Selection of new clones of linalool chemotype from genetic recombination in *Lippia alba*

Elcio Rodrigo Rufino ([1]); Walter José Siqueira ([1]*); Márcia Ortiz Maio Marques ([1]); Carlos Augusto Colombo ([1]); Joaquim Adelino Azevedo Filho ([2]); Antônio Lúcio Melo Martins ([3])

([1]) IAC - Centro de Pesquisa e Desenvolvimento de Recursos Genéticos Vegetais, Caixa Postal 28, 13020-902 Campinas (SP), Brasil.
([2]) APTA - Polo Regional de Desenvolvimento Tecnológico do Agronegócio do Leste Paulista, Caixa Postal 01, 13910-000 Monte Alegre do Sul (SP), Brasil.
([3]) APTA - Polo Regional de Desenvolvimento Tecnológico do Agronegócio do Centro-Norte, Caixa Postal 24, 15830-000 Pindorama (SP), Brasil.
(*) Corresponding author: walterjs@iac.sp.gov.br

Abstract

The aromatic and medicinal species *Lippia alba* is vigorous and rugged native to the South America (Atlantic Rainforest). Because it is an allogamous and self-incompatible species, natural populations have high morphological and chemical variability. This work had as objective to conduct a preliminary screening to identify new promising clones from a novel (recombinant) base population of *Lippia alba* with regard to its agronomic and phytochemical traits, using the linalool oil or chemotype as model. The two superior linalool clones, obtained by collection, were used as controls. Traits evaluated included: dry mass of leaves (DML), oil yield percentage (EOY%), oil production per plant (OP), and linalool percentage (LN%). Forty linalool chemotype clones were evaluated in three experiments, in a random block design with four replicates and four cuttings (clones) per plot. Besides means comparisons, multivariate analysis was used in order to aid in the preliminary selection of clones. There were positive correlations from moderate to strong for DML vs. EOY%, OP vs. EOY% and DML vs. OP. Linalool clones superior or similar to both controls were identified for the DML, EOY%, OP, and LN% traits (univariate analyses), aimed at further validating experimentation. Five distinct groups were defined in the cluster analysis (UPGMA), each containing subgroups as well.

Key words: Verbenaceae, oil linalool, genetic divergence, clone selection, plant breeding.

Seleção de novos clones de quimiotipo linalol em *Lippia alba* oriundos de recombinação genética

Resumo

A espécie *Lippia alba*, aromática e medicinal, é nativa da América do Sul (Mata Atlântica) e possui muito vigor e rusticidade. Por ser espécie alógama e autoincompatível, as populações naturais possuem elevada variabilidade morfológica e química. O presente trabalho teve por objetivo realizar uma triagem prévia para identificação de novos clones promissores, oriundos de uma população-base inédita (recombinante) de *Lippia alba* quanto às características agronômicas e fitoquímicas, tendo como modelo o óleo ou quimiotipo linalol. Como controles, utilizaram-se os dois melhores clones linalol, obtidos por coleta. As características avaliadas foram: massa seca de folhas (MSF), rendimento de óleo (RD%), produção de óleo por planta em g (PO) e porcentagem de linalol (LN%). Foram avaliados 40 clones de quimiotipo linalol em três experimentos em delineamento de blocos ao acaso com quatro repetições e quatro estacas (clones) por parcela. Todos os dados foram obtidos com médias por planta. As médias foram testadas por Scott e Knott a 5% de probabilidade. Para auxiliar na seleção prévia de clones também foi empregada a análise multivariada. Houve correlações positivas moderadas a fortes para MSF x RD%, PO x RD% and MSF x PO. Para as características MSF, RD%, PO e LN% (análises univariadas) identificaram-se clones linalol superiores e semelhantes aos dois controles visando às experimentações posteriores de validação. Na análise de agrupamentos (UPGMA) foram definidos cinco grupos distintos e cada um deles também com subgrupos.

Palavras-chave: Verbenaceae, óleo linalol, divergência genética, seleção de clones, melhoramento de plantas.

1. INTRODUCTION

Due to a wide range of edaphoclimatic conditions, Brazil has been considered as one of the most important biological diversity centers of the world.

Nowadays, the herbal medicine market is expanding in Brazil. It represents the incoming of US$ 160 millions, what means the increase of 15% per year, while the allopathic medicine reaches only 4% (Carvalho et al., 2008).

Plant Breeding | Article

The species *Lippia alba*, which belongs to the Verbenaceae family and has Brazil as one of its main countries of origin, stands out among the South American flora (Brandão et al., 2007). This family has about 200 species in three diversity centers, of which Brazil is the largest, with 111 species (Atti-Serafini et al., 2002; Silva et al., 2006). Reproduction is by allogamy, with inflorescences of hermaphrodite flowers, but marked by self-incompatibility and irregular germination of seeds. *L. alba* shows variability for several morphoagronomic traits (Rufino et al., 2010), including its essential oils, which are products of secondary metabolism. Essential oil is a product obtained from vegetable raw material either by distillation with water or steam, leaves, seeds, flowers or by a mechanical process or by dry distillation.

L. alba preparations have been used in home medicine, for the relief of several illnesses: stomach aches, constipations, colds and spasms (Di Stasi et al., 2002, Braga et al., 2005), as well as sedative and hypertension relieves (Gazola et al., 2004; Braga et al., 2005). Leaves and roots are the most used in several ways: infusions, baths, and alcoholics extracts (Julião et al., 2003; Scarpa, 2004). Among the several chemotypes occurring in the species, linalool, an open chain monoterpenic alcohol has been detected, able to give rise to isomers (Silva et al., 2003). So, a wide range of uses can be prescribed: antiseptic (Simões and Spitzer, 2003), sedative and insecticides (Silva et al., 2003; Luz et al., 2009) as well as in formulations of cosmetics and perfumes (Bozin et al., 2006; Martins et al., 2010).

Because it is used in the composition of perfum (Chanel n.° 5, commercially launched in 1921), linalool as a raw material is being obtained by extractivism, contributing toward the extinction of *Aniba rosaeodora* (rosewood), a hardwood tree from the Amazon region, whose bark abundantly produces the oil. Therefore, starting from a broad-base genetic population (recombination between five different chemotypes) of *L. alba*, obtained at Instituto Agronômico, State of São Paulo, 40 new linalool chemotype clones were identified. These clones were evaluated for several morphological, agronomic, and phytochemical traits in three experiments, as compared to the best linalool clones used as controls, obtained by sampling (Yamamoto et al., 2008).

2. MATERIAL AND METHODS

A base population of 296 individuals was obtained at Instituto Agronômico (IAC), Campinas, State of São Paulo, in January 2006, by means of open-pollinated recombination of 20 clones, representing five chemotypes (linalool, limonene/carvone, citral, myrcene/camphor, and myrcene). The plants were maintained on a commercial organic substrate for vegetables (HT) in 10 L pots under drip irrigation. When the adult stage was reached, plants of the linalool chemotype were identified by olfaction (three evaluators), since these are easily recognizable. Thirty-eight new linalool clones from that population (more two controls) were then identified and cuttings were collected to conduct three experiments, two at Centro Experimental de Campinas (CEC/IAC) and one at Polo Regional de Desenvolvimento do Leste Paulista, in Monte Alegre do Sul, SP. The two best clones (IAC2 and IAC8) of the linalool chemotype studied by Yamamoto et al. (2008) were collected and used as controls. The cuttings from the forty clones measured 20 cm on average, with four alternate nodes (two opposed buds per node) and a diameter of approximately 1.0 cm; cuttings were maintained in a greenhouse (02/22/2007) on HT organic substrate for sprouting and rooting.

A random block experimental design was adopted, with four replicates and four cuttings per plot, spaced at 1.0×0.80 m.

Two experiments were installed in Campinas, SP, Brazil (22°31'S, 47°02'W, 674 m), one on 03/06/2007 (EP1), in a medium fertility area, pH 5.3, with a base saturation level of 65.0%, while the other (EP2) was installed on 05/17/2007 in a more fertile area, previously cultivated with tomato, pH 6.4, and 89.9% of base saturation level. The third experiment (EP3), was installed on 06/02/2007 at Polo Regional do Leste Paulista (22°24'S, 46°24'W, 820 m), in the city of Monte Alegre do Sul; the region has lower temperatures, with fertility equivalent to the Campinas site, pH 5.4, and 62.0% of base saturation level. The plants were drip irrigated with a flow of 2.4 L h^{-1}. Traits were evaluated in the two central plants and means were obtained per plant.

During growth the plants were tutored to prevent them from touching the ground and becoming rooted, since this is a colonizing species. The above-ground part of both central plants was harvested separately per experiment, harvested between 01/08 to 08/21/2007 (EP1), 24/09 to 10/29/2007 (EP2) and 05/11 to 11/26/2007 (EP3), covering the period of spring. At harvest, the plants were evaluated for the following morphological, agronomic, and phytochemical traits:

a) Dry mass of leaves (DML) in g pl^{-1} – after removing the leaves from all branches collected in both plants of the plot, the leaves were distributed onto newspaper sheets in rooms maintained at room temperature for gradual drying until constant weight to obtain DML and, later, to extract the essential oil and obtain yield (Santos and Innecco, 2003).

b) Essential oil yield (EOY%) – this trait was only evaluated in the 30 most productive clones (28 recombinants + IAC) for DML. Dry leaves were macerated and placed in 2.0 L volumetric flasks containing 1.0 L distilled water; extraction was performed for 1 hour

and 30 minutes. After that period, the essential oil was collected into previously weighed glass flasks with glass stoppers and placed in freezer in order to later obtain yield and inject into a gas chromatograph attached to a mass spectrometer to get readings for the essential oil components. Since this trait has high experimental precision (Santos and Innecco, 2003), and to facilitate laboratory operations, we chose to obtain yield values in only two blocks selected at random per experiment.

After all extractions were performed, the water present in the flasks was removed with a micro pipet (200 mL), and then the essential oil was weighed in a semi-analytical balance (0.01 mg). EOY% was obtained by the g g^{-1} ratio between mass of oil and mass of dry leaves × 100.

c) Oil production per plant (OPP) in g – obtained by multiplying DML by EOY × 100^{-1}.

d) Linalool percentage in the essential oil (LN%) – for phytochemical analysis purposes, the five best clones for yield and oil production per plant were selected, in addition to both controls, as follows: 166, 201, 251, 280, 288, IAC 2 and IAC 8. Only the major compound linalool, present in the essential oil, was evaluated statistically.

The separation and quantification (method of normalization area) of the compounds was performed by gas chromatography equipment with detector flame ionization (GC-FID, Shimadzu GC-2010), capillary column DB-5 (J & W Scientific), 30.0 mm x 0.25 mm x 0.25 μm. The identification of substances was performed in chromatograph gas coupled to mass spectrometer (GC-MS, Shimadzu QP-5000), operating in electron impact (70eV), OV-5 capillary column (Ohio Valley Specialty Chemical, Inc.), 30.0 mm x 0.25 mm x 0.25 μm. The conditions of analysis in the GC-FID were as follows: injector: 220 ºC, detector: 230 ºC, carrier gas: helium with a flow rate of 1.0 mL min^{-1}, Split: 1/20, with sample dilution 1 μL of oil to 1 mL of ethyl acetate, the injection volume was 1 μL and the temperature program was 50 ºC - 165 ºC, 4 ºC min^{-1}, 165 ºC – 220 ºC, 10 ºC min^{-1}. Analysis in the conditions of GC-MS: injector: 220 ºC, detector: 230 ºC, carrier gas: helium with a flow rate of 1.0 mL min^{-1} Split 1/20. The dilution of the sample was 1 μL of oil to 1 mL of ethyl acetate, with 1 μL injection volume and the temperature program was: 60 ºC – 240 ºC, 3 ºC min^{-1}.

The identification of substances was performed by comparing their mass spectros with the database system GC-MS (62 NIST. lib.) And authentic standard of linalool (Sigma Aldrich, 95%) and the linear retention index (Adams, 1995). The retention rates of the substances were obtained by co-injection of the essential oil with a standard mixture of hydrocarbons (C$_9$-C$_{24}$), applying the equation Van Den Dool and Kratz (1963).

Scott and Knott's test at 5% (Scott and Knott, 1974) was used for all traits evaluated in the clone mean contrasts, which is less robust but without ambiguities, with well defined contrast classes (Zimmermann, 2004; Cruz, 2005). The single variation sources were tested against the clones × experiments interaction whenever the interaction was significant at 5% by the F test. As to the means comparison test, in case of interaction, the discussions on clone performance were prioritized for each experiment to the detriment of the joint analysis of means (Gomes, 1966). On the other hand, it must be pointed out that in the present work, the aim was not to make a final recommendation of clones or cultivars for planting, in view of the large number of treatments, but to select new, promising, and therefore more competitive linalool clones, with performance near or superior to the controls obtained by collection (Yamamoto et al., 2008), for later validating experimentation. Therefore, the means of the joint analysis of data were also taken into account even when interactions occurred.

Thus, in order to identify some superior genotypes in this preliminary screening, involving a large number of clones from recombination for later experimentation, selection was oriented toward both means comparison tests in a pairwise fashion, as well as towards their situation in relation to both controls (IAC 2 and IAC 8).

Pearson's correlations test (Morrison, 1976) was applied to determine the linear associations between the various traits evaluated, since early selection could be applied to aid genetic breeding, especially in relation to phytochemical traits, which are time-consuming, costly, and hard to obtain. Significance of correlations was checked by t test at 1% and 5%. High number of treatments and high degrees of freedom as well, have caused significant correlations valus (t test) even in the those of low values. Therefore, Shimakura and Ribeiro Junior (2007) criterion was applied, with classes separated according to the magnitudes of correlations obtained, as follows: 0.0–0.19 very weak; de 0.20–0.39 weak; de 0.40–0.69 intermediate; 0.70–0.89 strong and 0.90–1.00 very strong.

The following programs were used in the univariate statistical analyses and Pearson correlations: SANEST (SANEST Software Program – Machado and Zonta, 1995) and GENES (Cruz, 2001).

In order to supplement the identification of new linalool clones among the 38 analyzed by univariate statistics character by character, two multivariate analysis were employed as well: cluster analysis (dendrogram) and principal components analysis (PCA). It is important to point out that only clones that participated in essential oil extraction (30 clones), therefore those that possessed

phytochemical traits, were evaluated in the multivariate analyses. The GENES software was used in both multivariate analysis and the data input matrix was prepared from the overall means of the three experiments.

In the cluster analysis, the genetic divergence between genotypes was quantified based on the standardized Euclidean distance, as a dissimilarity measure. Based on the distance established between individuals, the genotypes were grouped using the UPGMA method. The clusters were thus established in bidimensional graphical form via PCA, using the Statistica software (STATSOFT INC. 1999).

3. RESULTS AND DISCUSSION

Pearson Correlations (r)

The results obtained for the simple correlations between traits essential oil yield (EOY%), oil production per plant (OPP), dry mass of leaves (DML) in *L. alba* as follows: EOY% x OPP: 0.506; EOY% x DML: 0.104 and OPP x DML: 0.868. The correlation between EOY% × OPP (0.506) was highly significant by the t test, but fell into the moderate class. However, EOY% did not show correlation with DML. In this respect, it seems that the occurrence of moderate correlation between EOY × OPP could be exclusively due to dry mass total per plant, since OPP was calculated by the product between EOY and DML.

Single and joint variance analyses

The DML trait can be considered more relevant to increase total oils and linalool yield during breeding for the selection of new clones. From table 1, facilitating the selection of more productive clones, even at high $CV_E\%$ values (26.0 to 32.5). SANGALLI et al. (2004) also found low experimental precision values for DML in studies on fertilization with organic residues and nitrogen. MARCO et al. (2006) also obtained a high coefficient of experimental variation working with different Citronella Java (*Cymbopogon winterianus* Jowitt) row spacing and harvesting seasons.

In EP1, clone 238 differed from both controls. Clone 280 was again the best in Ep2 and EP3, significantly differing from both controls at 5% (EP3). The clones selected for DML were as follows: 280, 238, 219, 199, 251, 201, 241, 144, and 288.

The EOY% levels of superior clones found in this work are within the range obtained by YAMAMOTO et al. (2008), whose lowest EOY% value observed in ten experiments was 0.93. CV% values were low, varying between

8.5% and 9.5% and revealing good experimental control (Table 2). The clones selected for this trait with values near those obtained in the controls were: 288, 166, 251, 80, 280, 70, 49, 201, 144, and 137.

There was broad variation in OPP contents among the three experiments (Table 3). EP1 was considered an unfavorable environment (soil compaction); for this reason, it had the lowest OPP mean (0.096 g pl^{-1}). Ep3 ranked second, at 0.396 g pl^{-1}, while the best experiment area was EP2, with 0.647 g pl^{-1}. Clone 280 stood out in EP2, statistically differing from the other clones evaluated, with a mean production of 2.36 g.

In EP3, the means were intermediate between EP1 and EP2; however, clone 280 again differed statistically from the other genotypes evaluated, with an average of 1.63 g. The clones selected for OPP considering their mean (joint) behavior and homogeneity in the three experiments were: 280, 251, 166, 288, 201, and 70.

Based on the analysis of the phytochemical profile of the five best clones for DML, EOY%, and OPP, in addition to both controls, significant mean variations in linalool contents were detected among the clones. Since no genotype × environment interactions occurred, the discussions focused only the means obtained in the joint ANOVA for all experiments.

The coefficient of variation for this analysis was very low, which allowed good separation of clones via Tukey's test. It was seen that clones 201 and 288 did not statistically differ from each other; however, they were statistically superior to both controls used, demonstrating the genetic potential of the species to be exploited for the selection of new clones by means of genetic recombination. TAVARES et al. (2005) analyzed the essential oil chemical composition of three *Lippia alba* chemotypes and identified linalool contents of 74.0% during the vegetative stage and 60.1% during flowering, which are much smaller values than those found in this work.

The best two clones in this study for LN% produced, on the average of three Exps, 85.3% and 84.8%, respectively, for clones 201 and 288, and were statistically superior to both controls. In turn, clone 280 showed statistical similarity with the controls, with LN% values of ~80.0 (Table 4). It can be therefore concluded that gains in linalool production and total essential oils can be obtained by direct selection for these traits *per se*, but the highest increases will be achieved by indirect selection, i.e., selecting for DML.

Multivariate analyses

The thirty-clone analysis (28 recombinants and 2 controls) generated the dendrogram shown in figure 1, forming five distinct groups, including subgroups,

according to the standardized Euclidean distance. Since the objective of multivariate analysis was to observe which recombining clones would be more similar to both controls, IAC2 and IAC8, an attempt was made to identify the group or subgroup that would contain those clones. Thus, it was seen that the fourth group consisted of recombining clones together with the collected clones (controls), including 129, 219, T2 (IAC 2), and T1 (IAC 8). The most similar genotypes within this group

were 219 and T2 (IAC 2). The clone with the greatest dissimilarity within the group was number 129. These genotypes had means very close to the controls for some of the traits evaluated. Special emphasis is placed on clone 280, whose superiority had already been statistically detected for some individual traits. In the multivariate cluster analysis, this clone formed the fifth and last group by itself, with 100% dissimilarity from all clones evaluated (Figures 1, 2 and 3).

Table 1. Evaluation of dry mass of leaves (DML) in 38 experimental clones and two controls in *Lippia alba*

Clones and experiments*											
CL	EP 1	SK	CL	EP 2	SK	CL	EP 3	SK	CL	CJ	SK
238	31.50	B	a	280	163.00	A	a	280	126.67	A	a	280	102.72	A
IAC 8	24.13	C	b	IAC 2	125.63	A	b	IAC 8	73.50	B	b	IAC 8	70.42	B
219	19.00	B	c	IAC 8	113.63	A	b	199	68.63	A	b	IAC 2	69.51	B
280	18.50	B	c	219	89.75	A	c	IAC 2	65.17	B	b	238	58.13	B
IAC 2	17.75	C	c	238	78.63	A	d	238	64.25	A	b	219	57.21	B
261	16.50	B	c	129	74.75	A	d	219	62.88	AB	b	199	46.13	C
144	16.50	B	c	201	72.00	A	d	251	42.38	AB	c	251	41.83	C
199	16.13	B	c	251	70.13	A	d	70	42.25	A	c	109	40.38	C
201	15.25	B	c	137	68.50	A	d	109	41.50	B	c	129	39.00	C
147	14.63	B	c	109	65.50	A	d	241	37.25	AB	c	201	38.25	C
109	14.13	C	c	135	64.88	A	d	129	36.75	B	c	241	37.88	C
241	13.75	B	c	241	62.63	A	d	288	36.13	AB	c	288	35.46	C
251	13.00	B	c	166	62.25	A	d	146	34.00	A	c	70	34.83	C
135	12.25	B	d	128	61.75	A	d	147	33.83	AB	c	166	34.63	C
9	11.25	B	d	288	60.63	A	d	166	33.25	B	c	135	34.60	C
70	11.00	B	d	144	60.00	A	d	44	31.63	A	d	137	34.21	C
120	9.75	C	d	9	59.63	A	d	212	28.88	AB	d	147	32.32	C
288	9.63	B	d	199	53.63	A	d	235	27.50	AB	d	128	30.33	C
47	9.50	A	d	31	52.75	A	d	201	27.50	B	d	144	30.11	C
43	8.88	B	d	80	51.63	A	d	213	27.38	A	d	44	29.33	C
166	8.38	C	d	70	51.25	A	d	137	27.13	B	d	9	29.24	C
235	8.13	B	d	44	50.13	A	d	135	26.67	B	d	146	27.67	C
136	7.75	B	d	147	48.50	A	d	2	26.17	B	d	120	26.19	C
212	7.63	B	d	120	45.00	A	e	27	25.25	A	d	235	25.63	C
213	7.63	B	d	146	42.88	A	e	128	24.13	B	d	213	25.54	C
80	7.50	B	d	32	42.75	A	e	120	23.83	B	d	32	24.29	C
32	7.50	B	d	213	41.63	A	e	32	22.63	AB	e	80	24.04	C
49	7.25	B	d	235	41.25	A	e	261	21.75	AB	e	212	23.75	C
137	7.00	B	d	196	41.25	A	e	22	19.83	A	e	31	23.72	C
22	6.50	B	d	216	39.25	A	e	49	18.75	AB	e	261	22.92	C
44	6.25	B	d	172	36.38	A	e	216	17.67	B	e	2	21.76	C
146	6.13	B	d	212	34.75	A	e	9	16.83	B	e	216	20.56	C
2	5.63	C	d	136	33.63	A	e	144	13.83	B	e	196	18.54	C
196	5.63	B	d	2	33.50	A	e	31	13.17	B	e	49	18.33	C
129	5.50	C	d	261	30.50	A	e	80	13.00	B	e	27	17.92	C
31	5.25	B	d	47	29.00	A	e	43	12.00	AB	e	136	17.38	C
128	5.13	C	d	49	29.00	A	e	47	10.75	A	e	172	16.61	C
216	4.75	B	d	27	24.50	A	e	136	10.75	B	e	22	16.57	C
172	4.13	B	d	22	23.38	A	e	172	9.33	B	e	47	16.42	C
27	4.00	B	d	43	14.88	A	e	196	8.75	B	e	43	11.92	C
Means	10.77	C			56.12	A			32.59	B			33.16	
CV(%)	32.24				24.78				26.53				29.15	

Means followed by the same minuscule letter (vertical) do not differ significantly between clones by Scott and Knott test (SK) at 5% of probability.
Means followed by the same capital letter (horizontal) do not significantly differ between experiments by Scott and Knott test (SK) at 5% of probability.
*CL = clones: CJ = joint analysis of experiments: CV(%) = environmental coefficent of variation: EP1 = first experiment: EP2 = second experiment: EP3 = third experiment.

According to the literature, the most important application of cluster methods in multivariate analyses is the identification of divergent parents for future hybridizations, in order to exploit heterotic gene effects. In the present work, the aim was exactly the opposite, i.e., this type of analysis is to be used by the breeder as an additional strategy for the preliminary identification of clusters of similar (less divergent) individuals or divergent individuals that are superior in relation to both controls used in this study.

Principal components analyses

Using principal components analyses, the distribution and formation of groups of individuals very similar to those shown in the cluster analysis was visualized on a bidimensional plane. The values absorbed for each component were 53.7; 15.3; and 9.5, respectively, for PCA 1, PCA 2, and PCA 3 (Figures 2 and 3). Together, the first two components absorbed 69% of the observed variation. In PCA1, among the variables that most influenced the distribution of clones, DML were prominent, with eigenvectors of 0.945 (Figure 2). By analyzing the spatial planes for PCA1 and PCA2, the selected clones (with redundancies in accordance with the distances and principal components involved) were as follows: 280 as the only clone with divergence higher than the controls, and the others in the same group, 238, 219, 70, 251, 199, 129 and 109 (PCA1) and 238, 219, 70, 109, 129, and 137 (PCA2). In PCA3, the selected clones were 280, 70, 109, 129, and 137 (Figure 3). Ten new linalool clones (70, 109, 199, 201, 219, 238, 241, 251, 280 and 288) were selected using through three different analysis approaches.

Table 2. Evaluation of essential oil yield (EOY%) in 28 experimental clones and two controls in *Lippia alba*

						Clones and experiments*					
CL	EP 1	SK		CL	EP 2	SK		CL	EP 3	SK	
166	1.24	A	a	166	1.60	A	a	288	1.65	A	a
280	1.11	A	a	201	1.38	A	a	166	1.47	A	a
201	1.11	A	a	49	1.37	A	a	251	1.45	A	a
251	1.10	A	a	80	1.34	A	a	5	1.43	A	a
80	1.09	A	a	280	1.29	A	a	80	1.39	A	a
70	1.08	A	a	288	1.24	A	a	49	1.29	A	a
5	1.08	A	a	251	1.23	A	a	280	1.23	A	a
IAC 8	1.07	A	a	147	1.20	A	a	IAC 2	1.21	A	a
49	0.98	A	a	70	1.12	A	a	201	1.20	A	a
288	0.96	A	a	5	1.09	A	a	IAC 8	1.18	A	a
147	0.94	A	a	IAC 8	0.98	A	b	137	1.17	A	a
IAC 2	0.89	A	a	146	0.97	A	b	70	1.16	A	a
144	0.87	A	a	144	0.97	A	b	144	1.07	A	a
146	0.79	A	b	IAC 2	0.94	A	b	2	1.00	A	b
135	0.78	A	b	137	0.92	A	b	22	0.96	A	b
22	0.76	A	b	128	0.91	A	b	128	0.96	A	b
2	0.75	A	b	135	0.88	A	b	9	0.95	A	b
120	0.74	A	b	9	0.86	A	b	146	0.93	A	b
128	0.74	A	b	22	0.82	A	b	199	0.86	A	b
137	0.72	A	b	2	0.78	A	b	129	0.82	A	b
129	0.68	A	b	120	0.77	A	b	147	0.81	A	b
9	0.67	A	b	129	0.73	A	b	241	0.77	A	b
212	0.56	A	c	235	0.69	A	c	135	0.74	A	b
235	0.53	A	c	238	0.66	A	c	120	0.73	A	b
109	0.50	A	c	109	0.65	A	c	109	0.70	A	b
199	0.47	A	c	199	0.64	A	c	238	0.68	A	b
241	0.43	A	c	212	0.58	A	c	44	0.63	A	b
44	0.36	A	c	241	0.44	A	c	212	0.62	A	b
219	0.35	A	c	219	0.35	A	c	235	0.57	A	b
238	0.33	B	c	44	0.35	A	c	219	0.54	A	b
Means	0.76	C			0.89	B			0.98	A	
CV(%)	8.48				8.92				9.47		

CL	CJ	SK
288	1.44	A
166	1.39	A
5	1.31	A
251	1.27	A
80	1.22	B
280	1.21	B
70	1.14	B
IAC 8	1.14	B
49	1.09	B
201	1.08	B
IAC 2	1.07	B
144	1.05	B
137	1.03	B
2	0.93	C
9	0.91	C
128	0.90	C
22	0.89	C
146	0.83	C
135	0.81	C
147	0.79	C
199	0.77	C
129	0.76	C
120	0.70	D
241	0.68	D
109	0.63	D
212	0.62	D
44	0.60	D
235	0.53	D
219	0.53	D
238	0.52	D
0.93		
9.50		

Means followed by the same minuscule letter (vertical) do not differ significantly between clones by Scott and Knott test (SK) at 5% of probability.
Means followed by the same capital letter (horizontal) do not significantly differ between experiments by Scott and Knott test (SK) at 5% of probability.
*CL = clones; CJ = joint analysis of experiments; CV(%) = environmental coefficient of variation; EP1 = first experiment; EP2 = second experiment; EP3 = third experiment.

Table 3. Evaluation of oil production per plant (OPP) in 28 experimental clones and two control clones in *Lippia alba*

					Clones and experiments*						
CL	EP 1	SK	CL	EP 2	SK	CL	EP 3	SK	CL	CJ	SK
IAC 8	0.2495	A a	280	2.3632	A a	280	1.6344	AB a	280	1.4100	A
280	0.2325	B a	IAC 2	1.2646	A b	iAC 8	0.8820	A b	IAC 2	0.7586	B
166	0.1863	A b	IAC 8	1.0345	A b	iAC 2	0.8288	AB b	IAC 8	0.7220	B
IAC 2	0.1826	B b	166	0.9649	A b	288	0.6395	A c	251	0.5625	C
201	0.1479	A b	251	0.9243	A b	251	0.6332	A c	166	0.5429	C
144	0.1367	A b	288	0.8629	A b	70	0.5109	B d	288	0.5266	C
147	0.1322	C b	201	0.8581	A b	238	0.4833	A d	201	0.4543	C
251	0.1300	B b	80	0.7990	A b	166	0.4776	A d	70	0.4415	C
70	0.1086	C c	144	0.7706	A b	5	0.4078	A d	238	0.3805	C
5	0.1016	A c	70	0.7051	A b	199	0.3649	A e	80	0.3615	C
238	0.0995	A c	137	0.6371	A c	201	0.3569	A e	144	0.3568	C
9	0.0926	B c	128	0.6220	A c	137	0.3381	A e	147	0.3507	C
120	0.0921	B c	135	0.6161	A c	146	0.3344	AB e	137	0.3430	C
135	0.0901	B c	147	0.6006	A c	147	0.3194	B e	129	0.3173	C
109	0.0805	C c	129	0.5914	A c	129	0.3192	A e	128	0.3077	C
80	0.0805	A c	9	0.5898	A c	219	0.3106	A e	49	0.2988	C
288	0.0773	B c	49	0.5709	A c	241	0.2846	A e	135	0.2927	C
199	0.0720	A c	238	0.5587	A c	109	0.2844	B e	199	0.2862	C
49	0.0591	A c	109	0.4638	A c	2	0.2768	A e	5	0.2805	C
219	0.0569	B c	146	0.4434	A c	49	0.2664	A e	9	0.2794	C
22	0.0561	A c	199	0.4217	A c	128	0.2561	B e	109	0.2762	C
241	0.0551	A c	219	0.4048	A c	44	0.2532	A e	146	0.2726	C
137	0.0538	A c	120	0.3662	A c	80	0.2051	A e	219	0.2574	C
235	0.0503	A c	241	0.3411	A c	22	0.2011	A e	241	0.2269	C
2	0.0471	B c	235	0.3333	A c	120	0.1854	AB e	120	0.2146	C
128	0.0452	B c	5	0.3321	A c	212	0.1851	A e	2	0.2010	C
212	0.0420	A c	2	0.2790	A c	135	0.1721	B e	235	0.1818	C
129	0.0413	A c	212	0.2570	A c	144	0.1632	A e	22	0.1656	C
146	0.0400	B c	22	0.2397	A c	235	0.1620	A e	212	0.1614	C
44	0.0257	B c	44	0.2007	A c	9	0.1558	B e	44	0.1598	C
Means	0.0955	C		0.6472	A		0.3964	B		0.3796	
CV(%)	29.29			36.11			20.30			37.82	

Means followed by the same minuscule letter (vertical) do not differ significantly between clones by Scott and Knott test (SK) at 5% of probability.
Means followed by the same capital letter (horizontal) do not significantly differ between experiments by Scott and Knott test (SK) at 5% of probability.
*CL = clones: CJ = joint analysis of experiments: CV(%) = environmental coefficient of variation: EP1 = first experiment: EP2 = second experiment: EP3 = third experiment.

Table 4. Linalool percentage of the five *Lippia alba* clones recombinants with higher leaf dry matter compared with two controls based on results of three experiments; Tukey's test at 5% for individual and joint variance analysis

					Clones and experiments*						
CL	EP 1	Tk	CL	EP 2	Tk	CL	EP 3	Tk	CL	CJ	Tk
201	87.02	A a	288	84.48	A a	288	84.48	A a	201	85.28	A
251	85.81	A a	201	84.38	A a	201	84.38	A a	288	84.82	A
288	85.51	A a	251	81.94	B b	251	81.94	B b	251	83.27	AB
IAC 8	83.12	A a	280	79.84	BC a	280	79.84	BC a	iAC 8	80.76	BC
IAC 2	79.00	A a	iAC 8	79.55	C a	iAC 8	79.55	C a	280	79.38	C
280	78.46	A a	iAC 2	77.03	D a	iAC 2	77.03	D a	iAC 2	77.69	CD
166	75.51	A a	166	73.47	E a	166	73.47	E a	166	74.15	D
Means	82.24	a		80.22	b		80.22	b		64.09	
CV(%)	3.45			0.60			0.59			2.08	

Means followed by the same minuscule letter (vertical) do not differ significantly between clones by Tukey's test (Tk) at 5% of probability.
Means followed by the same capital letter (horizontal) do not significantly differ between experiments by Tukey's test (Tk) at 5% of probability.
*CL = clones: CJ = joint analysis of experiments: CV(%) = environmental coefficient of variation: EP1 = first experiment: EP2 = second experiment: EP3 = third experiment.
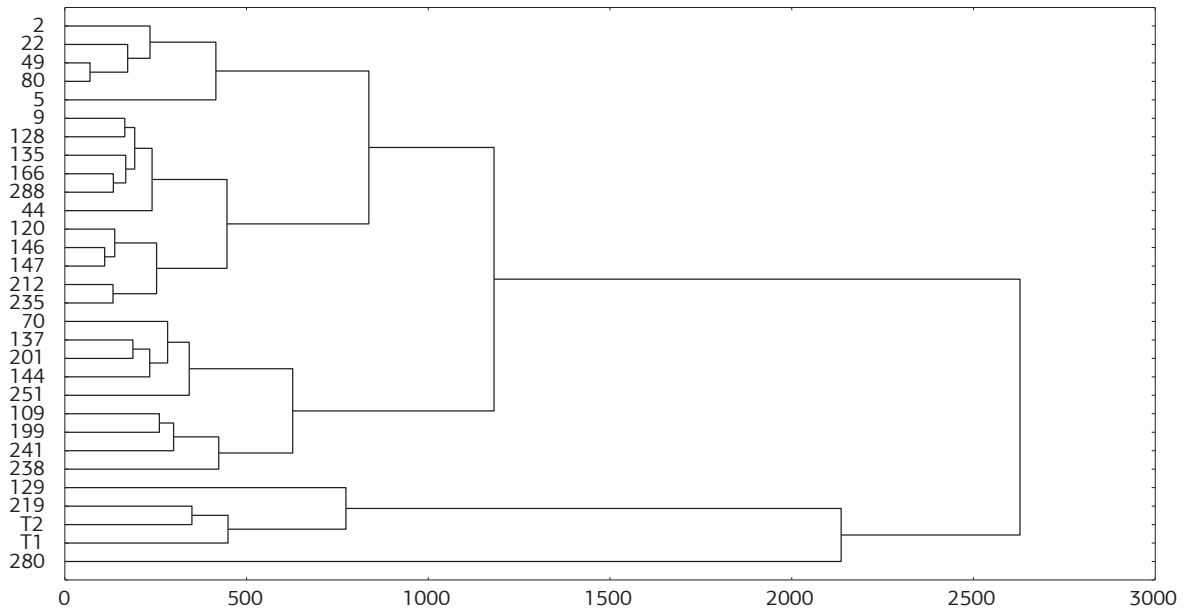
Figure 1. Genetic divergence analysis for 30 *Lippia alba* clones. Genetic distances were obtained from means of three experiments and were quantified by standardized Euclidean distance; clustering was made by the UPGMA method. T1: the best control IAC 2; T2: the best control IAC 8.
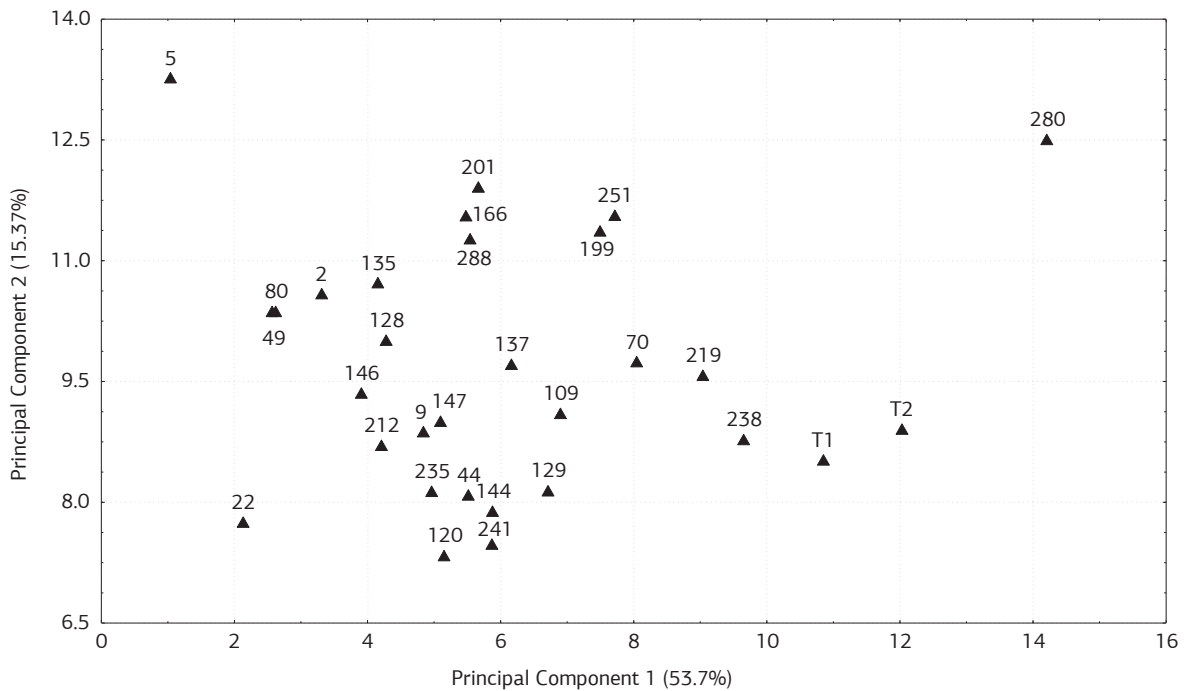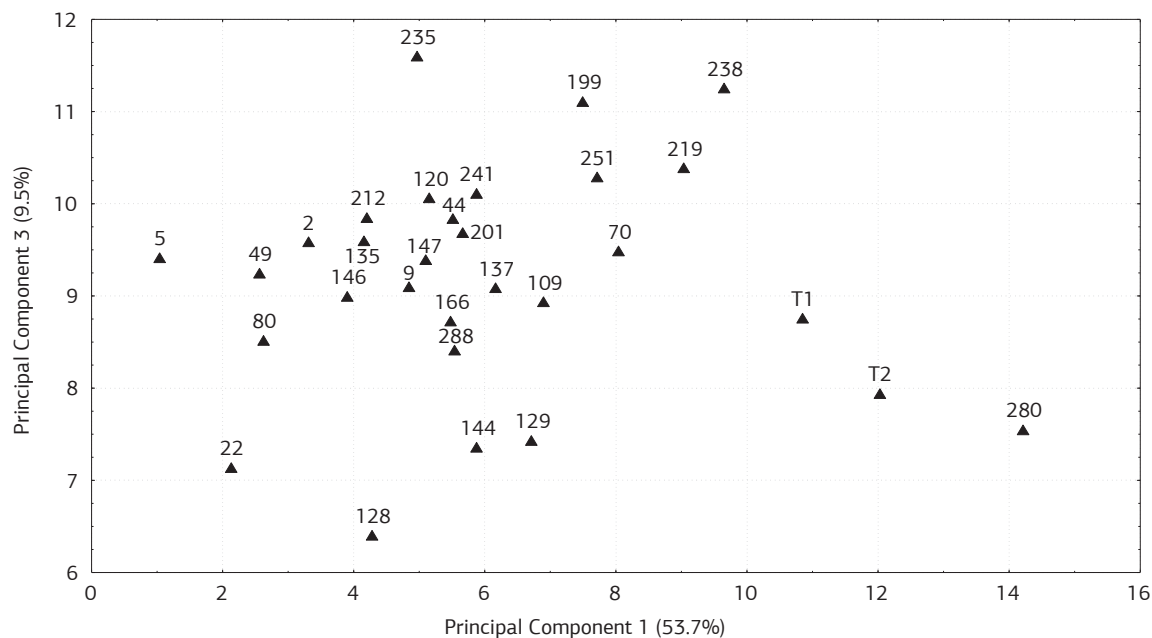

Figure 2. Principal components analysis (PCA1 and PCA2) for thirty *Lippia alba* clones based on studied traits and means from three experiments. T1: the best control IAC 2; T2: the best control IAC 8.

Figure 3. Principal components analysis (PCA1 and PCA3) for thirty *Lippia alba* clones based on studied traits and means from three experiments. T1: the best control IAC 2; T2: the best control IAC 8.

4. CONCLUSION

The clone 280 from two recombinations was higher than controls for most traits, including dry mass leaves (DML) and oil production per plant (OPP).

It was obtained within the clones evaluated, an individual "off type" (clone 201) for the characters of dry mass leaves, LN%, and this clone does not follow the trend established for this correlation variables.

Two clones were selected with higher production of linalool per plant: clone 201 (85.3%) and 251 (84.1%).

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