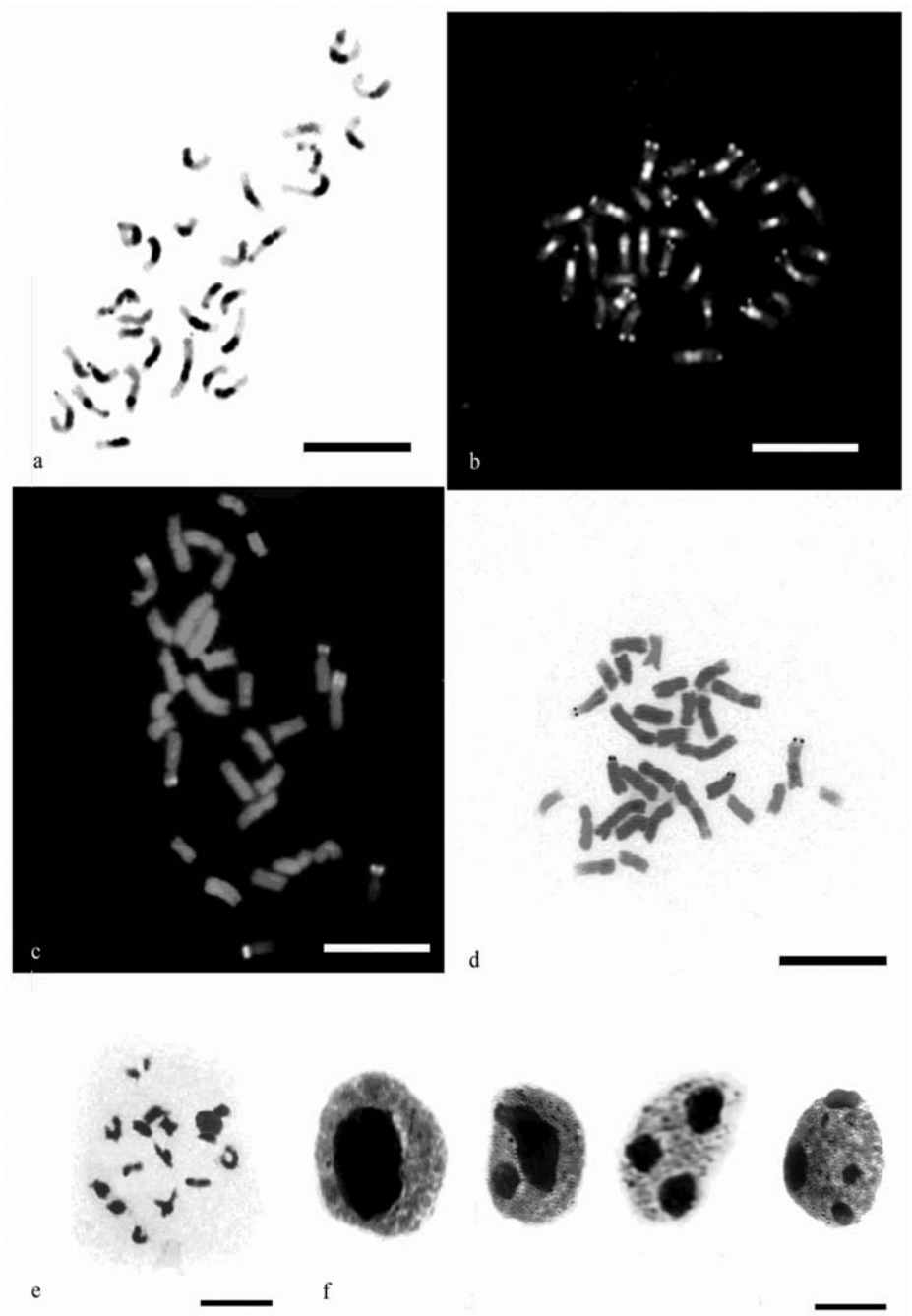

Fig. 2. *Lippia alba*. C-banding (a), DAPI banding (b), CMA banding (c), AgNOR banding (d), diakinesis stained with silver nitrate showing two bivalent associated with the nucleolus (e), interphasic nucleus stained with silver nitrate showing nucleus with one, two, three and four nucleoli (f). Scale bar 5 μm.

which ten were classified as metacentric (1–10) and five as submetacentric (11–15) (Levan et al. 1964; Fig. 1).

A maximum number of three chromosome pairs with satellites were observed in all accessions of the chromosomes 2, 5 and 11 (Fig. 1). However, using the Giemsa staining technique, it was possible to observe that the majority of the metaphases presented only one or two visible pairs of satellite chromosomes. A previous report showed secondary constrictions only in two pairs of chromosomes, but in the same study, the authors observed six sites for the 45S rDNA probes (Brandão et al. 2007). The secondary constrictions are formed in active sites with 45S rDNA (NORs) and sites with restricted NOR activation can, but don't have to, form secondary constrictions, thus hindering the real determination of their number and position through conventional techniques (Carvalho & Guerra 2002; Panzara et al. 1996; Marcon et al. 2005). Four marks with AgNOR banding (Fig. 2d), a technique used in the detection of active NORs, were detected in all metaphases of all *L. alba* accessions that presented two pairs of secondary constriction. These results suggest a partial inactivation of one pair of NORs and can explain the difficulty of observing the secondary constriction on chromosome

Table 3. Heterochromatin pattern of five different accessions of *Lippia alba*. ST = short arm telomere; C = centromeric region; LT = large arm telomere; + = DAPI and C coincidentes bands; * = only DAPI positive bands; □ = CMA_3 positive band and negative silver nitrate impregnation (AgNOR-); ■ = CMA_3 positive band and positive silver nitrate impregnation (AgNOR+)

Chromosome pair	LaCat			LaGua			LaJF			LaRJ			LaVC		
	ST	C	LT	ST	C	LT	ST	C	LT	ST	C	LT	ST	C	LT
1	+	+	*	+	+	*	+	+	*	+	+	*	+	+	*
2	□	+		□	+		□	+		□	+		□	+	
3		+			+			+			+			+	
4		+			+			+			+			+	
5	■	+	+	■	+	+	■	+	+	■	+	+	■	+	+
6		+			+			+			+			+	
7		+			+			+			+			+	
8		+			+			+			+			+	
9		+			+			+			+	+		+	
10		+	+		+	+		+	+		+	+		+	
11	■	+	+	■	+	+	■	+	+	■	+	+	■	+	+
12		+	+		+	+		+	+		+	+		+	
13		+			+			+			+			+	
14	*	+	+		+			+	+		+	+		+	
15		+	*		+	*		+			+			+	

two (Brandão et al. 2007). Furthermore, we always observed a maximum number of four nucleoli (Fig. 2f) in the interphasis nucleus of all accessions and never six (the expected number, if the three pairs of secondary constrictions are active), enhancing the partial inactivation hypothesis. In agreement with this data, when the Ag-NOR method was applied to meiotic chromosomes, it was possible to observe (in diakinesis) only two pairs of chromosomes in association with a great nucleoli (Fig. 2e).

In most species of plants, the number of active rRNA genes is very small. In *Pisum sativum*, only about 5% of the units are transcribed (González-Melendi et al. 2001). Thus, a small fraction of rRNA genes from only one locus seems to be sufficient to supply all the ribosomal machinery of a cell. Homologous NORs usually have identical behaviour in terms of their expression, and consequently both are either transcribed or silenced (Caperta et al. 2002). Previous studies about the regulation of rDNA transcription at intra and interspecific levels indicate that different rDNA loci can compete for essential transcription factors. Consequently, different NORs may present different degrees of activity (Pikaard 1999, 2000; Zurita et al. 1998, 1999). Although we can not explain the regulatory process in *L. alba*, this is the first report of NOR inactivation for any *Lippia* species.

Regarding the heterochromatic pattern, all accessions displayed an apparently identical C-band pattern, with large blocks near the centromere of all chromosomes and terminal bands in some chromopockajsomes (Figs 1b, 2a). The fluorochrome staining in all accessions revealed that the constitution of heterochromatin in *L. alba* accessions is rich in AT bases with great $DAPI^+$ blocks around the centromere of all chromosomes (Fig. 2b). These marks coincided with the C^+ blocks (Fig. 1b) observed in metaphases treated by the C banding technique (Table 3). CMA^+ bands were observed only in six chromosomes (Fig. 2c) always coinciding with satellites (Fig. 1b, Table 3) and no interstitial band was observed. Differences in heterchromatin pattern among the five accessions were observed only on chromosomes 9, 10, 12, 14 and 15 (Table 3).

Finally, a detailed map of heterochromatin was made for the first time in *Lippia alba*. In spite of the great chemotype variability and morphological plasticity, the chromosome constitution among different accessions is very similar in number, heterochromatin distribution and heterochromatin quality.

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