Phytochemical variability between Colombian accessions of *Lippia alba (Mill.)* N.E. Brown

Variabilidad fitoquímica entre accesiones colombianas de *Lippia alba* (Mill.) N.E. Brown

Variabilidade fitoquímica entre acessos colombianos de Lippia alba (Mill.) N.E. Brown

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Abstract

The physiological fluctuation of organic constituents about its environmental response has two components, intra-inter individual variation. This study assessed the components of biological variation based on a matrix of presence-absence, generating precision values inter-intra individual/population based on a sample of 58 accessions of *L. alba*. Chemical intra-individual variability was high (WI=2.06 [98%]) and the inter-population, was low (AP=0.033), with F_{ST}=0.023 p(rand>data=0.07) and 2% proportion. There were no high values of Estimates of variance [Est.Var.] leading to establish chemical variability among individuals and populations. The results generated by the test point to the presence of two sub-populations, defined each by local environmental conditions.

Keywords: WARDMLM.SAS, phytochemical screening, Verbenaceae, intra/interindividual variation

Resumen

La fluctuación fisiológica de constituyentes orgánicos alrededor de su respuesta ambiental, tiene dos componentes, variación intra e interindividual. Este estudio evaluó los componentes de variación biológica con base en una matriz de presencia-ausencia, generando valores de precisión inter-intra poblacional/individual a partir de una muestra de 58 accesiones de *L. alba*. La variabilidad química intra-individual fue alta (WI=2.06 [98%]) y la inter-poblacional, fue baja (AP=0.033), con F_{ST}=0.023 p(rand>data=0.07) y proporción 2%. No se observaron valores altos de estimativos de varianza [Est.Var.] que conduzcan a establecer diversidad genética-DG- entre poblaciones e individuos. Los resultados generados por la prueba apuntan a la presencia de dos sub-poblaciones, delimitadas cada una por condiciones ambientales locales.

Palabras clave: WARDMLM.SAS, tamizaje fitoquímico, Verbenaceae, variación intra/interindividual

Resumo

A flutuação fisiológica dos constituintes orgânicos ao redor de sua resposta ambiental têm dois componentes, variação intra e inter individual. Neste estudo, foram avaliados os componentes da variação biológica baseado em uma matriz de presença – ausência, gerando valores de precisão inter e intrapopulacional/ individual partindo de uma amostra de 58 acessos de *L. alba*. A variabilidade química intraindividual foi alta (WI=2.06 [98%]) e a interpopulacional, foi baixa (AP=0.033), com F_{ST}=0.023 p(rand>data=0.07) e proporção 2%. Não foram observados valores altos de Estimativos de Variância [Est.Var.] que conduzem a estabelecer Diversidade Genética-DG- entre populações e indivíduos. Os resultados gerados pelo teste apontam á presença de dois subpopulações, delimitadas (cada uma) por condições ambientais locais.

Palavras-chave: WARDMLM.SAS, peneiramento fitoquímico, Verbenaceae, variação intra/interindividual

Introduction

Plant essential oils and secondary metabolites. Essential oils are liquid oily aromatic fragrant [sometimes semi-liquid or solid], obtained from plant material [flowers, buds, seeds, leaves, branches, bark, herbs, Woods, fruits and roots] (UNCTAD, 2005). Take advantage mainly in the food industry as flavoring, the perfume-fragrances industry, and the pharmaceutical, to add taste and/or smell drugs (UNCTAD, 2005). Plant secondary metabolites production is genetic, performing functions of attraction-repulsion (Viccini et al., 2004). However, its expression is determined by pre-existing conditions in their habitat (presence or absence of phytophagous, other plant species, biophysical components, other). These (secondary metabolites), are being actively used as bioprotectants action _cida [suffix] sustainable in current systems of agricultural production in the world (Das & Das, 2005). The determinants of chemical variability and yield of essential oil (and its complex mix of secondary metabolites) in each species are important (Figueiredo et al., 2008). These includes: (a) physiological variations; (b) environmental conditions; (c) geographical variations; (d) factors genetic and evolutionary; (e) socio-cultural conditions and (f) quantity of plant material (Riccardi *et al.,* 2000; Jarvis et al., 2006; Figueiredo *et al.,* 2008; Palacio & López, 2008).

Composition of the essential oil of *Lippia alba*. ***In L. alba***, secondary metabolites are mainly flavonoids, produced by epidermal glands in the mesophyll cells. Essential oil of *L. alba* has a relative high content of carvone, between 30% and 35% (Castro *et al.,* 2002), turning it into a substitute for other essential oils that contain this same component. Another important chemical component of *L. alba* is limonene, making up more than 25% of essential oil (UNCTAD, 2005). The main compounds of the essential oil observed in leaves are: terpinene, p-cymene, caryophylene, myrcene, geraniol, neral and (Castro *et al.,* 2002.; Jarvis et al., 2006; Blanco *et al.,* 2007; Celis *et al.,* 2007; Mesa *et al.,* 2009; Escobar *et al.,* 2010).

Chemical variability of essential oils in *L. alba*. The chemical composition of the essential oil of *L. alba* depends on the geographical origin, the conditions of culture, age and part of the plant used for extraction, and geobotanical factors (Stashenko *et al.,* 2003). Genotypic determination (Montanari *et al*., 2004; Viccini *et al.,* 2004-2006; Dias *et al.,* 2005-2006; Suárez *et al.,* 2007; Yamamoto *et al.,* 2008; Martínez *et al.,* 2008), biochemical characterizations (Fischer *et al.,* 2004; Hennebelle *et al.,* 2006) and morphological descriptions (Montanari *et al.,* 2004; Hennebelle, 2007); they make up the bulk of studies reported in the species *L. alba*. Individuals (*L. alba*) collected in the northeastern region of Colombia, was shown to be a new chemotype, not previously described in the literature, containing carvone [40-57%] as a major component in oils, followed by limonene [24-37%], biciclosesquifelandreno [2-22%] piperitenona [1-2%], piperitone [0.8-1.2%] and the β-burboneno [0.6-1.5%]; the component main in

volatile fractions was limonene [27-77%], followed by carvone [14-30%]], biciclosesquifelandreno [1-33%] and the β-burboneno [0.5-6.5%] (Stashenko *et al.,* 2003). The analysis of essential oils obtained from leaves of samples of French Guiana, Martinique and Guadeloupe, showed seven different 'chemotypes' (Hennebelle *et al.,* 2006), with a possible connection between chemotype and morphotypes (Hennebelle *et al.,* 2006). The GxE interaction of 10 genotypes of four chemical compositions (chemotype) of *L. alba* from three regions of the State of São Paulo-Brazil, had a wide range of leaf production, while essential oils performance had a high and uniform genotypic determination. No qualitative variation was detected for chemical composition and quantitative variation was of low magnitude. Linalool and limonene genotypes / carvone were invariably more productive for performance of oil that the citral genotypes and myrcene/canfor (Yamamoto *et al.,* 2008).

Lippia alba (Miller) N.E. Brown is characterized by a great variability in morphology and chemical composition of the essential oil (Oliveira et al., 2006). Tavares *et al.* (2005), presented data on the quantitative variation of the major volatile components of linalol production in a chemotype of *L. alba*. The contents of [alpha] - pinene, (Z) - 3-hexenilo and [alpha] - gurjunene was higher in the mother plants cultivated in the ground that in the seedlings grew up in MS medium, while the contents of sabinene, myrcene, 1.8 - cineole and *p*-menta - l, 5, 8-trieno was lower. The addition of 0.2.3 μM of IAA in the middle was significantly higher for the myrcene content and sabinene. The addition of 0.92 μM kinetin increased significantly 3 (S) - (+) - linalool level (Tavares *et al.,* 2005). In Colombia, Camargo & López (2008) and Palacio López (2008) evaluated the response of *L. alba* to the availability of water and nitrogen (Antolinez & Rodriguez, 2008). The quantitative variations of the main volatile components of the production of linalool in *L. alba* are associated with nutritional and environmental factors (Tavares *et al.,* 2005; Camargo & López, 2008; Palacio & López, 2008; Antolinez & Rodriguez, 2008).

Chemical composition and antimicrobial activity of the essential oils in *L. alba* were investigated by Oliveira *et al.,* (2006) to relate them to their traditional uses. The analysis allowed the identification of two chemotypes of *L. alba*, a myrcene-citral as chemotype (15% and 37.1%, respectively) and *Lippia alba* f. intermedia as a chemotype citral (22.1%). The essential oils of both species were active against all the tested organisms (bacteria and fungi) by halos of inhibition testing with range from 1.1 to 5.0 cm; probably due to its high content of oxygenated monoterpenes (51.0% and 40.1%, respectively), mainly represented by aldehydes and alcohols. Chemical and pharmacological data of *L. alba* obtained by Oliveira *et al.,* (2006) agreed with the Ethnobotanic survey. In Colombia, "El Centro de Investigaciones en Biomoleculas-CIBIMOL"-from Universidad Industrial de Santander has made, among others, evaluative studies on tropical diseases, bioactivity and active ingredients of *Lippia alba* how antimicrobial (Bueno & Stashenko, 2009); antiviral [Ocazionez *et al.* (2010) in the dengue virus; [Meneses *et al.,* (2009) in the yellow fever virus], antifungal *Candida* and *Aspergillus* (Montiel *et al.,* 2007), in bacteria [Bueno *et al.,* (2009) in *M. tuberculosis*]; as anti-Protozoan (Escobar *et al.,* 2010). A complete list of reports places it in http://tux.uis.edu.co/quimica/ investigacion/centros/ cibimol.

The goal in the present investigation was to determine the chemical variability of 58 accessions of *L. alba* in such a way to admit an approach to the population structure of the species in Colombia. Been achieved, through the construction of an array of chemical data which allowed: a) determine chemical variability and b) forming a molecular and ecological data-compatible database that defined the genetic and spatial structure of the Colombian population of *L. alba*.

Materials and methods

Plant material. 200 g of aerial tissue samples (stems-leaves-flowers mix) of 58 accessions of *L. alba* collected in two agro-ecological zones of the Colombian Andean Region. Accessions formed part of a transient Bank *in vivo ex situ of L. alba* of the "Centro Experimental Universidad Nacional-Sede

Palmira (CEUNP)". The study considered two ecosystems of Bs-T [Chicamocha Region and Sumapaz Region]. Tropical dry forest corresponds to the Tropical Alterhidric zonobiom that develops in the lowlands between 0 and 1000 meters. Detailed information about zonobiomas on: http://www.humboldt.org.co/es/.

Phytochemical screening (García, 2003; García *et al.,* 2003; García, 2006; Baldizán *et al.,* 2006) **and estimates of variance** (Peakall & Smouse, 2006). Tests [twice] colorimetric with ethanol and chloroform as solvents, valued the presence (1)-absence (0) of seven groups of secondary metabolites in the work sample, encoded with the Munsell system (tone, value and chroma). Estimates of variance among and within populations (Chicamocha and Sumapaz) and individuals were estimated using the algorithm developed by Peakall & Smouse (2006). The GenAlex program (Peakall & Smouse, 2006) applied to the array of data from laboratory (presence/absence of metabolites) from dual tests with ethanol and chloroform as solvents, to assess the presence of seven secondary metabolites [1. derivatives of coumarins, 2. steroids and terpenoid, 3. flavonoids, 4. cardiac glycosides, 5. saponins, 6. glycosides and 7. tannin] in 58 Colombian accessions of *L. alba*. Tests were developed between December 2012 and January 2013, using the Protocol and the supervision of the team, from the Phytochemistry Laboratory at the Universidad National de Colombia-Sede Palmira. Bank samples, available in Lab. Biol. Molec. in UN-Palmira.

Analytical methodologies. Two groups of procedures were used, Group I (DISTANCE, CLUSTER-TREE) and group II (FACTOR, PRINQUAL-BIPLOT) SAS/STATv9.0; the WARDMLM.SAS strategy and the GenAlEx program (Peakall & Smouse, (2006). Step I: raw data were processed by DISTANCE+ CLUSTER-TREE procedures using complete linkage distance to find possible chemical groups. Step II. Laboratory data matrix was analyzed with procedures PRINQUAL and SAS/STAT FACTOR v9.0 to obtain an optimal number of common factors (continuous synthetic variables), the matrix conformative last for the joint final analysis (ecological, chemical and molecular data, available in Cardona (2014). A final matrix approved by common and unique factors was processed to determine conclusive analysis and number of groups. In addition, the WARD MLM.SAS strategy (supplemented with location data) was used to identify possible chemical groups, more a Biplot with SAS/STAT PRINQUAL-FACTOR.

The Analysis of Factors with Probability Maxims-AFPM, was the research base. The AFPM calculates estimates of preferential commonality with values of maximum likelihood with the use of the PRIORS = SAS/STATV9.0 MAX, in the presence of singular matrices. The usual form of the analysis is:

proc factor data=*SAS-data-set* method=principal scree mineigen=0 priors=max outstat=<libname>;
run;

The square of the multiple correlation (CCM) of each variable with all other variables was used as extremely preferential communality [or more important] (in: SAS/STATv9.0); and it was basic in the final formation of groups.

Interpretation of analysis. The array generated by the test data were analyzed using the algorithm developed by the program GenAlex v.6.5 (Peakall & Smouse, 2006) for binary data (1/0). The data matrix was formed by 58 accessions (35 of the Region I = Sumapaz and 23 Region II = Chicamocha). The results of the analysis of variance are obtained from a distance matrix to calculate the statistical F based on the formulas:

Fst = AP / (WI + AI + AP) = AP / TOT

Fis = AI / (WI + AI)

Fit = (AI + AP) / (WI + AI + AP) = (AI + AP) / TOT.

Inbreeding coefficient

Nm = [(1 / Fst) - 1] / 4 number effective

migrants per generation

Results

The analysis of variances (from phytochemicals data) shown in Table 1. Chemical variability within individual [WI = 2 060] had high and between populations [AP = 0. 033], indicator low. Among individuals, the analysis showed zero [AI = 0]. AP was the source of variation with more weight on the answer given by the ANOVA [MS = 2.567]. The variability between populations [AP = 0. 033] was low with a FST = 0.023 P(rand≥data=0.07) and 2% proportion. However, the estimate of variance with the largest proportion occurred within individuals WI = 98%. The greater variability occurred within individual. The maximum and minimum values for FST were 0.597 and 0.038, respectively.

Table 1. *Estimates of variance [Est.Var.] for 58 Colombian accessions of L. alba phytochemical screening, using program GenAlex v6.5.*

Source	Df	SS	MS	Est. Var.	Proporción
Among Pops(AP)	1	2.567	2.567	0.033	2%
Among Indiv(AI)	56	42.493	0.759	0.000	0%
Within Indiv(WI)	58	119.500	2.060	2.060	98%
Total	115	164.560		2.093	100%
F-Statistics	Value	P (rand >= data)			
Fst	0.023	0.070			
Fis	-0.462	1.000			
Fit	-0.429	1.000			
Fst máx.	0.597				
F'st	0.038				
Nm	10.819				

Main discussion. The degree of differentiation phytochemical (by presence-absence of secondary metabolites) between populations of 0.03, indicates a similarity between the two populations, 'probably' because of their form of dissemination of the species in Colombia (Anthropochory). However, this 2% indicates a minimum portion of individuals with maximum degree of differentiation caused by allogamy and low viability of seed; demonstrating sexual reproduction and differentiation among populations most likely because of allogamy, more local environmental factors. Estimates of variance between regions indicate similar populations and among individuals not differentiable chemotypes it shows. The estimate of variance within individual indicated high variability (98%) for secondary metabolites-*sm*-caused by physiological and environmental response content. The degree of differentiation for presence/absence of *sm* within and between individuals, had negative values (Table 1), indicating that individuals are chemically similar probably due to their low capacity of sexual reproduction and its form of propagation-dissemination (Anthropochory=active dissemination of reproductive material, due to human action). The possible chemical groups are displayed in the dendrogram of Figure 1. The range of values of F obtained our possibility: a) of identifying at least one special and b) discriminating chemotypes of the *sm* in the final formation of groups.

Number chemical groups present in the working sample. The WARDMLM.SAS strategy determined the presence of nine phytochemicals groups (Table 2). The final number of groups, shows nine phytochemical groups: Group I (accessions 189-359-360-362/3/4/5); Group II (174-201/2-209-373/4/5/6/7/8); Group III (300/1/2/3/4/5); Group IV (346-348/9-350/1/2/3/4/356/7/9); Group V (CEUN-308-310/1/2/3/4-320-322); Group VI (3-4-5); Group VII (369-370); Group VIII (187-366/7); Group IX (340/1/2/3/4/5-347). However, the Biplot of Figure 2 suggests a predominant majority chemical group [in Colombia] and the presence of at least three accessions with individual chemical characteristics by the presence-absence of *sm* in 201, 213, and 341 accessions. Its location within the Biplot indicated chemical properties own and different from the rest of accessions included in the test. This result suggests the 'possible' collection of individuals (sexually) propagated by seed. Group V, included the CEUN accession of UN-Palmira.

Table 2. *Final number of groups and individuals by Group [weight-Proportion] obtained with the WARDMLM.SAS strategy.*

Group	Frecuency	Weight	Proportion
1	7	7.0000	0.118644
2	10	10.0000	0.169492
3	6	6.0000	0.101695
4	11	11.0000	0.186441
5	9	9.0000	0.152442
6	4	4.0000	0.067797
7	3	3.0000	0.050847
8	3	3.0000	0.050847
9	6	6.0000	0.101695

The outstanding frequencies were, in order: Steroids & Terpenoids > Tannins> Flavonoids, the lower frequency was in glycosides and cardiac glycosides. The results of Table 3 show high frequencies to Tannins (chloroform-[C] = 0.8136 and ethanol [E] = 0.6949), Steroids & Terpenoids (C=0.8305 and E=0.6271) and Flavonoids (C=0.6271 and E=0.7458); and low for glycosides (C=0.0169 and E=0) and cardiac glycosides [C=0.0508 and E=0.0508]. Derivatives of coumarins and saponins showed intermediate values. Glycosides and heterosides were exceptionally low, 0.05 and 0.01 respectively (Table 3).

Table 3. *Proportion of individuals with value [1] presence of seven types of secondary metabolite in two types of solvent.*

Metabolites	Solvent	Frecuency
Derivates of Cumarins	Cloroformo	0.1186
	Etanol	0.1186
Esteroids & Terpenoids	Cloroformo	0.8305
	Etanol	0.6271
Flavonoids	Cloroformo	0.6271
	Etanol	0.7458
Glycosides inotropic agent	Cloroformo	0.0508
	Etanol	0.0508
Saponins	Cloroformo	0.3729
	Etanol	0.1356
Heterosides Cianogenics	Cloroformo	0.0169
	Etanol	0.0000
Tanins	Cloroformo	0.8136
	Etanol	0.6949

Discussion

The phytochemical screening showed the absence of alkaloids in *Lippia alba* (Morataya, 2006; Medina-Lopez et al., 2011). With nulls to alkaloids, WARDMLM.SAS strategy defined a final number of nine phytochemicals groups. For the number of groups technical support defined it strategy in step III, generating a list of the log-Likelihood corresponding to each of the possible numbers of groups and the graphic logL *versus* number of groups.

High frequency of steroids & amp; Terpenoides and flavonoids stock defines the chemotypes present in Colombia. Some medicinal properties of *Lippia alba* are due to the high content of lightweight terpenes as 1, 1,8-cineole, limonene, b-myrcene (Riccardi et al., 2000). The antioxidant activity and/or antimicrobial activity are attributed to flavonoids and phenols/ tannins (Martínez *et al.,* 2002; Núñez *et al.,* 2008)

Phytochemicals lower frequencies reveal the existence of other possible chemotypes for Colombia. Contrasting values of frequency indicate the presence of a dominant majority group in Colombia. The WARDMLM.SAS strategy shows three majority groups being more discriminating than the Biplot. The results obtained by the different tests suggest that variability Phytochemistry of the sample of *L. alba*, is a physiological-environmental and there are no involved epigenetic factors. Analysis genetic markers RAM, confirm it (Cardona, 2014).

Analysis of factors with maximum likelihood.
The cluster with the Ward method and the distance for Gower, formed two groups, one consisting of the 187-356-340-454-370-367-369-341 accessions and the second large group composed of three subgroups (Figure 2). Accession 341 were completely independent. The cluster shows that they are chemically equal each other: the accessions (1-351); (356-359-360-378); (362/3/4/5-374/5); (4-320); (174-189-373); (202-209-217-300-302/3/4/5-377); (301-346-344). The Biplot generated by factors of commonality-FC is shown in Figure 2.

AFPM (analysis of factors with high probability) procedure used the option PRIORS = MAX at presence of unique correlation matrices. The eigenvalues of the reduced matrix of correlations (Total=10.2698937, average=0.51349468) defined 11 factors with the resembles criteria explaining the 1.1464 variation found in the test. The matrix of 11 factors including region, altitude and coordinates (North and West) decimal places, is part of the final matrix of joint markers that defined the genetic structure and phytogeographic samples of Lippia alba used in the test. This output generated by the AFPM matrix markers phytochemicals that are generated from the respective Biplot and phytochemical cluster is considered. The Biplot and cluster generated by the AFPM are shown in Figures 1 and 2.

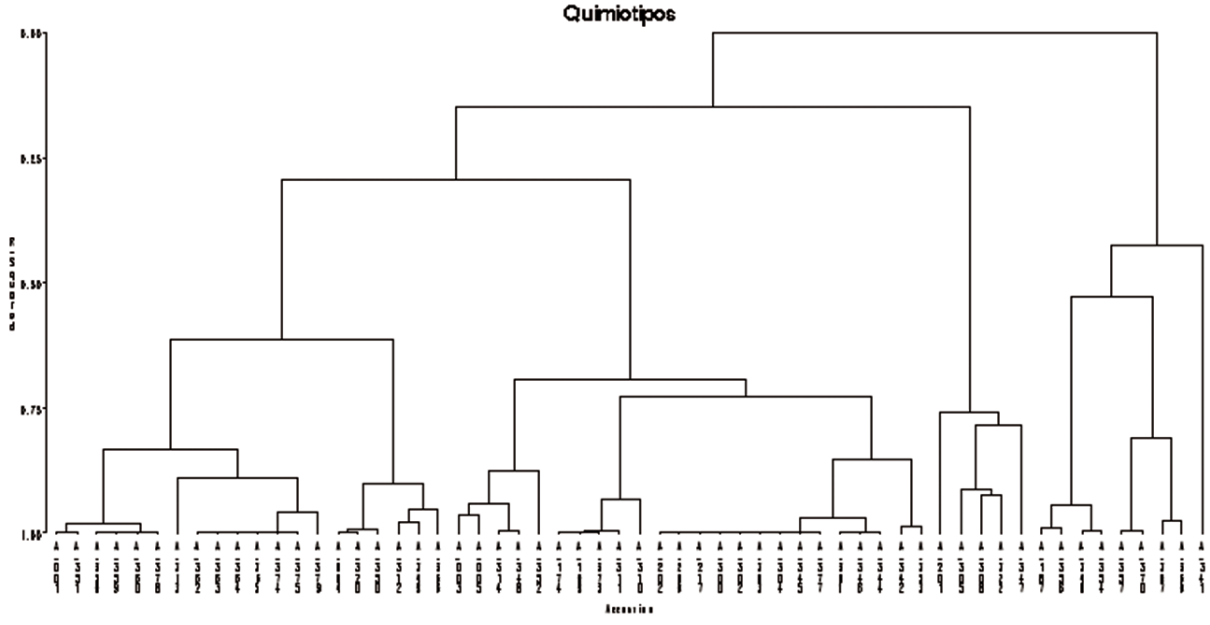

Figura 1. Phytochemical groups based in SAS/STATv9.0 AFPM for net laboratory data.

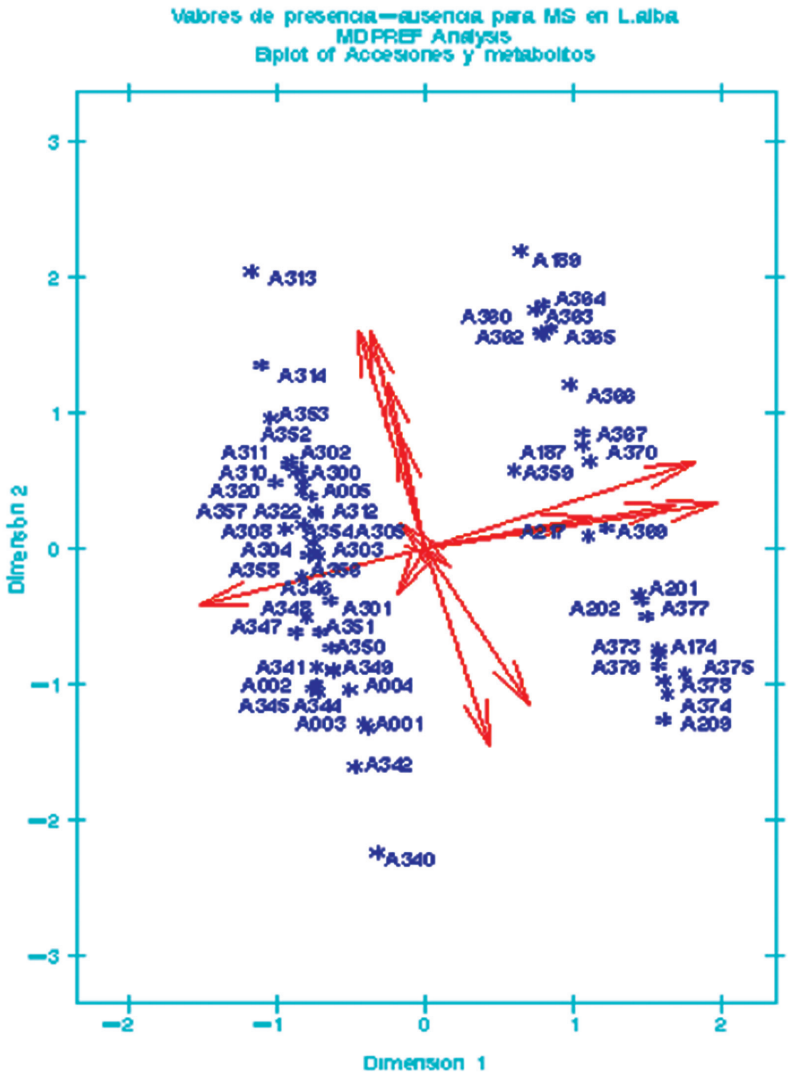

Figure 2. Biplot for factors with values of maximum-likelihood [FPM] presence of ms 58 Colombian accessions of *Lippia alba*, including data from site [region, altitude, coordinates].

Discussion. The Biplot and cluster shows four major trends, separating chemotypes into two large groups: Region Chicamocha and Sumapaz region. There are homologies within region and possible division into two sub-populations, determined by environmental conditions. Taking into account region, altitude, and coordinate strategy wardmlm.sas determined for the Region Chicamocha, four groups and to the Sumapaz Region five groups. The cluster shows the variability (WI) within and between sub-populations. The two sub-populations were determined based on location data and the values of Est.Var. obtained by the ANOVA. The environmental factor that predominates in *Lippia alba* and has been supported by several authors including Stashenko *et al.* (2003). the net chemical variability, is fully defined in Table 1.

Conclusions

There were no high values of Estimates of variance [Est.Var.] leading to establish chemical variability among individuals and populations. The results generated by the test point to the presence of two subpopulations, defined (each) by local environmental conditions.

The high value of WI indicated the possible presence of individuals with important chemical variants. The Verbenaceae tend to have chemical variations, particularly *Lippia alba*, without showing morphological differences among provenances (Riccardi et al., 2000). These variations occur even in plants located at short distances (Riccardi et al., 2000). 201, 213, and 341 accessions are variants important phytochemicals.

Biological variability can be original epigenetic, genetic, environmental, both, in response to Herbivory or mechanical damage and latrogenic (by pollution, especially chemical agents). Assuming, that the Individual variability [WI] must be less than the variability between individuals [AI], [WI] individual biological variability and maximum biological variability to estimate total [WT], allowed get population reference values and set values of variability for working sample.

The variables of location [including the environmental] explain the phytochemical variability of *Lippia alba* in large proportion. The working sample reported very wide range of adaptability, from 0 in Buenaventura-Valle, up to 2800 meters altitude in the Department of Cauca; revealed by the value of Phytochemical high intra variability (of 98%) found in the test (WI = 2.06).

The phytochemical characteristics of the subpopulations correspond to those reported by Colombian authors, with important chemical variants and value of extremely of variance [Est. Var.] also important. The examination of essential oils showed components lightweight terpenes as majority, alongside tannins and flavonoids. Thus, a) the sample of work corresponds to the chemotypes already documented by other authors for Colombia, and most likely others not yet reported, validating the laboratory data obtained; and (b) the similarity observed in the sample determines the accessions phytochemical kinship, which allows the formation of a large chemical group dominated by tannins-Terpenoids-flavonoids.

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Conflicto de Intereses
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