Environmental Influence on Phenols and Essential Oils of Myrciaria cauliflora Leaves

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O conteúdo foliar de fenóis totais e de taninos, bem como a composição química dos óleos essenciais em populações de Myrciaria cauliflora cultivadas em seis diferentes sítios de amostragem indicou a presença de quatro grupos de amostras de acordo com as características químicas da folha e do solo de cultivo. O grupo I incluiu as amostras oriundas do solo franco-arenoso (S1), caracterizado pela maior e menor percentagem de γ-eudesmol (11,55%) e germacrene D (20,48%), respectivamente, e alto conteúdo de fenóis totais (136,68 mg g⁻¹) e taninos (60,72 mg g⁻¹). O grupo II, rico em elemol (4,61%), incluiu as amostras cultivadas nos solos franco-arenoso-argilosos (S2, S3 e S6), enquanto que as amostras dos solos argilosos S4 (grupo III) e S5 (grupo IV) apresentaram as mais elevadas percentagens de germacrene D (III: 27,20%; IV: 26,83%) e os mais baixos teores de elemol (2,12–2,55%), fenóis totais (79,69 e 111,77 mg g⁻¹) e taninos (34,04 e 44,51 mg g⁻¹).

A análise de redundância canônica revelou como o balanço de nutrientes do solo e das folhas influenciou a distribuição dos constituintes químicos nos diferentes agrupamentos. A variação química parece ser determinada por fatores ambientais.

Keywords: Myrciaria cauliflora, essential oil, phenol, chemical variability, environmental influence, multivariate analysis

Introduction

Jaboticaba, also known as guaperu or sabara, is a small native Brazilian fruit of the cauliflorus Myrtle (Myrtaceae). It is grape-like in appearance and texture, although its skin is thicker and tougher, and its color varies from grown to deep purple or black due to high anthocyanin content.¹ The ripened berries are highly perishable – postharvest decay occurs within 2-3 days – which is a problem for both transport and storage.² The plant is distributed in southern and central Brazilian regions and consists of about nine species of trees and shrubs, among which Myrciaria cauliflora (Mart.) O. Berg. and M. jaboticaba (Vell.) O. Berg. are the most widely cultivated species.³ However, some of them have been included in the group of threatened Brazilian species due to population decline.³ Recently, cryopreservation by somatic embryogenic cultures has been successfully applied to the conservation of Myrciaria spp.⁴

In the Brazilian Cerrado, Myrciaria cauliflora grows wild or it is cultivated and its berries are consumed in natura or processed to produce different kinds of jams, ice creams, vinegar, liquor, and wines; it can be found in local markets

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and in other parts of South America. Citadin et al. have reported that natural shade or full sunlight tree development have not shown differences as regards physico-chemical features of fruit quality. The jaboticaba bagasse flour, which is a good source of fiber, proteins and minerals is made up of fine particles which have the fruit’s aroma and its taste is slight salty and bitter. Traditionally, an astringent decoction of sun-dried skins is used as a treatment for hemoptysis, asthma, diarrhea, and chronic inflammation of the tonsils. Previous chemical investigations have been restricted to phenolic compounds, including flavonoids, organic acids, anthocyanins, and depsides in fruits, which have also shown strong antiradical activity in DPPH assays. Leaf ethanolic extract has revealed antimicrobial activities on Streptococcus in the oral cavity responsible for dental plaque and anthocyanin-rich extracts of crushed skins have been reported as a natural source of dye sensitizer and may be a less costly alternative for the production of solar cells.

Despite the great potential and growing regional market for M. cauliflora, including cosmetics and fragrance, the essential oil compositions from its leaves and fruits as well as the environmental influence on phenolic contents have not yet been investigated.

We now report on the results obtained for the composition of volatiles and phenolic contents of M. cauliflora leaves. They were collected from cultivated populations on six different soils of Jabuticabal Winery, located in the Central Brazilian Cerrado. For this purpose, total phenols and tannins and the essential oils of representative population samples of each soil origin were analyzed by colorimetric assays and GC-MS. Soil parameters and foliar nutrients in representative samples from each site origin were also determined and regarded as environmental variables. To study environmental influence on chemical variability, chemical constituents were submitted to canonical redundancy (RDA) and linear discriminant (LDA) analyses in order to detect samples’ distribution pattern and to identify which chemical constituents are able to distinguish between these groups of individuals.

Results and Discussion

In this study, M. cauliflora leaf oils and total phenol and tannin contents were obtained from cultivated plants grown in six different soil types, which form two spatially discontinuous sampling sites at Jabuticabal Winery (S1-S4 and S5-S6 sampling sites). This winery is currently one of Brazil’s biggest producers of jaboticaba, and 11,000 of its 31,000 trees are used in production; furthermore, it commercializes the fruit on an exclusive basis, turning it into various industrial products. The cultivated plants were made up of 10 to 40 year-old individuals obtained by seed propagation from indigenous populations.

The sampling sites differed considerably with regard to texture and mineral nutrients. Soil textures range from sandy loam (S1) and clay sand loam (S2, S3, and S6) to clay (S4 and S5). The last two soil types are fertilized with organic fertilizer (S4) or a dark purple soil known as terra roxa (S5), whereas S1 and S6 soils showed lower nutrient balance (see Supplementary Information, SI, Table S1). Similarly, foliar nutrients from representative samples of each site showed that samples growing on S1 and S6 soils had the lowest balance (see SI, Table S2).

The essential oils of M. cauliflora collected in the six sampling sites had an average yield of 0.37 ± 0.13 wt%. The mean yield of oils from plants growing on the S5 site (0.26 ± 0.04%) was lower than that of plants from soil types S1 (0.48 ± 0.15%) and S6 (0.48 ± 0.08%) (Table 1). As regards the content of essential oils, some species have shown higher content in soils fertilized with chemical or organic fertilizers, such as basil (Ocimum basilicum L.), Mentha arvensis L. and M. piperita L. (Lamiaceae). Other species are indifferent to fertilization, such as Thymus vulgaris L. (Lamiaceae). Quite different results were observed for M. arvensis and M. villosa Huds., for which the content of essential oils was progressively reduced as manure amounts increased.

A total of 28 compounds were identified, accounting for 97-100 of volatile constituents. All essential oils predominantly reveal sesquiterpene hydrocarbon compositions (47.50-57.22%), even though the oxygenated sesquiterpene content for some sample sites is over 49% (Table 1).

Furthermore, important differences in the amounts of major oil constituents and phenolic contents were found according to samples’ site origin (Table 1). The analysis of variance (ANOVA) indicated that S1 samples had the lowest percentage of germacrene D, bicyclogermacrene and sesquiterpene hydrocarbons, although it showed the highest percentages of oxygenated sesquiterpenes, α-, β- and γ-eudesmol. On the other hand, samples from fertilized soils S4 and S5 showed the lowest amount in elemol, total phenols and tannins, despite showing the highest sesquiterpene content. Despite the high percentage of β-caryophyllene, this constituent did not reveal any significant differences between samples growing on different sites. The oil constituents were also grouped according to their carbon skeletons on each sampling site. Data were standardized in accordance with the total percentage identified on each site. The same chemical variations were observed in the ANOVA as regards oil constituents (see SI, Table S3).
Similarly to the *M. cauliflora* fruit, whose oils did not differ markedly from leaf oils, the Amazonian camu-camu (*M. dubia* (Kunth) McVaugh) showed higher relative abundance of two monoterpene hydrocarbons, α-pinene (17.5-74.3%) and limonene (10.8-40.8%), in both fruit and leaf oils. Among the sesquiterpenes, β-caryophyllene had the highest relative abundance (1.1-15.9%). On the other hand, leaves of *M. tenella* (DC.) O. Berg. (*cambut*) from the Brazilian Northeast revealed higher contents of β-caryophyllene (32.0%) whereas samples from the Brazilian Southeast showed α-pinene (31.5%) and β-pinene (19.5%) as principal constituents. In addition, leaf oils of *M. trunciflora* O. Berg (*jaboticaba de cabinho*) showed high contents of curcumene (18.1%), benzyl tiglate (12.9%), *cis*-calamenene (12.4%), and α-humulene (11.7%), whereas *M. glazioviana* (Kiaersk) G. M. Barroso & Sobral (*cabeludinha*) suffered clear seasonal influence, with its highest variations in germacrene B (6.5-32.6%), caryophyllene oxide (3.7-13.2%), aromadendrene (0-10.7%), and β-caryophyllene (4.5-8.6%).

### Table 1. Percentages* and yields in essential oils and phenolics (mg g⁻¹) from *M. cauliflora* leaves according to sampling sites

<table>
<thead>
<tr>
<th>Constituent</th>
<th>RP</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
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<tbody>
<tr>
<td>α-Pinene*</td>
<td>931</td>
<td>0.24 a</td>
<td>0.47 a</td>
<td>0.40 a</td>
<td>t</td>
<td>t</td>
<td>0.35 a</td>
</tr>
<tr>
<td>β-Pinene*</td>
<td>975</td>
<td>1.32 a</td>
<td>1.91 a</td>
<td>1.56 a</td>
<td>0.89 a</td>
<td>1.25 a</td>
<td>1.60 a</td>
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<tr>
<td>Limonene*</td>
<td>1026</td>
<td>0.53 abc</td>
<td>1.00 a</td>
<td>1.01 ab</td>
<td>0.36 bc</td>
<td>0.22 c</td>
<td>0.90 ab</td>
</tr>
<tr>
<td>1,8-Cineole*</td>
<td>1029</td>
<td>0.48 a</td>
<td>0.81 a</td>
<td>0.78 a</td>
<td>t</td>
<td>0.45 a</td>
<td>0.76 a</td>
</tr>
<tr>
<td>δ-Elemene*</td>
<td>1337</td>
<td>1.28 b</td>
<td>1.31 b</td>
<td>1.71 a</td>
<td>1.70 a</td>
<td>1.54 ab</td>
<td>1.75 ab</td>
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<tr>
<td>α-Copaene*</td>
<td>1376</td>
<td>1.99 b</td>
<td>2.17 ab</td>
<td>2.35 ab</td>
<td>2.70 a</td>
<td>2.32 ab</td>
<td>2.28 ab</td>
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<tr>
<td>β-Bourbonene</td>
<td>1385</td>
<td>1.54 a</td>
<td>1.73 a</td>
<td>1.67 a</td>
<td>0.22 c</td>
<td>0.74 bc</td>
<td>1.12 ab</td>
</tr>
<tr>
<td>Unknown (M = 204)*</td>
<td>1390</td>
<td>0.37 ab</td>
<td>0.75 ab</td>
<td>1.40 a</td>
<td>0.91 ab</td>
<td>0.80 ab</td>
<td>0.19 b</td>
</tr>
<tr>
<td>β-Elemene*</td>
<td>1392</td>
<td>0.83 ab</td>
<td>0.67 ab</td>
<td>0.16 b</td>
<td>0.07 b</td>
<td>0.59 ab</td>
<td>1.24 a</td>
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<td>β-Caryophyllene*</td>
<td>1421</td>
<td>7.55 a</td>
<td>8.58 a</td>
<td>8.95 a</td>
<td>8.13 a</td>
<td>7.92 a</td>
<td>8.34 a</td>
</tr>
<tr>
<td>β-Copaene</td>
<td>1429</td>
<td>0.41 a</td>
<td>0.44 a</td>
<td>0.50 a</td>
<td>0.20 a</td>
<td>0.18 a</td>
<td>0.30 a</td>
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<tr>
<td>6,9-Guaiadiene</td>
<td>1443</td>
<td>0.39 a</td>
<td>0.12 a</td>
<td>0.77 a</td>
<td>0.34 a</td>
<td>t</td>
<td>0.61 a</td>
</tr>
<tr>
<td>α-Humulene*</td>
<td>1454</td>
<td>1.27 a</td>
<td>1.36 a</td>
<td>1.52 a</td>
<td>1.33 a</td>
<td>1.28 a</td>
<td>1.40 a</td>
</tr>
<tr>
<td>allo-Aromadendrene*</td>
<td>1461</td>
<td>0.51 a</td>
<td>0.63 a</td>
<td>0.56 a</td>
<td>0.32 a</td>
<td>0.51 a</td>
<td>0.67 a</td>
</tr>
<tr>
<td>Germacrene D*</td>
<td>1484</td>
<td>20.48 c</td>
<td>22.23 bc</td>
<td>23.59 abc</td>
<td>27.20 a</td>
<td>26.83 a</td>
<td>24.24 ab</td>
</tr>
<tr>
<td>δ-Selinene</td>
<td>1492</td>
<td>0.09 a</td>
<td>0.08 a</td>
<td>0.12 a</td>
<td>0.30 a</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>Bicyclogermacrene*</td>
<td>1498</td>
<td>6.29 c</td>
<td>6.72 bc</td>
<td>7.06 abc</td>
<td>7.82 a</td>
<td>7.33 ab</td>
<td>7.55 ab</td>
</tr>
<tr>
<td>α-Muurolene</td>
<td>1501</td>
<td>0.42 a</td>
<td>0.37 a</td>
<td>0.31 a</td>
<td>0.34 a</td>
<td>0.21 a</td>
<td>0.22 a</td>
</tr>
<tr>
<td>Germacrene A*</td>
<td>1506</td>
<td>0.07 b</td>
<td>0.31 ab</td>
<td>0.86 ab</td>
<td>0.52 ab</td>
<td>t</td>
<td>0.82 a</td>
</tr>
<tr>
<td>δ-Cadinene*</td>
<td>1524</td>
<td>2.69 b</td>
<td>2.75 b</td>
<td>2.81 ab</td>
<td>3.32 a</td>
<td>2.96 ab</td>
<td>2.78 b</td>
</tr>
<tr>
<td>Elemol</td>
<td>1550</td>
<td>3.69 b</td>
<td>4.39 a</td>
<td>4.77 a</td>
<td>2.12 c</td>
<td>2.55 c</td>
<td>4.73 a</td>
</tr>
<tr>
<td>Germacrene B</td>
<td>1558</td>
<td>1.34 d</td>
<td>1.42 cd</td>
<td>1.49 bc</td>
<td>1.79 a</td>
<td>1.59 b</td>
<td>1.58 bc</td>
</tr>
<tr>
<td>Unknown (M = 220)*</td>
<td>1578</td>
<td>1.04 b</td>
<td>1.52 a</td>
<td>1.13 ab</td>
<td>0.38 c</td>
<td>0.46 c</td>
<td>1.15 ab</td>
</tr>
<tr>
<td>Guaiol*</td>
<td>1601</td>
<td>0.29 a</td>
<td>0.15 a</td>
<td>0.33 a</td>
<td>0.32 a</td>
<td>t</td>
<td>0.27 a</td>
</tr>
<tr>
<td>Eremoligenol</td>
<td>1630</td>
<td>0.54 a</td>
<td>1.07 a</td>
<td>0.57 a</td>
<td>0.91 a</td>
<td>0.97 a</td>
<td>0.36 a</td>
</tr>
<tr>
<td>γ-Eudesmol</td>
<td>1634</td>
<td>11.55 a</td>
<td>9.64 ab</td>
<td>8.12 b</td>
<td>7.81 b</td>
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<td>8.81 b</td>
</tr>
<tr>
<td>β-Eudesmol*</td>
<td>1653</td>
<td>19.20 a</td>
<td>15.68 ab</td>
<td>14.91 b</td>
<td>16.91 ab</td>
<td>17.46 ab</td>
<td>14.84 b</td>
</tr>
<tr>
<td>α-Eudesmol*</td>
<td>1656</td>
<td>12.72 a</td>
<td>11.24 ab</td>
<td>10.28 ab</td>
<td>12.41 ab</td>
<td>12.94 a</td>
<td>10.31 b</td>
</tr>
</tbody>
</table>

Monoterpene hydrocarbons  
2.37 ab| 4.19 a| 3.75 ab| 1.27 b| 1.93 ab| 3.61 ab|
Oxygenated monoterpene hydrocarbons  
2.09 ab| 3.39 a| 2.97 ab| 1.26 b| 1.48 ab| 2.85 ab|
Sesquiterpenes  
0.48 a| 0.81 a| 0.78 a| t   | 0.45 a| 0.76 a|
Sesquiterpene hydrocarbons  
96.54 ab| 95.33 b| 95.96 ab| 98.08 a| 97.98 a| 95.54 b|
Oxygenated sesquiterpenes  
49.04 a| 43.70 ab| 40.13 b| 40.86 b| 43.15 ab| 40.46 b|
Oil yield (%)  
0.48 a| 0.32 ab| 0.35 ab| 0.29 ab| 0.26 b| 0.48 a|
Total phenols  
136.68 b| 120.91 c| 119.94 c| 79.69 e| 111.77 d| 145.04 a|
Tannins  
60.72 a| 57.60 b| 54.53 ab| 54.04 c| 44.51 c| 63.08 a

*Average based on original data. *Retention index. *Co-injection with standard. *Co-injection with clove essential oil. *Co-injection with ylang-ylang essential oil. *Ranke and aracine-transformed in ANOVA analysis (see Experimental section). t = trace (<0.05%). Averages followed by the same letter in the rows did not share significant differences at 5% probability by Tukey’s test.
The oil constituents including biosynthetic classes and total phenol/tannin contents (treated as species data set) and soil parameters and foliar nutrients (environmental data set) were analyzed jointly via canonical redundancy analysis (RDA), a multivariate treatment that assesses the way environmental variables may account for species' data sets. In RDA, each canonical ordination axis corresponds to a direction in the multivariate scatter of species data, which is maximally related to a linear combination of explanatory environmental variables. The species-environmental correlation equals the correlation between sampled site scores that are weighted sums of species and site scores, which in turn are a linear combination of environmental variables. RDA canonical axis is thus similar to principal component analysis, but it has a restriction on sampled site scores.21

Figure 1 shows RDA's ordination results of leaf oils and phenol content data set, whose soil and foliar nutrients were treated as environmental variables (6 sites (36 samples) × 36 oil/phenol contents × 26 environmental variables). Oil/phenol-environmental correlations were higher for the first two canonical axes (0.943 and 0.620), explaining 84.7% of the cumulative variance in the oil/phenol-environmental relation. These results suggest a strong association between oil composition/phenol content and the measured soil/foliar nutritional parameters (environmental factors) shown in the data set.

According to the triplot shown in Figure 1, RDA's axis 1 clearly correlated to nutrient balance in clayey soil S4 (Mg, Ca, K, Zn, Mn, P, organic matter, and cationic change capacity), which shows a strong relationship with total sesquiterpenes, germacrene B, germacrene D, δ-cadinene and α-copaene, whereas total monoterprenes, β-bourbonene, elemol, total phenols, and tannins load fairly strongly onto the sand soils (S1-S3 and S6) rich in Al and Fe. On the other hand, an increase in the value of RDA's axis 2 is associated with an increase in foliar micronutrients (Cu, Mn) of S1 sample origin, which also showed high contents of total oxygenated sesquiterpenes and eudesmols. In addition, the value increase of axis 2 is also highly linked with a reduction in foliar macronutrients (N, P, S, Mg, and Fe) of S3/S6 samples, which show high amounts of total sesquiterpene hydrocarbons, germacrene A, and limonene. Thus, whereas axis 1 shows changes in soil fertility, axis 2 describes a differential nutrient accumulation in sampled leaves from different sites. A significance test with an unrestricted Monte Carlo permutation test (9,999 permutations) found Fischer's F-ratio for the eigenvalues of RDA axes 1 (F-value = 11.215; p < 0.0001) and 2 (F = 5.017; p < 0.0179). Trace statistics were highly significant, giving signs that patterns did not arise by chance.21

Several studies have reported the involvement of Cu and Mn in the shikimic acid pathway leading to the biosynthesis of several phenols, such as flavonoids, tannins, and lignin.23,24 In plants with copper and manganese deficiency, lignification is impaired and phenolics accumulate in plant tissues.23 A dose-response effect of copper on foliar tannins has been reported in seedlings of Aegiceras corniculatum (L.) Blanco (Myrsinaceae).24 At first tannins decreased with an increase in copper supply; however, when copper reached toxic levels the tannin concentration also increased. These results show that the biosynthesis of phenolic compounds is dependent on Cu and Mn levels: lignification is inhibited in deficient tissues and the production of other phenolics is enhanced, which is probably the case of cluster I. When Cu and Mn achieve sufficient levels, lignin biosynthesis increases, most likely using other phenolics as intermediates.

The observed positive and negative correlation between foliar Mg and Mn, respectively, and δ-cadinene is in agreement with the requirement of sesquiterpene synthases for a divalent metal ion as cofactor.26 In peppermint, the only by-product (δ-cadinene) produced by (E)-β-farnesene synthase in the presence of Mg2+ was entirely absent in the presence of Mn2+ ions.23 In addition, the formation of sesquiterpenes such as germacrenes D and B and bicyclogermacrene from farnesyl diphosphate by germacrene D synthase in ginger (Zingiber officinale Roscoe; Zingiberaceae) is favored with Mg2+ as cofactor, but it is inactive in the presence of Cu2+ ions.28 Similar negative effects of foliar Mn on δ-cadinene and foliar Cu on germacrenes D and B, and bicyclogermacrene...
are in agreement with the negative correlation observed in this study.

As regards foliar phenolic distributions, we found correlations with some volatile constituents in samples from low fertilized sand soils. An explanation for this correlation is that phenolics – especially flavonoids – were protecting leaves by acting as antioxidants and a higher concentration was required to protect leaves from abiotic stresses. Phenolic contents in plant tissues have been related to nutrient availability. In most studies, phenolic production decreases at high nitrogen availability and increases under nitrogen deficiency. This trend was not followed by *M. cauliflora*, which showed no correlation in total phenol/tannin contents with nitrogen levels; this phenomenon has also been reported for tannins in *Colophospermum mopane* (J. Kirk ex Benth.) J. Léonard (Fabaceae). In our experiment, phenols and foliar nutrients revealed significant relationships with three foliar micronutrients (Mn, Cu, and Zn) and only one foliar macronutrient (K).

When the LDA analysis was applied to the data set, samples from the six soils were grouped as shown in Figure 2. The fitted model has elemol, α-eudesmol, β-eudesmol, total phenols, and tannins as predictor variables. The first discriminant function (F1) accounts for 96% of total variability and distinguishes (*F*-value = 43.944; degree of freedom, *DF* = 15 and 85; *p* < 0.0001) cluster I (S1) and II (S2, S3, and S6) samples from clusters III (S4) and IV (S5) due to the former’s high negative scores of total phenols and tannins. On the other hand, F2 highlights (*F* = 10.564; *DF* = 8 and 64; *p* < 0.0001) cluster III (S2, S3, and S6) as a result of elemol’s high negative scores.

In addition, the two discriminant functions make it possible to correctly classify 98.6% of samples in the original clusters by means of a cross-validation approach. This involves making a number of slightly reduced modifications to the parent data set, estimating parameters from each of these modified data sets and then calculating the accuracy of predictions by each of the resulting models. The only mismatched classification was a sample originating from an S3 soil (cluster II) which had been classified as belonging to cluster I (S1). Such misclassification could be caused by a higher level of α-eudesmol and β-eudesmol in the sample, which is a feature of the S1 sampling site. Percentages of oil constituents/total phenol and tannin contents, as well as soil parameters in clustered samples are shown in a supplementary information file (see SI, Tables S4 and S5, respectively).

Previous studies have also revealed that the chemical polymorphism of essential oils of *Hyptis*, *Thymus*, *Baccharis*, *Lychnophora* and *Eugenia* species vary significantly under different environmental abiotic factors, such as temperature, latitude, moisture, and chemical soil composition. It has also been reported that abiotic conditions such as soil nutrients, water stress, pollution, light, and altitude can affect the production of plant phenols and tannins.

Although most of the total variance in this study was accounted for by environmental relations among populations, a significant amount (about 15.3% of total variability) could be associated with genetic variations in the sampled populations.

**Conclusions**

The chemical variability in *M. cauliflora* leaves determined by multivariate chemometric techniques may reflect environmental influence on oil compositions and phenol/tannin contents, although it may also have been caused by genetic factors in cultivated samples. In this work, the percentage of oil constituents or the accumulated percentage of grouped volatiles based on carbon skeletons afforded similar results in chemical polymorphism.

**Experimental**

**Plant material**

Cultivated *M. cauliflora* leaves were collected in November 2008 at Jabuticabal Winery, located in Hidrolândia, Goiás State, Brasil. Leaf samples were obtained from 39 trees grown in six different soils (sampling sites): S1 (S 16° 55′ 22.9″, W 49° 21′ 49.9″), 7 sampled trees; S2 (S 16° 55′ 24.5″, W 49° 21′ 53.3″), 7 samples; S3
(S 16° 55’ 25.9″, W 49° 21’ 41.0″), 5 samples; S4 (S 16° 55’ 24.3″, W 49° 21’ 36.0″), 7 samples; S5 (S 16° 54’ 40.6″, W 49° 21’ 26.4″), 7 samples; S6 (S 16° 54’ 43.9″, W 49° 21’ 25.4″), 6 samples. Trees were from 10 to 40 years old and originated from the seeds of the same progenies.

To assess chemical composition, leaf samples were dried for 7 days at 30 °C until constant weight. After having been powdered, each dried phytomass (ca. 50 g) was submitted to hydrodistillation (4 h) by means of a modified Clevenger-type apparatus. At the end of each distillation oils were collected, dried with anhydrous Na₂SO₄, transferred to glass flasks, and kept at a temperature of −18 °C until analysis. Oil yields (%) were based on the dried weight of plant samples.

**Soil and leaf analyses**

Soil samples were collected at a depth of 40 cm in all localities. Three soil samples were collected around the canopy of each tree and pooled together to form a composite sample for each site (S1-S6); they were subsequently air-dried, thoroughly mixed, and sieved (2 mm). The portion finer than 2 mm was kept for physical and chemical analysis.³⁹ The pH was determined in a 1:1 soil/water volume ratio. Ca, Mg, and Al were extracted with KCl 1M, and P, K, Zn, Cu, Fe, and Mn were extracted with Mehlich solution. Organic matter, cationic exchange capacity (CEC), potential acidity (H+Al), and soil texture were determined by the usual methods.³⁹ The dried and powdered leaves of each tree were pooled to form a composite sample for each site and foliar nutrients (N, P, K, Ca, Mg, Mn, Zn, Cu, and Fe) were also determined by the usual methods.³⁹ Soil parameters and foliar nutrients were ordered in an environmental data matrix with 26 variables for each sampling site.

**Oil analyses**

Oil sample analyses were performed on a GC-MS Shimadzu QP5050A instrument under the following conditions: a CBP-5 (Shimadzu) fused silica capillary column (30 mx0.25 mm i.d., 0.25 µm film thickness) connected to a quadrupole detector operating in the EI mode at 70 eV with a scan mass range of 40-400 m/z at a sampling rate of 1.0 scan s⁻¹; carrier gas: He (1 mL min⁻¹); injector and interface temperatures of 220 °C and 240 °C, respectively, with a split ratio of 1:20. The injection volume was 0.4 µL (ca. 20% in hexane) and the oven temperature was raised from 60 to 246 °C with an increase of 3 °C min⁻¹, then 10 °C min⁻¹ to 270 °C, holding the final temperature for 5 min. Individual components were identified by a comparison of their linear retention indices,⁴⁰ which were determined by a co-injection with a C₅-C₃₀ n-alkanes series,⁴¹ co-injection with standards, clove (Syzygium aromaticum (L.) Merrill & Perry, Myrtaceae), ylang-ylang (Cananga odorata (Lam.) Hook. F. & Thoms., Annonaceae) and sage clary (Salvia sclarea L., Lamiaceae) essential oils,⁴² mass spectra with those of the literature,⁴² and a computerized MS-database using NIST libraries.⁴²

**Total phenolic content**

The powdered and dried leaves (0.2 g) of each soil site were extracted at room temperature with 50% v/v aqueous ethanol in an ultrasonic bath. Samples were extracted twice with 10 mL of solvent, first during 30 min and later during 15 min, and then with 5 mL for 15 min. The extracts were combined to a final volume of 25 mL. Total phenolic analysis was performed by the Folin-Ciocalteu method.⁴² Extracts or tannic acid (Merck) (0.5 mL) and 0.5 mL of 2 mol L⁻¹ Folin-Ciocalteu reagent (Sigma, St. Louis, MO, USA) diluted 10-fold were mixed in a 25 mL volumetric flask. After 5 min, 10 mL of 20% Na₂CO₃ solution were added and the volume reached 25 mL with distilled water. This mixture was then allowed to stand for 60 min at room temperature and the absorbance was determined at 750 nm. The standard curve was constructed with tannic acid at the following dilutions: 0.02, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mg mL⁻¹. The correlation coefficient was \( r = 0.9987 \). Total phenolic content was calculated as tannic acid equivalents (TAE) per g of dry weight. All solutions were analyzed in triplicate.

**Tannin content**

The extract solutions (1.0 mL) were precipitated with 2.0 mL of bovine serum albumine (BSA; fraction V, Sigma) solution (1.0 mg mL⁻¹) in 0.2 mol L⁻¹ acetate buffer (pH 4.9).⁴³ After centrifugation the precipitate was dissolved in sodium dodecyl sulfate (Sigma)/trihanolamine (Merck) solution (4.0 mL) and the tannins were complexed with 1.0 mL of FeCl₃ solution; the colored complex was then read at 510 nm. Measurements were made in the range 0.2 < A < 0.9. All solutions were analyzed in triplicate. The standard curve was constructed with tannic acid at the following dilutions: 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0 mg mL⁻¹. The linearity range went from 0.2 to 0.6 mg mL⁻¹. The correlation coefficient for this range was \( r = 0.9964 \).

**Chemical variability**

For statistic purposes, the multivariate statistical software CANOCO for Windows (Canonical Community
Ordination) version 4.5 was used jointly with CanoDraw for Windows 4.1 packages. Oil compositions (28 volatile constituents in addition to 6 biosynthetic classes) and total phenol/tannin contents were ordered in a species data matrix with rows (36) = localities (samples from six sites) and columns (36) = oil/phenolic variables. Soil parameters and foliar nutrients were ordered in an environmental data matrix with rows (36) = localities (samples from six sites) and columns (26) = ecological variables.

The preliminary analyses applied the default options of the detrended canonical analysis (DCA) to CANOCO to check the magnitude of change in oil/phenolic composition along the first ordination axis (i.e., gradient length in standard deviation units, SD). In this study, DCA estimated the compositional gradient in the species data to be shorter than 0.4 SD units, thus canonical redundancy analysis (RDA) was the appropriate ordination method to perform linear direct gradient analysis.

Redundancy analysis was applied to elucidate the patterns of the only explained variation of interrelationships between oil composition/phenolic content and the variation within populations as a function of soil parameters and foliar nutrients, treated as environmental variables. An unrestricted Monte Carlo permutation test (9,999 permutations) was used to test the significance of the eigenvalues of the first two canonical axes. Intra-set correlations from the RDA were therefore used to assess the importance of environmental variables.

Linear discriminant analysis (LDA) via CANOCO was used to differentiate populations in which the set of environmental variables involved single nominal variables defining a priori recognized clusters. Thus, clusters were coded as dummy environmental variables based on RDA analyses. Forward stepwise procedure on the oil/phenol data set was used as variable selection. Partial Monte Carlo permutation tests (999 permutations) adjusted by Bonferroni corrections were used to calculate the statistical significance of variables’ effects. The predictive ability of linear discriminant functions was evaluated by a cross-validation approach. Prior to the multivariate analysis the data was preprocessed by autoscaling and mean centering.

In all tables, average multiple comparisons were established by one-way ANOVA using SAS GLM analyses. All data was checked for homoscedasticity with the use of Hartley’s test. This test revealed significant deviations from the basic assumption for oil constituents and, respectively. Whenever a difference was established a post-hoc Tukey test was performed. Results are shown as mean values and are joined by the standard deviation of independent measurements in some cases. P-values below 0.05 were regarded as significant.

**Supplementary Information**

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as a PDF file.

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**References**


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