Essential Oils of New *Lippia alba* Genotypes Analyzed by Flow-Modulated Comprehensive Two-Dimensional Gas Chromatography (GC×GC) and Chemometric Analysis

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**Abstract.** *Lippia alba* (Mill.) N. E. Br. (Verbenaceae) is an aromatic shrub whose essential oils have stood out as a promising source for application in several industrial fields. In this study, the essential oils chemical characterization of eight new *L. alba* genotypes was performed. The selected materials were collected from the Active Germplasm Bank of the Agronomic Institute and the essential oils were extracted by hydrodistillation. Flow-modulated comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GC×GC-MS) was employed for chemical characterization and evaluation of possible co-eluted compounds. In addition, the chemical analyses were submitted to multivariate statistical analyses. From this investigation, 73 metabolites were identified in the essential oils of the genotypes, from which α-pinene, β-myrcene, 1,8-cineole, linalool, neral, geranial, and caryophyllene oxide were the most abundant compounds among the accessions. This is the first report disclosing α-pinene in higher amounts in *L. alba* (19.69%). In addition, sabinen, *trans*-verbenol, myrtenol, (*E*)-caryophyllene, α-guaiaene, germacrene D, and α-bulnesene were also found in relevant quantities in some of the genotypes, and myrtenal and myrtenol could be well separated through the second dimension. Such results contributed to the understanding of the chemical composition of those new genotypes, being important to drive a future industrial applicability and studies in genetic breeding.

**Keywords:** *Lippia alba*; lemon balm; essential oils; flow-modulated; comprehensive two-dimensional gas chromatography

1. **Introduction**

*Lippia alba* (Mill) N. E. Brown (Verbenaceae) is a vigorous and rustic shrub originated from South and Central Americas, occurring with wide distribution in Brazil. The species, which is popularly known as lemon balm, is largely used in folk medicine, characterized as being one of the most important medicinal plants used in Brazil [1,2]. The species is consumed fresh, being prepared in the form of teas, sweets, extracts, syrups, and tinctures. Tea preparations of its leaves, for example, are popularly employed in the treatment of gastrointestinal, diarrhea and dysentery disorders and as having tranquilizing, sedative, and analgesic actions [1,3]. Moreover, as an aromatic plant, its essential oils (EOs) have also been used in preparations of cosmetics, perfumes, and hygiene products, already available to consumers [4].

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**L. alba** is a plant that presents great agronomic potential, with rapid and aggressive development and easy cultivation [2]. All the features previously mentioned have stood out the species as a promising plant for an economic exploration that might be applied as a natural source with different purposes and in varied fields, such as pharmaceutical, cosmetic, perfumery and agricultural industries [5]. However, as higher is the demand of compounds extracted from natural resources, the higher is the over-exploitation of native species that is also threatened by habitat transformation, pollution, and climate changes, endangering the extinction of the same. This is the case of some species of *Lippia* genus from Minas Gerais and Goias states (Cadeia do Espinhaço region) in Brazil, that are at risk of extinction due the vulnerability of that region [6]. Hence, to reach the sustainable cultivation of species with economic potential, genetic resources maintained in germplasm banks have been established as a fundamental role for biodiversity conservation and to provide genetic diversity [7–9]. In addition, studies of genetic breeding programs are important for the standardization of the chemical phenotype and can be used to select the well adapted and potential genotypes of the existing germplasm, improving quality and productivity as well as developing varieties with enhanced value to the market [1,9,10]. These researches become a powerful complement to fulfill the worldwide demand for quality products besides being a strategic tool for the development of new raw materials for industrial use (pharmaceutical, perfumery, and beverages) [9,10].

Previous studies of *L. alba* reported the evaluation of genetic parameters such as performance for environment stability, adaptability, and Genotype × Environment interactions. These studies resulted in a bank of aromas and fragrances with over 100 new combinations of plants belonging to the Agronomic Institute (IAC) in Campinas, Brazil [5,11], affording relevant information that subsequently led to the genetic breeding program from IAC to perform biparental crosses of the most promisor cultivars, aiming to obtain plants with different genetic variability. Hence, crossings between the genotypes linalool, myrcene/camphor, limonene/camphor, and citral resulted in new hybrids with different combinations of major constituents [11,12]. As far as we know, it was the first genetic breeding of *L. alba* in Brazil and worldwide. However, none of these materials were evaluated regarding their chemical composition so far.

In addition, although the species has been extensively a target of scientific studies regarding its chemical composition and evaluation of its biological profiles [13], a deeper investigation of its constituents and analysis of possible co-eluted compounds (quite common observed in natural matrices) is still lacking in studies. A previous study with the plant by Multidimensional Gas Chromatography (MDGC) showed the evaluation of the enantiomeric ratios of α-pinene, sabinene, limonene, and linalool. However, it was described the investigation of only one genotype and the employment of a heart-cut system of the enantiomeric fractions instead of the full sample analysis [14].

Hence, comprehensive analyses of the full sample are essential to reach the requirements of quality assurance, safety, and efficacy of a product, once it makes possible to discriminate beneficial compounds, as well as monitoring important parameters as adulterations and variability prior to the development for a subsequent commercialization of any natural formulations[15]. Moreover, a comprehensive identification of the substances in the sample can also contribute to the understanding of synergistic or antagonistic effects that might mediate some relevant biological activity [16].

Analytical tools that provide high resolution and sensitivity for untargeted analysis of the plant metabolome are desperately needed. Among these tools, comprehensive two-dimensional gas chromatography coupled to mass spectrometry (GC×GC-MS) is characterized for the increased peak capacity [17–19], increasing the identification power of plants volatiles and semi-volatiles composition [20,21].

Additionally, chemometrics is a versatile and useful tool to explore the diversity of metabolic profile in a dataset, being helpful to retrieve more valuable chemical
information from natural matrices due the combination of mathematics, statistics, and computer science [22,23]. Hence, the combination of the GC×GC-MS with multivariate analysis provides a better information on the similarity and relationship among the EOs chemical composition, enabling the visualization of the clustering patterns within the samples [24,25].

Thus, the objective of this work was to perform the chemical characterization of new genotypes combinations obtained from biparental crosses of L. alba by flow-modulated GC×GC-MS (FM-GC×GC-MS) combined to multivariate data analyses using principal component analysis (PCA) and hierarchical cluster analysis (HCA). This is the first investigation of Lippia species employing this modern technique. This study aims to contribute for a well understanding of these new plant crossings that might represent materials with highlighted agronomic value, as well as driving future genetic breeding studies and consequently obtention of different combinations of chemical phenotype for a future industrial applicability.

2. Results and Discussion

Extraction Yield and Chemical Composition of the Essential Oils

The hydrodistillation of the leaves of the eight studied genotypes yielded in yellowish oils with characteristics of citrus odor. The yields of volatiles oils from the leaves of each genotype ranged from 0.29 to 1.03%, as described in Table 1. The highest yield was found in the X6MIA genotype (1.03%), whereas X6M6 genotype disclosed the lowest yield (0.29%).

| Table 1. Yield of EOs of the eight studied L. alba genotypes. |
|--------------------|----------------|------------|------------|----------------|----------------|----------------|----------------|
| Extraction Yield (%) | X(2) | X2M1 | X6M6 | X6MIA | X6M9 | X6M13 | X6M15 | X10M37 |
|----------------------|------|------|------|-------|------|-------|-------|-------|
| 0.42                 | 0.38 | 0.29 | 1.03 | 0.30  | 0.54 | 0.47  | 0.57  |

The analysis of the essential oil chemical composition from eight genotypes of L. alba allowed the identification of 73 secondary metabolites, as disclosed in Table 2. In general, the constituents identified in oils belong to the terpenoids class, with the following predominance: monoterpenic hydrocarbons (1.53–59.79%) and oxygenated monoterpenes (9.99–86.44%), sesquiterpenic hydrocarbons (3.07–30.49%), and oxygenated sesquiterpenes (3.96–19.49%), from which α-pinene, β-myrcene, 1,8-cineole, linalool, citral (neral + geranial), and caryophyllene oxide were the most abundant compounds among the accessions, enabling the separation of the genotypes into different chemotypes of L. alba. Figure 1. In addition, an overlap of the chemical composition of all genotypes displayed the compounds sabinene, trans-verbenol, myrtenol, (E)-caryophyllene, α-guaiene, germacrene D, and α-bulnesene in relevant quantities in some of the genotypes, as disclosed in Figure 2A (chemical structures) and Figure 2B (identified compounds in the essential oils). The relative percentage of all identified compounds is shown in Table 2.
Figure 1. Major compounds and its relative percentage (%) in the genotypes of L. alba.
Figure 2. (A) Structure of the main compounds found in the new genotypes of *L. alba*. (B) Identified compounds and comparison of their distribution (relative abundance) among all genotypes.
Table 2. Chemical composition of essential oils from eight new L. alba genotypes analyzed by GC×GC-MS.

| Compound a | LTPRI b | Relative Content (%) c |
|------------|---------|------------------------|
|            | Exp. d | Similarity (%) e       | X(2) | X2M1 | X6M6 | X6M1A | X6M9 | X6M13 | X6M15 | X10M37 |
| 1 α-thujene | 924    | 920                  | 92   | -    | -    | -     | -    | -     | 0.24  |
| 2 α-pinene  | 932    | 926                  | 97   | 6.91 | 0.27 | 0.16  | 4.32 | 19.69 | 4.36  | 1.02  |
| 3 camphene  | 946    | 941                  | 90   | 0.16 | -    | -     | -    | 0.24  | -     | -     |
| 4 thuja-2,4(10)-diene | 953    | 947                  | 87   | -    | -    | -     | -    | 0.12  | -     | -     |
| 5 sabinene  | 969    | 965                  | 94   | 1.35 | 1.17 | 0.11  | 1.37 | 0.33  | 2.62  | 3.43  | 7.10  |
| 6 β-pinene  | 974    | 968                  | 94   | 0.60 | -    | -     | 0.24 | 0.75  | -     | -     |
| 7 myrcene   | 988    | 981                  | 95   | 19.17| 51.11| 34.5  | -    | 19.79 | 31.59 | 14.87 | 6.57  |
| 8 p-cymene  | 1020   | 1015                 | 92   | -    | -    | 0.05  | -    | 0.27  | -     | 0.21  |
| 9 limonene  | 1024   | 1018                 | 95   | 1.79 | 0.31 | -     | 0.71 | 3.55  | 1.71  | 1.07  |
| 10 1,8-cineole | 1026   | 1021                 | 95   | 3.60 | 2.15 | -     | 15.05| 5.95  | 29.36 | 3.58  |
| 11 (E)-β-ocimene | 1044   | 1040                 | 92   | -    | -    | 0.10  | -    | 0.11  | -     | -     |
| 12 (Z)-sabinene hydrate | 1065   | 1055                 | 86   | 0.87 | 0.57 | -     | 0.17 | 0.12  | 0.81  | 0.53  | 0.10  |
| 13 (Z)-linalool oxide (furanoid) | 1067   | 1061                 | 92   | 0.15 | -    | -     | 1.13 | -     | -     | -     |
| 14 (E)-linalool oxide (furanoid) | 1084   | 1077                 | 87   | 0.10 | -    | -     | 0.79 | -     | -     | -     |
| 15 6,7-epoxymyrcene linalool | 1090   | 1080                 | 84   | -    | 0.10 | 0.21  | 0.42 | 0.14  | -     | -     |
| 16 (E)-sabinene hydrate perillene | 1095   | 1086                 | 93   | 19.83| 5.59 | 0.28  | 68.15| 3.39  | 2.83  | 2.02  | 4.28  |
| 17 1,3,8-p-menthatriene α-campholenol | 1102   | 1095                 | *    | -    | -    | 0.06  | -    | 0.40  | -     | -     |
| 18 trans-pinocarveol | 1108   | 1100                 | 80   | -    | -    | -     | 0.30 | 0.50  | -     | -     |
| 19 α-pinolenol | 1122   | 1114                 | 88   | 0.50 | 0.11 | -     | 0.30 | 0.85  | -     | -     |
| 20 exo-isocitral | 1135   | 1129                 | 89   | 0.77 | 0.09 | -     | 0.18 | 0.83  | -     | -     |
| 21 δ-terpineol | 1140   | 1132                 | 80   | -    | -    | 0.11  | -    | -     | -     | -     |
| 22 trans-verbenol | 1140   | 1134                 | 93   | 10.71| 0.42 | 0.20  | 0.68 | 9.55  | 2.88  | 0.17  |
| 23 (Z)-isocitral | 1160   | 1150                 | 83   | -    | -    | 0.13  | -    | -     | -     | -     |
| 24 α-pinocarveol | 1160   | 1152                 | 87   | 0.40 | 0.48 | -     | 0.27 | 0.48  | -     | -     |
| 25 terpinen-4-ol | 1173   | 1161                 | 80   | -    | -    | 0.18  | -    | -     | -     | -     |
| 26 2,3-epoxy-geraniol | 1188   | 1179                 | 85   | 0.20 | -    | 0.17  | -    | 0.09 | 0.12  | 0.11  |
| 27 terpen-4-ol | 1194   | 1184                 | 94   | 1.84 | 0.28 | -     | 0.46 | 1.47  | 1.91  | 0.10  |
| 28 myrtenol | 1195   | 1184                 | 94   | 4.23 | 0.20 | -     | 0.16 | 1.15  | 1.52  | -     |
| 29 myrtenol | 1204   | 1197                 | 89   | 0.64 | -    | -     | 0.44 | -     | -     | -     |
| 30 trans-carveol | 1215   | 1205                 | 82   | 0.32 | -    | -     | 0.20 | -     | -     | -     |
| 31 α-pinolenol | 1227   | 1220                 | 80   | -    | -    | 0.10  | -    | -     | -     | -     |
| 32 2,3-epoxy-geraniol | 1234   | 1224                 | 80   | -    | 0.06 | -     | -    | -     | -     | -     |
| 33 carvone | 1235   | 1225                 | 93   | -    | 21.04| -     | 17.53| -     | 13.34 |
| 34 carvone | 1239   | 1232                 | 80   | -    | -    | -     | 0.10 | -     | -     | -     |
| 35 isobornyl acetate | 1264   | 1254                 | 93   | -    | 27.89 | -    | 22.24| -     | 10.81 |
| 36 myrtenyl acetate | 1283   | 1271                 | 80   | 0.13 | -    | -     | -    | 0.15  | -     | -     |
| 37 α-copaene | 1324   | 1309                 | 86   | 0.34 | -    | -     | -    | 0.10  | -     | -     |
| 38 geranyl acetate | 1374   | 1367                 | 89   | 0.08 | 0.05 | -     | 0.28 | 0.46  | 0.04  | 0.05  | 0.61  |
| 39 geranyl acetate | 1379   | 1370                 | 80   | -    | 0.21 | -     | -    | -     | 0.18  | -     |
| 40 β-bourbonene | 1387   | 1376                 | 87   | 0.13 | 0.09 | 0.05  | 0.04 | 0.05  | 0.14  | 0.32  | 0.56  |
| Compound \(^a\) | LTPRI \(^b\) | Relative Content (%) \(^f\) |
|---------------|----------------|--------------------------|
|               | Lit. \(^c\) | Exp. \(^d\) | Similarity (%) \(^e\) | X(2) | X2M1 | X6M6 | X6M1A | X6M9 | X6M13 | X6M15 | X10M37 |
| 45 β-elemene   | 1389 | 1380 | 92 | 0.59 | 1.28 | 0.39 | 0.38 | 0.56 | 0.28 | 0.33 | 1.99 |
| 46 (E)-caryophyllene | 1417 | 1411 | 94 | 3.20 | 5.12 | 1.67 | 1.22 | 1.03 | 1.29 | 10.8 | 5.11 |
| 47 β-copaene   | 1430 | 1421 | 83 | -   | 0.08 | -   | 0.09 | 0.19 | -   | -   | -   |
| 48 α-guaiene   | 1437 | 1429 | 93 | 0.14 | 5.42 | 4.22 | -   | -   | -   | 8.26 | 0.80 |
| 49 α-humulene  | 1452 | 1446 | 92 | 0.18 | 2.33 | 0.53 | 0.18 | 0.21 | -   | 1.51 | 1.45 |
| 50 (E)-β-farnesene | 1454 | 1438 | 87 | -   | 0.38 | 0.11 | -   | 0.18 | -   | 0.37 | 0.46 |
| 51 allo-aromadendrene | 1458 | 1454 | 88 | 0.08 | -   | -   | 0.11 | 0.16 | 0.10 | -   | 1.27 |
| 52 9-epi-(E)-caryophyllene | 1464 | 1460 | 90 | -   | -   | -   | -   | -   | 0.09 | 0.40 |
| 53 γ-murolene  | 1478 | 1465 | 88 | -   | -   | -   | 0.10 | 0.09 | 0.05 | 0.05 | -   |
| 54 germacrene D | 1480 | 1473 | 93 | 1.19 | 4.70 | 0.12 | 0.48 | -   | 0.83 | 1.84 | 1.89 |
| 55 γ-amorphene  | 1495 | 1494 | 86 | 0.07 | -   | -   | 0.10 | 0.37 | 0.10 | -   | 0.83 |
| 56 α-murolene  | 1500 | 1495 | 85 | -   | 0.49 | -   | 0.11 | 0.11 | -   | -   | -   |
| 57 (E)-β-guaiene | 1502 | 1497 | 80 | -   | 0.57 | -   | -   | 0.24 | 0.54 | 2.46 |
| 58 β-bisabolene | 1505 | 1500 | 80 | -   | -   | -   | -   | -   | 0.47 |
| 59 α-bulnesene | 1509 | 1502 | 91 | 0.12 | 4.80 | 1.23 | -   | -   | -   | 6.22 | 0.28 |
| 60 δ-amorphene  | 1511 | 1504 | 83 | 0.53 | 1.26 | -   | 0.36 | 1.61 | -   | -   | -   |
| 61 δ-cadinene  | 1522 | 1510 | 84 | -   | 0.10 | -   | 0.12 | -   | -   | -   | -   |
| 62 germacrene B | 1559 | 1549 | 90 | -   | 0.19 | -   | 0.32 | 0.08 | -   | 0.11 | 3.94 |
| 63 (E)-nerolidol | 1561 | 1551 | 88 | 0.32 | 0.50 | 0.05 | 0.14 | 0.15 | 0.06 | 0.07 | 0.52 |
| 64 germacrene D-4-ol | 1574 | 1563 | 80 | 0.23 | 1.35 | -   | -   | -   | -   | -   | -   |
| 65 spathulenol  | 1577 | 1570 | 85 | -   | -   | 1.1 | -   | 0.20 | 0.33 | 0.25 |
| 66 caryophyllene oxide | 1582 | 1575 | 88 | 13.42 | 3.88 | 2.76 | 4.48 | 15.28 | 10.50 | 3.23 | 15.96 |
| 67 humulene epoxide II | 1608 | 1600 | 88 | 1.11 | 1.05 | -   | 0.48 | 1.76 | 0.38 | 0.42 | 1.74 |
| 68 1.10-di-epi-cubenol | 1618 | 1604 | 86 | -   | 1.04 | -   | 0.15 | -   | 0.18 | 0.46 |
| 69 allo-epoxide aromadendrene | 1639 | 1630 | 80 | -   | -   | 0.05 | -   | -   | -   | 0.18 |
| 70 khusilal     | 1647 | 1639 | 80 | 0.41 | 0.54 | -   | -   | 0.11 | -   | -   | 0.38 |
| 71 pogostol     | 1651 | 1643 | 80 | -   | 0.54 | -   | -   | -   | -   | -   | -   |
| 72 (E)-14-hydroxy-9-epi-caryophyllene | 1668 | 1660 | 80 | -   | -   | 0.16 | 0.50 | -   | -   | -   | -   |
| 73 cis-thujopenol | 1708 | 1698 | * | -   | 0.15 | -   | -   | -   | -   | -   | -   |

| Monoterpenes | Oxygenated Monoterpenes | Sesquiterpenes | Oxygenated Sesquiterpenes |
|--------------|--------------------------|----------------|--------------------------|
| Descriptive | Hydrocarbons | Hydrocarbons | Hydrocarbons |
| Monoterpenes | 29.98 | 52.86 | 34.66 | 1.53 | 25.39 | 59.79 | 24.37 | 16.21 |
| Oxygenated Monoterpenes | 44.63 | 9.99 | 49.94 | 86.44 | 45.93 | 25.08 | 40.02 | 34.24 |
| Sesquiterpenes | 6.31 | 26.29 | 8.89 | 3.89 | 5.10 | 3.07 | 30.49 | 22.52 |
| Oxygenated Sesquiterpenes | 15.49 | 9.05 | 3.96 | 5.26 | 17.95 | 11.14 | 4.23 | 19.49 |
| Total Identified | 96.41 | 98.19 | 97.45 | 97.12 | 94.37 | 99.08 | 99.11 | 92.46 |

* Compounds identified comparing the substance mass spectra with NIST 14 data base, literature [26] and filtered by the retention index (LTPRI); \(^b\) LTPRI: Linear temperature programmed retention indices; \(^c\) Exp.: LTPRI experimental obtained by the injection of a homologous series of C8–C20 n-alkanes using the Van den Dool and Kratz equation [27]; \(^d\) Lit.: LTPRI obtained from literature [26]; \(^e\) Similarity of compounds based on NIST 14 database (National Institute of Standards—Gaithersburg, MD, USA). \(^f\) Compounds identified based on literature [26]; \(^f\) Concentration of the metabolites were obtained by area normalization.

The essential oil of the X2M1 genotype showed the highest abundance of β-myrcene (51.11%), also observed to the genotypes X6M6 (34.50%) and X6M13 (31.59%, Figure 1), and the values described in this work are higher than those that are commonly reported in literature to this species, ranging mainly from 0.30 to 25.81% among the distinct chemotypes analyzed from different geographical origin [28–31]. Marques, (2018), e.g., observed an increase of 1.30% of the production of β-myrcene after cultivation of the plant in green manures in succession [32]. However, the highest abundance of this compound reached
only 8.04%. A second work disclosed 15.00% of β-myrcene of a myrcene-citral chemotype of *L. alba* [33]. Our results, however, were similar to that reported by Jannonuzi, (2010) [34], which one among the sixteen analyzed chemotypes revealed the abundance of 47.60% of β-myrcene. Additionally, our findings are also comparable to those matrices already known of having higher quantities of this compound, as, e.g., in hops essential oils. Studies of the essential oils of hop Polish cultivars afforded from 29.90 to 67.00% of this monoterpane [35].

In a general way, β-myrcene was present in all analyzed accessions of *L. alba*, except in X6M1A. β-myrcene is a colorless or light-yellow oily monoterpane with an important industrial value. Due to its pleasant smell with a woody, spicy, peach, sweet, vanilla, and wine-like odor description, this compound is a value intermediate for the preparation of flavor and fragrance chemicals. Furthermore, myrcene is also described as a versatile starting material for vitamins and pharmaceuticals due to its reactive diene structure [36–39], and as having significant pharmacological properties, such as anti-inflammatory and anticytotoxic effects for deceleration of osteoarthritis progression [40], and as an activator for TRPV1 as target for treating pain [41].

The essential oil of the X6M13 genotype in addition to β-myrcene also showed a high abundance of α-pinene (31.59% and 19.69%, respectively, Figure 1). Usually, the abundance of α-pinene in *L. alba* essential oils, when reported, is extremely low, reaching less than 1.0% [1,33,42]. Our results pinpoint to a new myrcene-α-pinene chemotype. However, further seasonality investigations are still needed to confirm this chemotype. α-pinene is a colorless and water-insoluble volatile plant metabolite, found in many essential oils and being the major monoterpane of pine trees. As a safe food additive approved by the U.S. Food and Drug Administration, this compound has been widely used as a food-flavoring ingredient [43,44]. In addition, several biological activities have been attributed to α-pinene, including antibacterial and antifungal [45], apoptotic and antimitostatic effect [46], anti-inflammatory and chondroprotective [47], and gastroprotective [48] effects.

The essential oil of the X6M6 and X6M9 genotypes in addition to β-myrcene, also showed a high abundance of citral (neral + geraniol) as their major component. The X10M37 genotype, however, disclosed citral and caryophyllene oxide as its major components (13.00–15.00% and 37–53% of citral [33,34]. Citral is an open chain monoterpenoid formed by the neral and geraniol isomers found in several medicinal plants and widely used as additives in foods, beverages, and cosmetics, due its intense lemon aroma and flavor [50]. A plethora of pharmacological properties have already been reported to citral, such as antibacterial [51], antifungal [52], insecticide [53], antioxidant [16], anticancer [54], anti-inflammatory [55], and anti-nociceptive [56] activities.

The essential oil of the X6M1A genotype disclosed linalool and 1,8-cineole as its major constituents (68.10% and 15.0%, respectively, Figure 1). Similar results of these compounds were also described by Barros, 2009 (63.70% of linalool and 10.40% of 1,8-cineole) [57]. The genotype X(2), however, disclosed linalool and myrcene (19.80% and 19.20%, respectively) as its major compounds (Figure 1). Linalool is an acyclic monoterpane alcohol widely used in cosmetics and flavoring ingredients [58]. Moreover, previous works have demonstrated linalool to have a comprehensive range of biological properties, such as potent antibacterial [59], anti-inflammatory [60,61], antioxidant, anticancer [62], antinociceptive [58], anxiolytic, and neuroprotective [63] agent.

The essential oil of the X6M15 genotype disclosed 1,8-cineole and myrcene as its major constituents (29.40 and 14.70%, respectively, Figure 1). The existence of a 1,8-cineole:myrcene chemotype was reported by Ricciardi, 2009 [31], in which the amounts of these compounds reached only 14.70% and 10.40%, respectively. 1,8-cineole is a saturated
monoterpene with pleasant aroma and taste, widely used in food, fragrances, and cosmetics [64]. Plenty studies have reported the substances as having benefits for respiratory tract infection, such as bronchitis, sinusitis as well as exhibiting secretolytic and bronchospasmolytic properties, and anti-inflammatory, antimicrobial, and antiseptic efficacy [64].

Although major constituents present in the genotypes might be effective to drive a future biological application of the samples, it should be noted that different ratios of these components can affect the final behavior of the same. In addition, a deeper investigation and knowledge of other minor constituents are pivotal since synergistic or antagonistic effects might influence the overall performance [16,65]. Into this perspective, the analyses of the L. alba essential oils allowed the identification of 26 compounds in low concentrations (up to 0.1%) at least in one of the eight analyzed genotypes, considered as trace. In addition, 66 compounds were identified in concentrations up to 0.5% in at least one of the eight genotypes Table 2. The typical GC×CG-MS total ion chromatograms for the most representative essential oils are disclosed in Figure 3. In addition, it is also relevant the evaluation of possible co-eluted components in the samples for their chemical composition discrimination. Hence, the analyses of the GC×GC-MS allowed to verify the presence of co-eluted compounds in five genotypes. Whereas the coelution of humulene epoxide II with a second unknown compound Figure 4A was noticed to the X6MIA genotype, myrtenal and myrtenol were well separated and identified in the X(2), X2M1, X6M13, and X6M15 genotypes Figure 4B.

**Figure 3.** GC×GC-MS total ion chromatograms (TIC) of essential oils of new L. alba genotypes: (A) X6M6, (B) X6M13, (C) X6M15, and (D) X10M37.
In order to investigate the similarity and relationship among the EOs chemical composition of *L. alba* genotypes, hierarchical cluster analysis (HCA) and principal component analysis (PCA) were constructed to the oil components. From the HCA analysis, it was observed a separation among the samples, which the X(2) and X6M13 genotypes (group I) were the most dissimilar, followed by the X10M37 genotype (group II). The remaining genotypes (X6M6, X6M9, X6M15, and X6MIA, group III) showed a greater chemical similarity to each other.

For PCA analysis, a three-component PCA model expressed 63.67% of the total variance, with the first principal component (PC1) being responsible for 27.62%, the second principal component (PC2) for 19.46% and the third principal component (PC3) for 16.59% of the total variance, making it possible to determine the most significant substances in the data set.

Thus, PC1 positively correlated to α-pinene (2), found in a higher relative proportion in the X6M13 genotype, camphene (3), β-pinene (6), trans-pinocarveol (21), trans-verbenol (23) and isobornyl acetate (40), and negatively correlated to (E)-β-farnesene (50), allo-aromadendrene (51), 9-epi-(E)-caryophyllene (52), β-bisabolene (58), γ-amorphene (55), (E)-β-guaiene (57), germacrene B (62), and allo-epoxide aromadendrene (69). PC2, in addition, positively correlated sabine (5), p-cymene (8) and β-bourbonene (44) and negatively correlated to γ-nerol (53) and δ-cadinene (61). PC3, on the other hand, showed positive correlations to germacrene D (54), cis-thujopsenol (73), pogostol (71), germacrene D-4-ol (64), α-bulnesene (59), α-humulene (49), and pinocarvone (25), and a negative correlation to (E)-14-hydroxy-9-epi-caryophyllene (72), neral (37), geranial (39), and 6,7-epoxyy myrcene (15).

The dendrogram from the HCA analysis is illustrated in Figure 5A, whereas the scores graph from the PCA analysis is illustrated in Figure 5B. The compounds responsible for the observed clustering among the samples is disclosed in Figure 5C.

These analyses allowed to distinguish the samples in four main groups: (X(2) and X6M13, group I), (X10M37, group II), (X6M15 and X2M1, group III), and (X6M6, X6M9, and X6MIA, group IV). This classification had been previously supported by the dendrogram from the HCA analysis, mainly allowing the distinction of the groups I and II.

However, a greater information could be observed from the PCA analysis, which resulted in an efficient subdivision of the group III (previously obtained from HCA) in two
groups (III and IV, Figure 5B). A higher similarity between the genotype X2M1 and X6M15 was observed Figure 5B and the compounds α-guaiene (48), α-bulnesene (59), α-humulene (49), germacrene D (54), and (E)-caryophyllene (46) could be pinpointed from the PCA biplot graph as the responsible for such similarities Figure 5C. Indeed, those genotypes disclosed the highest percentage of sesquiterpenes hydrocarbon among the samples Table 2.

![Dendrogram, scores plot, and loadings plot](image)

**Figure 5.** Dendrogram (A) obtained from HCA, scores plot (B) and loadings plot (C) obtained from PCA of the chemical composition data of the *L. alba* essential oils. Numbers in loadings plot (C) represent the identified compounds as described in Table 2.

### 3. Materials and Methods

#### 3.1. Plant Material Collection

Leaves of eight *L. alba* genotypes coded as X(2), X2M1, X6M6, X6MIA, X6M9, X6M13, X6M15, and X10M37 were collected from the germplasm bank located at Agronomic Institute (IAC), Campinas, Brazil (22°54' latitude S and 47°05' longitude W). The branches were collected in January 2018 and the leaves were manually separated from the branches and subsequently dried in an oven with air circulation at 40 °C for 48 h up to constant weight.
3.2. Essential Oils Extraction

About 80 g of the dried leaves from each L. alba genotype were submitted to a hydrodistillation using Clevenger apparatus for two hours. Obtained essential oils were then separated from aqueous phase and kept in sealed vials at −20 °C in the dark for further analysis. The yield (%) of EOs was calculated based on plant material dry in grams.

3.3. Essential Oil Chemical Characterization

Samples were diluted in ethyl acetate (Tedla, chromatographic grade, Fairfield, OH, USA) at the concentration of 1 mg/mL and 1 μL of each solution was injected.

The GC×GC-QMS experiments were conducted on a TRACE 1310 gas chromatograph coupled to a fast-scanning ISQ single transmission quadrupole mass spectrometer (MS) (Thermo Scientific—Waltham, MA, USA). The GC was equipped with a split/splitless injector (SSL) (ThermoFisher Scientific—Austin, TX, USA). A Topaz 4.0 mm-id split precision inlet liner (Restek Corporation—Bellefonte, PA, USA) was used for sample vaporization. A TriPlus RSH autosampler (ThermoFisher Scientific) fitted with a 10 μL syringe (Trajan Scientific—San Diego, CA, USA) was used to inject the liquid sample. The rinsing solvents were isopropanol and methylene chloride.

Differential flow modulation was performed in reverse fill/flush (RFF) configuration using the INSIGHT flow modulator (SepPlex Analytical—Waterloo, ON, Canada). ChromSpace software (1.9 version, SepPlex Analytical) was used to control the INSIGHT modulator. Instrument control and data acquisition were performed using Xcalibur software (ThermoFisher Scientific). The column arrangement consisted of two wall-coated open tubular (WCOT) capillary columns. The primary column was a 30 m × 0.25 mm-id × 0.25 μm (β = 250) HP-5MS (Agilent Technologies—Santa Clara, CA, USA). Secondary column was a 5.0 m × 0.25 mm-id × 0.25 μm (β = 250) HP-50+ (Agilent Technologies). A 23 cm × 0.53 mm-id sampling loop of 50 μL (part N. 70058) (Restek Corporation) and a 3.0 m × 0.10 mm-id deactivated fused silica restrictor were used for RFF. An unpurged SilFlow GC 3-port splitter (part N. 123725) (Trajan Scientific) was used for passive division of the secondary column effluent for parallel detection (MS/FID). Two 5.0 m × 0.18 mm-id and 5.0 m × 0.32 mm-id deactivated capillaries (Restek Corporation) were used to connect the three-way splitter to the MS and FID, respectively. A reproducible division of approximately 1:6 was achieved throughout the experiments. The interested reader is directed elsewhere for more details on the instrument setup [17–19].

A modulation period of 5 s with a re-injection (flush) pulse of 200 ms was used in all FM-GC×GC analyses. Ultrapure Helium was used as carries gas and auxiliary gas at constant flow rates of 1 mL/min and 20.0 mL/min, respectively. The GC inlet was kept at 250 °C and operated with a split ratio of 1:20. The oven temperature was programed from 60 to 240 °C at 3 °C min⁻¹. The ion source and MS transfer line were kept at 220 °C and 250 °C, respectively. Electron ionization was performed at 70 eV and 150 μA emission current. The mass range was set from 45 to 400 Da at 42 scans s⁻¹.

Data processing was performed using GC Image software (2020r1.2 version, Zoex—Houston, TX, USA). Compounds were tentatively identified comparing the substance mass spectra with NIST 14 database (National Institute of Standards—Gaithersburg, MD, USA) and filtered by the retention index (LTPRI), adopting minimum similarity match of 80% from NIST and ± 25 LTPRI deviation as those reported by Adams (2017) [26]. GC retention index of each compound was calculated based on injection of a homologous series of C₃–C₇ n-alkanes (Merck-St. Louis, MO, USA) using the Van den Dool and Kratz equation [27], and the concentration of the metabolites were obtained by area normalization.
3.4. Statistical Analyses

The results of the chemical analyses were submitted to multivariate statistical analyses, such as principal components analysis (PCA) and hierarchical clustering analysis (HCA). The models were built using the software XLSTAT-2020 version (Addinsoft—Bordeaux, France).

4. Conclusions

In this work, the chemical characterization of eight essential oils from new L. alba genotypes was performed by GC×GC-MS combined to multivariate data analyses. From this investigation, the new genotypes could be distinguished in four main groups according to the variance observed through the PCA analysis, indicating a variation in their chemical composition. Higher amount of β-myrcene was observed to the X2M1 genotype, indicating this accession as a natural source to obtain this compound. In addition, higher amounts of sesquiterpenes were observed in the genotypes X2M1 and X6M15. This is the first report disclosing α-pinene in higher amounts in L. alba (19.69%, X6M13 genotype), suggesting the existence of a new α-pinene/myrcene chemotype. The use of GC×GC-MS for L. alba essentials oils was reported in this work by the first time, allowing the identification of 73 metabolites in the accessions, and the technique was also efficient to identify compounds in low abundance, which 26 compounds were identified in concentrations up to 0.1% in at least one of the eight analyzed genotypes. Furthermore, the compounds myrtenal and myrtenol could be well separated through the second dimension, whereas humulene epoxide II could be identified and well separated from a second unknown compound. The results found in this work contributed to the understanding of the chemical composition of new L. alba genotypes and highlight the relevance of these materials as natural sources for a future flavor, fragrance, and pharmaceutical applicability. In addition, this work also emphasizes the relevance of genetic breeding studies to potentially improve the essential oils quality and obtention of different combinations of chemical phenotype.

Author Contributions: L.G., J.C.R.L.S., and M.O.M.M. planned the experiments; L.G. and J.C.R.L.S. acquired and analyzed the chemometric analyses; L.G., J.C.R.L.S., R.F., and L.W.H. acquired the GC×GC-MS experiments; L.G. and J.C.R.L.S. analyzed all the data; W.J.S. supervised the genetic breeding experiments; L.G. and J.C.R.L.S. wrote the paper with input from all authors. Supervision: M.O.M.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Plants of Lippia alba were used in this study. All collected material belongs to the Germplasm Bank of the Agronomic Institute, Campinas, SP, Brazil.

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