

Artigo

CG-MS/SPME as a Complimentary Tool to Histochemistry in the Study of the Influence of Water Regime on the Physiology of *Callistemon viminalis*

Oliveira Júnior, A. H.; Mendonça, H. O. P.; Augusti, R.; Melo, J. O. F.*; Silva, C. J.

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CG-MS/SPME como Uma Ferramenta Complementar à Histoquímica no Estudo da Influência do Regime Hídrico sob a Fisiologia de *Callistemon viminalis*

Resumo: Os óleos essenciais do gênero *Callistemon* possuem diversas propriedades bioativas. Condições ambientais e as alterações às quais as plantas são expostas influenciam sobre o metabolismo e a fisiologia das plantas. Assim, os óleos essenciais produzidos por *C. viminalis*, biossintetizados em suas folhas, estão sujeitos às alterações edafoclimáticas. Analisou-se possíveis distinções entre perfis anatômicos, histoquímicos e químicos de populações localizadas em regiões com diferentes regimes hídricos a fim de caracterizar a influência do regime hídrico sobre a fisiologia e síntese de metabólitos secundários da espécie. A micromorfometria não mostrou diferença entre os parâmetros nas populações estudadas, o que também foi o caso para análises histoquímicas. Utilizando microextração em fase sólida (SPME) e cromatografia gasosa acoplada a espectrometria de massa (CG-MS), foi possível verificar variações entre populações e seus perfis químicos. A influência do regime hídrico no perfil químico de compostos voláteis foi demonstrada através de variações na proporção de monoterpenos e sesquiterpenos. Embora os estudos anatômicos não demonstrem influência do regime hídrico, as análises químicas fornecem uma ferramenta poderosa para aprimorar os resultados preliminares, identificando a influência do regime hídrico sobre a fisiologia de *C. viminalis*.

Keywords: Water stress; volatile compounds; CG-MS/SPME.

Abstract

Essential oils from the genus *Callistemon* are known to possess bioactive properties. Oftentimes, environmental conditions and changes to which plants are exposed reflect on the plant metabolism and physiology. As such, essential oils produced by *C. viminalis* biosynthesized in its leaves, are subject to edaphoclimatic changes. This work analysed possible distinctions between anatomical, histochemical, and chemical profiles of populations located at regions with different water regimes. Micromorphometry did not show any difference between parameters in studied populations, this was also the case for histochemistry, analysed chemical compounds did not differ. Using solid phase microextraction (SPME), and gas chromatography coupled with mass spectrometry (CG-MS), it was possible to ascertain variations between populations and their chemical profiles. Influence of the water regime on the chemical profile of volatile compounds was shown through variations in the proportion of monoterpenes and sesquiterpenes detected when comparing the two populations. Though anatomical studies did not demonstrate influence of the water regime, chemical analyses proved a powerful tool in fine tuning results, positively identifying the influence of water regimen on the synthesis and profile of essential oils of *C. viminalis*.

Palavras-chave: Estresse hídrico; compostos voláteis; CG-MS/SPME.

* Universidade Federal de São João del-Rei, Departamento de Ciências Exatas e Biológicas, Campus Sete Lagoas, Rodovia MG 424 Km 47, CEP: 35701-970, Sete Lagoas-MG, Brasil.

 onesiomelo@gmail.com
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CG-MS/SPME as a Complimentary Tool to Histochemistry in the Study of the Influence of Water Regime on the Physiology of *Callistemon viminalis*

Afonso H. de Oliveira Júnior,^a Henrique de O. P. Mendonça,^a Rodinei Augusti,^b Júlio O. F. Melo^{a,*}, Cleber J. da Silva,^a

^a Universidade Federal de São João del-Rei, Departamento de Ciências Exatas e Biológicas, Campus Sete Lagoas, Rodovia MG 424 Km-47, CEP 35701-970, Sete Lagoas - MG, Brazil.

^b Departamento de Química, Universidade Federal de Minas Gerais, CEP 31270-901 Belo Horizonte - MG, Brazil.

*onesiomelo@gmail.com

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1. Introduction

Callistemon viminalis (Sol. ex Gaertn.) G. Don (Myrtaceae) is an exotic species of Australian origin¹ introduced in Brazil for urban landscaping purposes. Locally known as “bottle brush”, due to its inflorescence, *C. viminalis* can reach up to 7 m in height with its woody shrub-like habit.

Its leaves are often small, lanceolate, presenting translucent points and characteristic aromas, due to volatile compounds which make up the essential oils within cavities.¹

The genus *Callistemon* stands out with species producing essential oils whose properties confer bactericide effects to strains which are cited in literature as presenting resistance to conventional antibiotics such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*.²⁻⁴

The essential oil of the leaves of *C. viminalis* is mainly composed of 1,8-cineol, α -terpineol and α -pinene, which are related to antioxidant, anti-inflammatory and antimicrobial activities.^{6,7}

Leaves are one of the first organs which respond to stress in plants, exhibiting early signs and symptoms of physiological alterations caused by environmental change which impact their metabolic processes. Water, and the lack thereof, is one of the most common factors which can reflect on the metabolic processes in plants, greatly affecting physiological development.^{8,9}

As such, the objective of this work was to analyse possible anatomical, histochemical and volatile compound profile differences in *C. viminalis* leaves from populations in sites with contrasting water availability so as to highlight the impact of the water regime on the development of this species as well as its influence on the synthesis of secondary metabolites.

2. Materials and Methods

2.1. Samples

Fully expanded leaves from the 3th and 4th nodes were collected from individuals from two distinct populations located in sites with contrasting water availability: Site A – species on *campus* Sete Lagoas of the Federal University of São João del-Rei (UFSJ) with strict water regime and Site B – lakeside from Lagoa Paulino, Sete Lagoas, Brazil, with constant available water. Both sites were georeferenced.

2.2. Anatomical studies

Samples were fixed in FAA 70% (formaldehyde, acetic acid, 70% ethanol, 1:1:18, v/v) under vacuum for two hours, and later stored in 70% ethanol. In order to obtain cross and longitudinal sections, samples were embedded in methacrylate (Historesina Leica Instruments, Heidelberg, Germany).

Cross and longitudinal sections of 8 μ m were made using an automatic rotary microtome (Carl Zeiss™, RM55) and stained in toluidine blue for structural characterization.¹⁰ The slides were mounted permanently in synthetic resin (Permount®, Fisher).

The sections were analysed with a camera (AxioCam ERc5s) coupled to a light microscope (Zeiss™), using the software Axio Vision Documentation®.

2.3. Micromorphometry

Micromorphometrical analyses were carried out in which measurements of the thickness of mesophyll (MF), palisade parenchyma (PP), lacunose parenchyma (PL) tissues, the height of epidermis cells and cuticle thickness of both sides of the leaf. There were twenty-seven measurements for each tissue, repetition and parameter. The data was statistically analysed with ANOVA, Student's t-test, $\alpha = 5\%$.

2.4. Histochemistry

Samples from populations were processed and cross sectioned in order to carry out histochemical tests for lipids (Sudan IV, Nile blue sulfate), essential oils (NADI reagent), steroids (antimony trichloride), phenolic compounds (potassium dichromate), tannins (hydrochloric vanillin), starch (Iugol), polysaccharides (PAS), proteins (Xylidine Ponceau) and alkaloids (Wagner's reagent) following the guidelines laid out by the respective authors of each test.^{10,11-17}

2.5. Chemical analysis

Polydimethylsiloxane/Divinylbenzene (PDMS/DVB, 65 μ m) fibers were used for solid-phase microextraction (SPME).

In order to carry out headspace SPME (HS-SPME), 1.0 g samples were transferred to 20mL headspace vials, which were sealed and placed in aluminium blocks. After pre-heating for 5 minutes, fibers were inserted in the vials where the temperature was kept at 60 °C for 10 minutes. The fibers were then transferred to the CG/MS injector, where they remained in the equipment for 5 minutes.^{18,19,20}

A gas chromatograph (Trace GC Ultra) equipped with a mass spectrometer (Polaris Q, Thermo Scientific, San Jose, USA) and an injector split/splitless on splitless mode was used as an ion-trap type analyser. Helium gas (1 mL min⁻¹ flow) was used with a HP-5 MS capillary column (5% phenyl and 95% methylpolysiloxane); 30 m x 0,25 mm x 0,25 μ m (Agilent Technologies INC, Munich, Germany). Settings used in the mass spectrometer were: fragment ions of 35 and 300 m z⁻¹, ionizing mode with electron impact on 70 eV, transference line temperature at 275 °C and ion source temperature at 200 °C. Volatile compounds were identified using the National Institute of Standards and Technology library as well as data gathered from literature.

All experiments were carried out three times. Chromatograms resulting from the analysis of the volatile compounds were analysed on Xcalibur 1.4 (Thermo Scientific, San Jose, CA, USA) and Excel 2013 (Microsoft, WA, USA).

Mass spectra were evaluated on Xcalibur 2.1 (Thermo Scientific, San Jose, CA, USA). Principal Component Analysis (PCA) models were plotted with the centered average of the data, using MatLab 7.9.0.529 (Mathworks, Natick, Massachusetts, USA) and PLS Toolbox 5.2.2 (Eigenvectors Research, Manson, Washington, USA).

3. Results

3.1. Morphology

C. viminalis leaves from both populations (Region A and B) are lanceolate, with translucent punctuations. Venation pattern is pinnately parallel, with a large midrib and secondary ribs sharing similar girth. The apex and base of the

leaves taper towards the end, and the leaf blade is glabrous, with no division pattern.

3.2. Anatomy

Samples from both sites presented similar anatomical characteristics: uniseriate epidermis, with square cells covered by a thick, smooth cuticle (Figure 1.b-c, f). Anomocytic stomata are found on both sides of the leaf blade along with epidermis cells forming an epistomatic chamber (Figure 1b-c). Secretory cavities in cross and longitudinal sections occur on the interface between the palisade and spongy parenchyma, below the epidermis, with epithelial secretory tissue formed by flattened cells (Figure 1d-f). Mesophyll is compact and isobilateral (Figure 1a). The palisade parenchyma is formed by long, dense cells, with two layers; spongy parenchyma tissue is compact and formed by irregularly shaped cells (Figure 1a). The vascular system is composed of collateral bundles (Figure 1e).

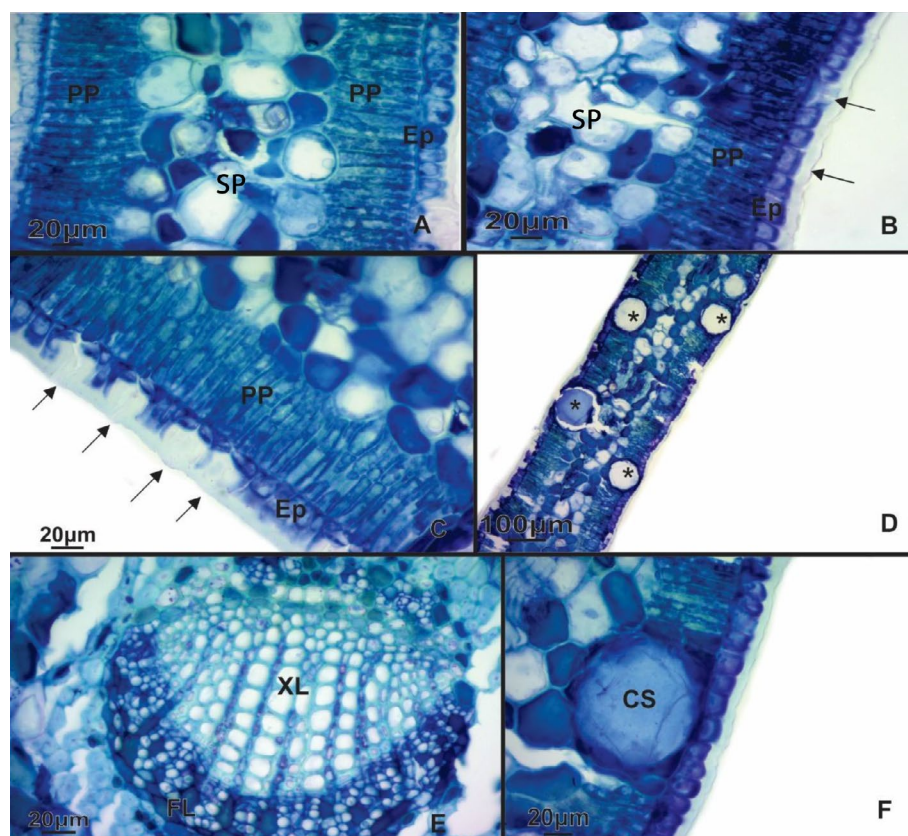


Figure 1. Cross section of leaf blade embedded in historesin. A. Isobilateral mesophyll. B. Adaxial epidermis, cuticle and stoma (arrows), Ep epidermis, PP palisade parenchyma, SP spongy parenchyma. C. Abaxial epidermis, stomata shown by arrows. D. mesophyll, secretory cavities (*). E. Xylem (XL) and phloem (FL) vascular bundle. F. secretory cavity (CS)

3.3. Micromorphometry

There were no statistically significant differences for the parameters analysed in either population of *C. viminalis* as shown on Table 1.

3.4. Histochemistry

Histochemical tests for both sampled populations showed no difference in results, as shown on Table 2.

Lipid deposits on the cuticle and in cavity secretions reacted with Sudan IV (Figure 2a). Blue Nile Sulphate helped identify the neutral characteristic of the lipids in both regions analysed (Figure 3b).

Production of essential oils within the secretory cavities was confirmed through the positive reaction to NADI reagent (Figure 2c). Furthermore, it was possible to identify steroid and alkaloid compounds which reacted with antimony trichlorate and

Wager's reagent, respectively (Figure 2d,g). Phenolic compounds were detected in idioblasts located throughout the lacunous parenchyma by their reaction with potassium dichromate (Figure 2e-f).

3.5. Volatile compounds profile

Thirty-five organic compounds were identified between the four leaf samples acquired on the sites A (lakeside, population of plants L1 and L2) and B (campus CSL/UFSJ, population of plants C1 and C2).

Plants C1 and C2 presented, respectively, 59% and 53% of monoterpenes and 25% and 32% of sesquiterpenes among the identified molecules, whereas for plants L1 and L2, the ratio was 72% of monoterpenes and 14% and 15% of sesquiterpenes comprising its essential oil. Table 3 shows the comparison between the volatile compounds found in the essential oils of *C. viminalis* on the two evaluated locations.

Table 1. Thickness of anatomical parameters (average; n = 10) of *C. viminalis*. Values in μm

	CAD	EAD	PPAD	SP	CAB	EAB	PPAB	MF
Site A	10.2	18.52	52.47	130.16	10.03	17.36	48.52	296.2
Site B	11.08	19.87	60.99	137.43	9.56	19.07	55	316.69
CV (%)	13.28	7.01	9.78	12.39	9.95	4.68	7.71	6.22
Average	10.64	19.19	56.73	133.79	9.79	18.22	51.76	306.44

Average values are statistically equal, Student's t-test, $\alpha = 0.05$. CAD: Adaxial Cuticle; EAD: Adaxial Epidermis; PPAD: Adaxial Palisade Parenchyma; SP: Spongy parenchyma; CAB: Abaxial Cuticle; EAB: Abaxial Epidermis; PPAB: Abaxial Palisade Parenchyma; MF: Mesophyll; Site A: campus CSL/UFSJ; Site B: Lagoa Paulino (Sete Lagoas, Brazil).

Table 2. Histochemical characterization of secretory cavities and mesophyll in *C. viminalis*

	Site A		Site B	
	Cavity	Mesophyll	Cavity	Mesophyll
Sudan	+	-	+	-
Blue Nile S	+	-	+	-
NADI Reagent	+	-	+	-
Antimony Trichloride	+	-	+	-
Potassium dichromate	-	+	-	+
Hydrochloric Vanillin	-	-	-	-
Floroglucinol	-	-	-	-
Lugol	-	-	-	-
PAS	-	-	-	-
Ruthenium Red	-	-	-	-
Xylidine Ponceau	-	-	-	-
Wagner's Reagent	+	-	+	-

Site A: campus CSL/UFSJ; Site B: Lagoa Paulino (Sete Lagoas, Brazil)

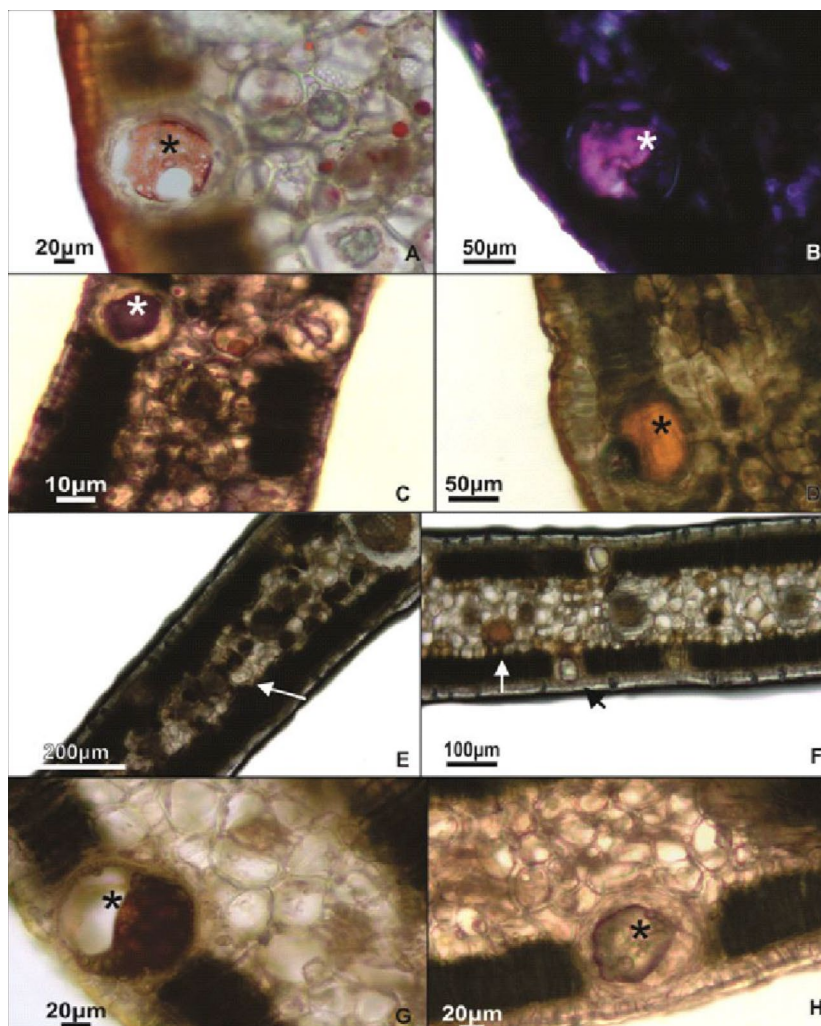


Figure 2. Sections of *C. viminalis* leaves. Aspects of secretory cavity secretions (*) A-D. E. Phenolic compounds in idioblasts (arrow). F. Secretory cavity showing no phenolic compounds (arrow). G. Secretory cavity showing the presence of alkaloids (*). H. Control section, secretory cavity (*)

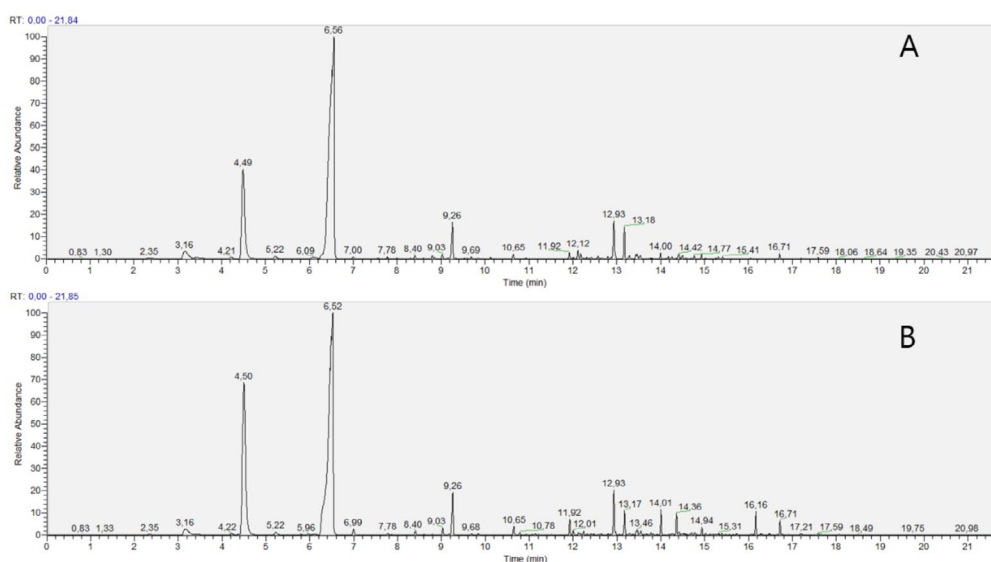


Figure 3. Comparative chromatogram representation of the volatile compound profile of lakeside (A) and UFSJ/CSL (B) populations

Table 3. Characterization of the volatile compound profiles of *C. viminalis*

RT	Name	Class	C1	C2	L1	L2
2.34	3-methylhexane	Hydrocarbon	+	+	+	+
3.16	e-hex-2-enal	Aldehyde	+	+	+	+
4.21	alilpenthylesteroxalyc acid	Esther	+	+	+	+
4.50	3,7,7-trimethylbicyclo[4.1.0]het-2-ene	Monoterpene Hydrocarbon	+	+	+	+
6.10	3-methylbutane 2-methylbutanoate	Esther	+	+	-	-
6.53	α -pinene	Monoterpene Hydrocarbon	+	+	+	+
7.78	7,7-dimethyl-2-metenilbicyclo[2.2.1]heptane	Monoterpene Hydrocarbon	+	+	+	+
8.80	6,6-dimethyl-2-metenilbicyclo[2,2,1]heptan-3-one	Oxygenated Monoterpene	+	+	-	-
9.03	1-methyl-4-isopropilcyclohex-2-en-1-ol	Oxygenated Monoterpene	+	+	+	+
9.26	α -terpineol	Monoterpene Alcohol	+	+	+	+
9.59	1,8-cineol	Ether	+	+	+	+
9.69	trans-p-mentha-6,8-dien-2-ol	Oxygenated Monoterpene	+	+	-	-
10.65	e-pent-3-en-2-one	Ketone	+	+	+	+
11.92	2-metoxi-4-(2-propenil)-phenol	Oxygenated Monoterpene	+	+	+	+
12.01	α -camfolenal	Oxygenated Monoterpene	+	+	+	+
12.12	1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-metenil-1-isopropilnphthalene	Hidrocarboneto Sesquiterpeno	+	+	+	+
12.24	Z-3,7-dimethyl-2,6-octadien-1-ol	Oxygenated Monoterpene	-	-	+	+
12.42	α -bisabolene epoxide	Oxygenated Sesquiterpene	+	+	-	+
12.64	Selinene	Sesquiterpene Hydrocarbon	-	+	+	+
12.93	1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methyletenil)-[1R-(1 α ,7 β ,8 $\alpha\alpha$)]-naphthalene	Sesquiterpene Hydrocarbon	+	+	+	+
13.18	decahydro-1,1,7-trimethyl-4-metenil-[1aR-(1 $\alpha\alpha$,4 $\alpha\beta$,7 α ,7 $\alpha\beta$,7 $\beta\alpha$)]-1H-cycloprop[e]azulene	Sesquiterpene Hydrocarbon	+	+	+	+
13.29	1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methyletenil)-[1R-(1 α ,7 β ,8 $\alpha\alpha$)]-naphthalene	Sesquiterpene Hydrocarbon	+	+	+	+
13.46	cis-(-)-2,4a,5,6,9a-hexahydro-3,5,5,9-tetramethyl(1H)benzocycloheptene	Sesquiterpene Hydrocarbon	+	+	+	+
13.54	guaia-1(10),11-diene	Sesquiterpene Hydrocarbon	+	+	+	+
14.18	cubenol	Oxygenated Sesquiterpene	+	+	-	-
14.26	1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methyletenil)-[1R-(1 α ,7 β ,8 $\alpha\alpha$)]-naphthalene	Sesquiterpene Hydrocarbon	+	+	+	+
14.36	(-)-spathulenol	Oxygenated Sesquiterpene	-	-	+	+
14.42	1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methyletenil)-[1R-(1 α ,7 β ,8 $\alpha\alpha$)]-naphthalene	Sesquiterpene Hydrocarbon	+	+	-	-
14.50	guaia-1(10),11-diene	Sesquiterpene Hydrocarbon	+	+	-	-
14.54	3,3-dimethylcyclohexanoacetaldehyde	Oxygenated Monoterpene	-	-	+	+
14.75	3,4,7-trimethylbicyclo[4,3,0]non-3-ene	Sesquiterpene Hydrocarbon	+	+	-	-
14.77	1-(5-hidroxi-4a,8-dimethyldecahydro-2-naftalenil)etanone	Ketone	-	-	+	+
14.93	5-methyl-2-(1-methyletilideno)cyclohexanone	Sesquiterpene Hydrocarbon	+	+	+	+
16.16	2-methyl-5-isopropil-cyclohex-1,3-diene	Monoterpene Hydrocarbon	-	-	+	+

RT: retention time; C: UFSJ/CSL population; L: lakeside population; +: present; -: absent

3.6. Principal Component Analysis

Figure 4 presents the principal component analysis demonstrating the water stress effect over the volatile compound profile of the essential oils in *C. viminalis*. The PCA model was able to explain 96.98% of variation between data.

4. Discussion

Micromorphometric analyses of *C. viminalis* in different water regimes did not indicate differences between the parameters for which comparisons were statistically evaluated. Considering the fact that this species occurs naturally along the margins of watercourses, it also appears to be able to adapt to places where the water supply is restricted, suggesting at first an adaptive plasticity for regions under different environmental conditions.²¹

Species which occur in heterogeneous regions oftentimes show a potential for plasticity which grants them an adaptative advantage in colonizing different habitats while adjusting morphological and physiological processes to local conditions.²² Such changes in functional and structural characteristics can be attributed to environmental selective pressure to which individual populations of the same species are exposed.²³

In this study, however, there were no observable anatomical or histochemical variations between the populations as would have been expected, at first glance pointing towards the conclusion that the water regime does not influence, chemically, the composition of the essential oils in *C. viminalis*.

Similarly, to these findings, in other Myrtaceae species, few changes have been observed in similar contrasting conditions, with literature citing exclusively morphological differences. In *Eugenia calycina* out of all analysed parameters, height was the only parameter with statistically

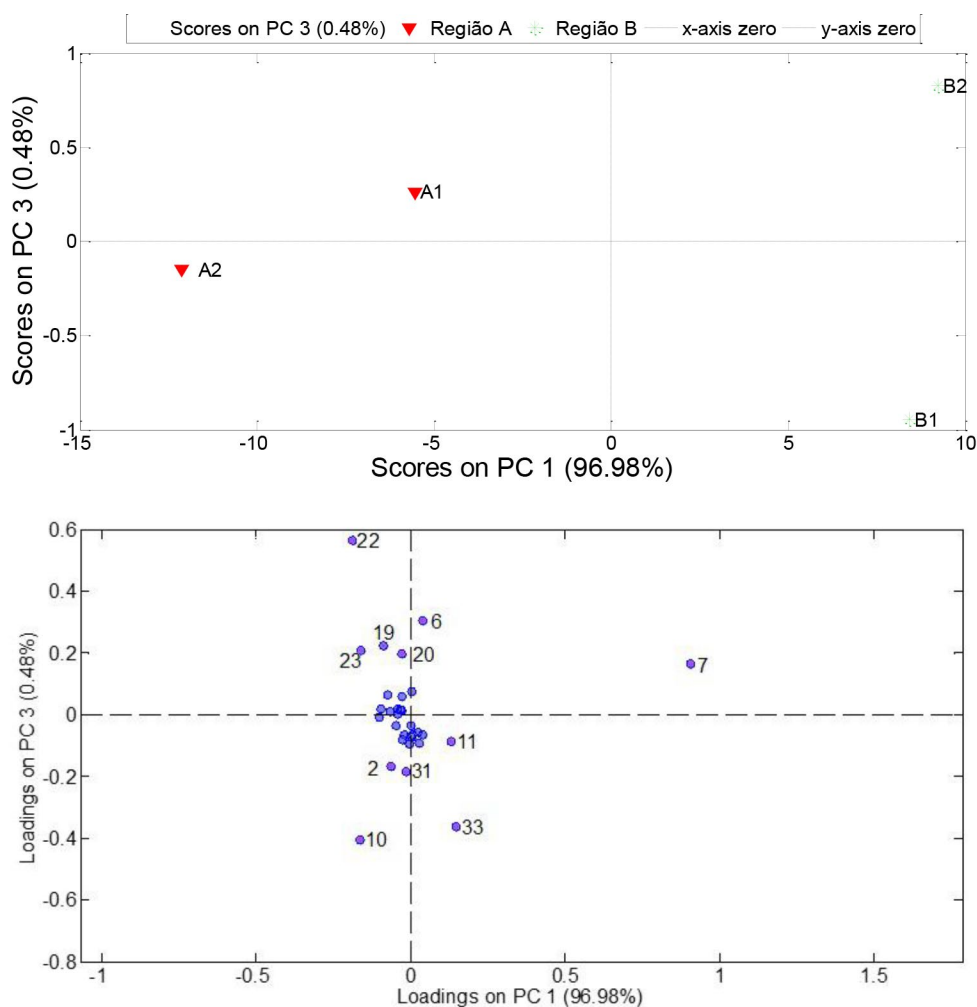


Figure 4. Principal component analysis (PCA) of *C. viminalis* samples

significant variation due to different environmental conditions.²⁴ Authors refer these results to phenotypical plasticity which would imply genetic variability between sampled individuals. However, *C. viminalis*, did in fact show a physiological variation between studied populations: flowering. The population along water margins were able to flower throughout the year.

In the same way that morphologically there were no differences observed between populations, histochemical characterization also did not, present significant changes owing to the contrast in water regime in relation to histolocation, nor class of secondary metabolite synthesized, which would again propose no water stress effect for the composition of essential oils.

Water stress effect can vary between species, affecting individual populations differently in regard to their production of essential oils as well as their composition.⁹ Volatile compound profiles show, in contrast with the morphoanatomical studies, significant variation in the concentration of monoterpenes and sesquiterpenes. Plant samples from populations coming from site B (L1 and L2) showed a higher number of monoterpenes in the makeup of their essential oils.

On the principal component analysis, generated by volatile organic compounds (VOC), the composition of plants in the region of *Campus* CSL/UFSJ differed significantly from those sampled around the water margins of Lagoa Paulino, mainly due to 2-carene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-[1R-(1 α ,7 β ,8 $\alpha\alpha$)]-naphthalene, 1-methyl-3-(1-methylethenyl)cyclohex-1-ene and (-)-spathulenol. Among these chemical compounds, only (-)-spathulenol was identified in a single location, whereas other compounds differed in concentration.

5. Conclusion

Anatomical and histochemical analyses were able to qualitatively classify the aspects of essential oils; with the integration of chemical analyses through the method of solid phase microextraction it was possible to refine and identify compounds and qualitatively between populations. This presents GC/SPME as an extremely efficient tool in the identification of the influence of different environmental conditions, namely, in this study, water regime, in the makeup of

the profiles of volatile organic compounds between analysed populations.

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