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Comprehensive two-dimensional gas chromatography combined to multivariate data analysis for detection of disease-resistant clones of *Eucalyptus*

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ABSTRACT

In this paper it is reported the use of the chromatographic profiles from volatile fractions of plant clones – in this case, hybrids of *Eucalyptus grandis* × *Eucalyptus urophylla* – to determine specimens susceptible to rust disease. The analytes were isolated by headspace solid phase microextraction (HS-SPME) and analyzed by comprehensive two-dimensional gas chromatography combined to fast quadrupole mass spectrometry (GC × GC-qMS). Parallel Factor Analysis (PARAFAC) was employed for estimate the correlation between the chromatographic profiles and resistance against *Eucalyptus* rust, after preliminary variable selection performed by Fisher ratio analysis. The proposed method allowed the differentiation between susceptible and non-susceptible clones and determination of three resistance biomarkers. This approach can be a valuable alternative for the otherwise time-consuming and labor-intensive methods commonly used.

1. Introduction

Over the last decade, extensive research and development has been performed in the field of multidimensional separations in gas chromatography [1,2], to tackle the analysis of complex samples such as those currently found in metabolomics [3,4]. Metabolomics, for instance, deals with fractions, as representative as possible, of the cellular metabolites which requires powerful instrumentation to isolate and identify specific analytes [5]. Amongst these analytes, several chemically distinct biogenic volatile organic compounds (BVOC) can be found in the metabolome – such as aliphatic alcohols, aldehydes, methylketones, acids, lactones, esters, terpenoids, furanones and pyrones [6]. In this sense, modern-day mass spectrometers (MS) interfaced with gas chromatography (GC) has allowed the unprecedented detection of several hundreds of peaks from a single biological sample [7].

Naturally, the instruments used to obtain a proper metabolic profile generate very large and complex data, such as those originated from comprehensive two-dimensional gas chromatography hyphenated with mass spectrometry (GC × GC–MS) [8]. Consequently, because of the exceedingly complex nature of the experimental measurements, intuitive data interpretation is often difficult [7]. Thus, powerful data analysis techniques are often necessary in order to glean the most useful information from these measurements [5].

In this context, amongst the several applications in metabolomics, a niche that is currently expanding is the study of plant-pathogen interactions, focusing, especially, on the pathosystems with economical interests. More specifically, the current research will focus on the rust disease in *Eucalyptus* because it is an important commercial plant, extensively used for production of cellulose, paper, coal and as a source of essential oils used for the production of aroma and fragrance products [9]. Rust fungi are amongst the most important plant pathogens worldwide, such as the biotrophic fungus *Puccinia psidii* [10]. This pathogen can cause severe damage to *Eucalyptus* that considerably reduces biomass accumulation and plant reproduction, which can lead to major negative economic impact [10]. Consequently, analytical methods for the early, and fast, diagnosis of the disease have been proposed and are currently under development. Several methods are based on analysis of the genetic material of the pathogen, often accomplished by the use of polymerase chain reaction (PCR), [11] or by specific interaction between antigens and antibodies, such as enzyme-linked immunosorbent assay (ELISA) [11], or by the analysis of the BVOC from the host [9].

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Alternatively, the selection and growth of *Eucalyptus* clones that are resistant, or at least more tolerant, to rust disease is potentially interesting and is currently being explored. The traditional method for screening of the resistance is based on the evaluation of a rating scale of the disease [12]. Typically, the pathogen is inoculated and the phenotype of various genetic materials is then evaluated with the rating scale, which defines if the plant is resistant or susceptible to the disease [12]. However, the inoculation and the evaluation of the rating scale is not a trivial task and too often it becomes a very laborious and time-consuming process [12].

Among plant secondary metabolites, the terpenoids function as phytoalexins in plant direct defenses, or as signals in indirect defense responses which involves pathogens [13]. In this context, recently, several terpenoids, found in the volatile fraction of *Eucalyptus globulus*, were successfully used as disease biomarkers for screening the presence of infection, caused by neurotropic fungus *Teratosphaeria nubilosa*, in *E. globulus* [9]. Hence, in this context, the current study proposes the screening of the BVOC for the assessment of potential resistance biomarkers in hybrids of *Eucalyptus grandis* and *Eucalyptus urophylla* against *Eucalyptus* rust, caused by *Puccinia psidii* fungus. It is hoped that the current method can be a potentially interesting alternative for otherwise time-consuming and labor-intensive methods conventionally used in plant biology.

1.1. Fisher ratios

Frequently in metabolomic studies the amount of information contained in the experimental measurements are often unrelated with the property of interest [9,14,15]. Hence, when unsupervised exploratory analysis is performed, such as principal component analysis (PCA) or parallel factor analysis (PARAFAC) the scores, or the loadings from the sample mode, may cluster incorrectly [14]. Consequently, in these cases, the analyst cannot extract any meaningful information from data analysis for chemical and biological interpretation. Thus, prior multivariate data analysis, "filtering" methods are commonly applied to the set of instrumental responses, which either eliminates the uncorrelated information [9,15] – hence, it can provides optimal platform for multivariate modeling – or it is able to find specific portions in the raw data, that are statistically relevant for the analysis [14] – such as the case of the Fisher ratio. In addition, the selection of specific segments of the data, also, decreases significantly the computational time for data modeling.

The Fisher ratio method was originally described almost 90 years ago [16] and first applied for third order chromatographic data in 2006 [14]. Because the Fisher ratio calculation differentiates regions of the chromatogram with large class-to-class variation (σ^2_{cl}) from regions containing large within-class variation (σ^2_{err}), the method can be used to select specific regions of the data array for multivariate data analysis, which allows for more efficient extraction of biologically relevant information from the experimental measurements.

Briefly, the class-to-class variation, σ^2_{cl}, and within-class variation, σ^2_{err}, are calculated as [14]:

$$\sigma^2_{cl} = \sum \frac{(\bar{x}_i - \bar{x})^2 n_i}{(k-1)} \quad (1)$$

$$\sigma^2_{err} = \frac{\sum(\sum(\bar{x}_{ij} - \bar{x})^2) - (\sum(\bar{x}_i - \bar{x})^2 n_i)}{(N-k)} \quad (2)$$

where n_i is the number of measurements in the ith class, $\bar{x}_i$ is the mean of the ith class, $\bar{x}$ is the overall mean, k is the number of classes, $\bar{x}_{ij}$ is the ith measurement of the jth class and N is the total number of sample profiles.

The Fisher ratio analysis is calculated at every point in the separation space, and is the σ^2_{cl} of the detector signal divided by the sum of the σ^2_{err} of the detector signal, as illustrated in Eq. (3) [14]. The indexing scheme to reduce the four-dimensional array into a two-dimensional array was performed according to the method proposed by Synovec et al. [14]. A more detailed description has been reported elsewhere [14].

$$\text{Fisher ratio} = \sigma^2_{cl}/\sigma^2_{err} \quad (3)$$

1.2. Parallel factor analysis

A GC × GC-qMS instrument is capable of generating a set of four-way data. Naturally, these arrays require more sophisticated data analysis methods in order to extract all the chemical information from these chromatograms. In this sense, parallel factor analysis (PARAFAC) is a high-order method capable of evaluating simultaneously the information from the four modes of data set [17,18,19,20]. This technique has been successfully used for exploratory and peak deconvolution investigations of data generated from hyphenated and multidimensional chromatographic instruments [3,18,21].

In this case, considering a four-way array, $\underline{\mathbf{X}}(I, J, K, L)$, the PARAFAC model decomposes it into the four loading matrices **A**, **B**, **C** and **D.** This decomposition is performed as to minimize the quadratic sum of the residues in $\underline{\mathbf{E}}$ and is represented by Eq. (4). The loading matrices are related to each of the four dimensions: $\mathbf{A}(I \times F)$ is related to the sample mode, $\mathbf{B}(J \times F)$ and $\mathbf{C}(K \times F)$ are related to the second and first dimensional chromatographic profiles and $\mathbf{D}(L \times F)$ is related to the mass spectra mode – where F is the number of factors su for and $|\otimes|$ represents the Khatri–Rhao product [17,22].

$$\underline{\mathbf{X}} = \mathbf{A}(\mathbf{D}| \otimes |\mathbf{C}| \otimes |\mathbf{B})^{\mathrm{T}} + \underline{\mathbf{E}} \quad (4)$$

2. Materials and methods

2.1. Samples

All samples were collected from trees kept at the Faculty of Agronomic Sciences greenhouse (Botucatu, SP, Brazil). Leaves from several hybrids of *E. grandis* × *E. urophylla* were harvested, yielding samples covering 17 different genetic profiles. The leaves were immediately frozen under liquid nitrogen (LN_2) to cease all physiological processes and kept under refrigeration (dry ice, −78 °C) until analysis.

2.2. Reagents and materials

For the determination of the ^{1}D linear temperature programmed retention index (LTPRI), a mix of C7-C20 *n*-alkanes was used (Sigma-Aldrich – St. Louis, MO, USA). For the HS-SPME procedure, septum-sealed glass v-vials, (Wheaton Science Products – Millville, NJ, USA), magnetic stirrers and appropriate screw caps with PTFE/silicon septa (Sigma-Aldrich) were employed, as well as SPME fibers coated with 50/30 μm divinylbenzene/Carboxen on poly(dimethylsiloxane) (DVB/CAR/PDMS) (Sigma-Aldrich).

2.3. HS-SPME procedure

Prior to the isolation of the volatiles, c.a. 5–7 frozen leaves were ground in a sterile mortar in the presence of LN_2 and kept on sealed flasks to thaw until ambient temperature. Aliquots of (300 ± 5) mg of ground leaves were weighted directly in the v-vials and 2 mL of 18.5% (m/v) aqueous sodium chloride was added.

After 10 min stirring at 45 °C for sample/headspace equilibration under magnetic stirring (800 rpm), a 50/30 μm DVB/CAR/PDMS SPME fiber was exposed to the headspace of the suspension for 30 min. The extracted analytes were immediately desorbed, separated and detected using GC × GC-qMS.

2.4. GC × GC-qMS

All analyses were performed on a lab-made GC × GC-qMS prototype assembled on a QP2010+ (Shimadzu Corp. – Tokyo, Japan) fitted with a split/splitless injector and a miniaturized sealed two-staged cryogenic modulator (supplementary material) [23]. The modulator was controlled by a low cost 8-bit Duemilanove microcontroller board (Arduino – Ivrea, Italy) [24]. The column set consisted of a 25 m × 0.25 mm × 0.25 μm HP-5 MS (Agilent Technologies – Palo Alto, CA, USA) column fitted to a 200 cm × 0.10 mm × 0.10 μm SupelcoWax 10 (Sigma-Aldrich) using a SilTite zero-volume union (SGE Inc. – Austin, TX, USA). The modulation period for all analyses was 5.0 s. Injection port and MS transfer line were kept at 250 °C and H_2, at a constant pressure of 31.9 psi (220 kPa), was used as carrier gas. For all runs, the oven temperature programming was set from 60 °C to 171 °C at 3 °C min^{-1} followed by a ramp of 20 °C min^{-1} to 250 °C. The MS ionization source was kept at 200 °C and the scan range was set from m/z=40–380 units, resulting a data collection rate of 25 spectra s^{-1}. Peak identification was performed by matching against NIST 2010 (NIST – Gaithersburg, MD, USA) and FFNSC (Chromaleont – Messina, Italy) spectra libraries, combined with LTPRI inspection. Complementary data analysis and figure generation was performed using GCImage software (Zoex Corp. – Houston, TX, USA). The samples were analyzed in duplicate, except for six samples where the amount available was limited.

2.5. Estimation of plant resistance to disease

For the determination of the resistance or susceptibility of the 17 hybrids to *Eucalyptus* rust, a rating scale was used. The samples were classified as either resistant or susceptible, with different tolerances to the disease, based on this scale [25].

2.6. Multivariate analysis

The data set consisted of 28 chromatograms from 17 hybrids of *E. grandis* × *E. urophylla*. Two classes were defined according to their resistance against the disease: resistant (10 samples) and susceptible (18 samples) trees. Fisher ratio with weighting was used to select the regions of the four-dimensional data arrays to be considered for multivariate processing [14]. After removal of the non-significant sections, the reduced four-way data was analyzed by PARAFAC, which was initialized by the singular value decomposition (SVD). Core consistency diagnosis test (CORCONDIA) was used in order to find the appropriate number of factors for the model [17,26].

All data analysis was performed in MATLAB environment version R2009b (MathWorks, Natick – MA, USA). The chromatographic data was converted to the NetCDF format using GCMS Solution software (Shimadzu Corp.). Two MATLAB scripts were written to create the three dimensional arrays from the NetCDF files and to perform the Fisher ratio calculations. The former combined two functions – "iCDF" [27] and "mzcdfread", from the Bioinformatics Toolbox. The PARAFAC algorithm was obtained from the N-way Toolbox 3.00 [28].

3. Results and discussion

Through the rating scale it was found that 5 hybrids were resistant to *Eucalyptus* rust, while 12 were susceptible, with varying tolerance to the disease. Hence, these results were be used to evaluate the proposed method.

A preliminary screening of the BVOC detected in the headspace of the *Eucalyptus* leafs by HS-SPME-GC-qMS showed that most analytes had similar polarities, ranging from low to medium-polarity. Therefore, for the GC × GC-qMS analyses a longer second dimension capillary column was adopted to increase peak resolution. The modulation period was set to 5.0 s, since it was adequate for separation of the analytes in the ^{2}D without jeopardizing resolution in the ^{1}D.

Prior multivariate data analysis, the chromatograms were inspected to check for retention time shifts. It was not necessary to align the chromatograms prior processing. Fig. 1(A) and (B) illustrate typical chromatograms obtained from leaves of two different rust-resistant clones of *Eucalyptus*. In other hand, Fig. 1(C) shows a chromatogram from a highly-susceptible clone and (D) a chromatogram of a moderately susceptible clone. Visual inspection of these figures shows no obvious correlations between chromatographic profiles and resistance/susceptibility of the corresponding sample to rust disease; therefore, it is clear that more sophisticated data mining strategies have to be adopted in order to find possible links between chromatographic data and biological behavior.

For the multivariate analysis, the three-way GC × GC-qMS chromatograms were combined in a four-dimensional array, $\underline{\mathbf{X}}$ (28 × 125 × 459 × 344), but only after a preliminary selection of the relevant sections of the raw chromatographic data. The data hypercube resulting from assembling raw chromatograms would be massive, highly demanding on terms of computational resources and resulting on an excessively large processing time. However, a significant fraction of the chromatograms is not relevant for this study, since it only contains baseline or signals not associable to the biological property being assessed. Therefore, a preliminary operation using Fisher ratios approach was employed to select specific regions of the chromatogram for the PARAFAC analysis. Fisher ratio cutoff value was empirically. A cutoff value of 1×10^3 signal units was selected, as illustrated in Fig. 2(A). Fig. 2(B) shows a plot of the sum of weighted 2D Fisher ratios; the regions of the chromatogram indicated in this figure were pointed as statistically different between samples, indicating the regions of the chromatogram to be selected for multivariate data analysis. Remarkably, conventional PARAFAC modeling of the entire raw data was performed in 94 min, while after variable selection by Fisher ratio analysis the computational time was reduced to 5 min.

After the masking of non-relevant signals on the data tensor, PARAFAC was applied for pattern recognition. For the determination of the number of factors, CORCONDIA was performed, in triplicate, by varying the factors from 1 to 10 to evaluate the adequacy of the resulting model. The number of factors was optimized by verifying the $\underline{\mathbf{X}}$ quadratic sum of residues, the trilinear consistency and the number of iteration needed [26]. It was observed that the most suitable model was obtained with two factors describing 37.45% of the total variance and a CORCONDIA value of 99.81%.

The loadings graph for Mode I (samples) obtained from the two-component PARAFAC model is represented in Fig. 3. A visual inspection of the loadings graph shows distinct clustering of the samples originated from clones resistant to *Eucalyptus* rust (□) apart from clones susceptible to the same disease (×) on Factor 1. In addition, it was found that Factor 2 did not contain any chemical information relevant for the biological analysis, based on the clustering of the samples in Fig. 3. Hence, the information

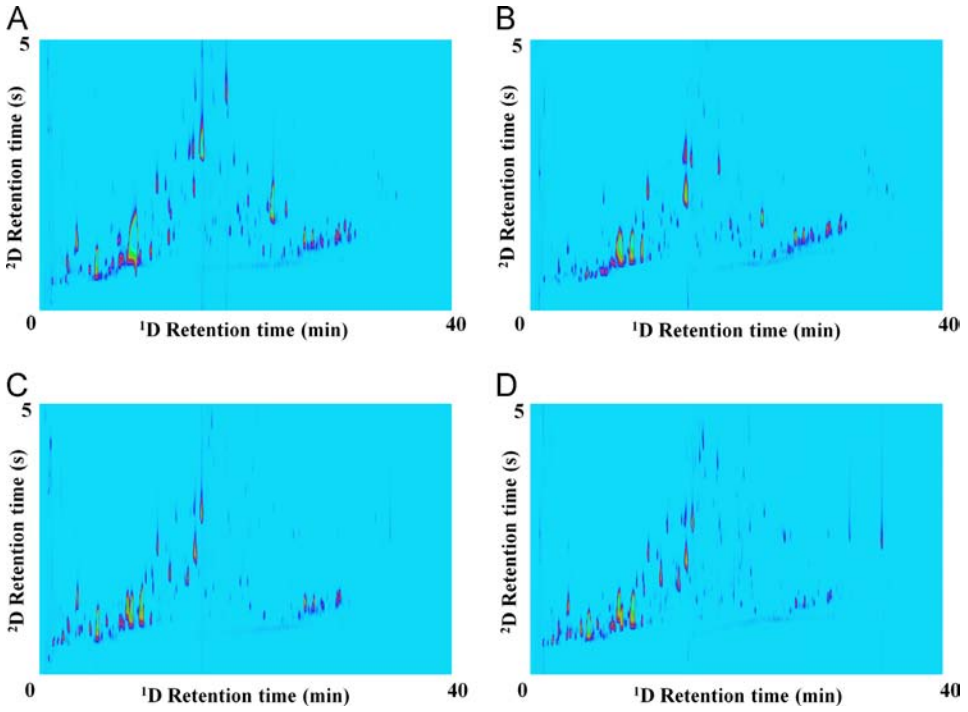

Fig. 1. Typical GC × GC-qMS total ion chromatograms for BVOC of leaf samples of hybrids of *E. grandis* × *E. urophylla*: (A), (B): hybrid clones resistant against *Eucalyptus* rust; (C): very susceptible clone and (D): clone susceptible but more tolerant to the disease.

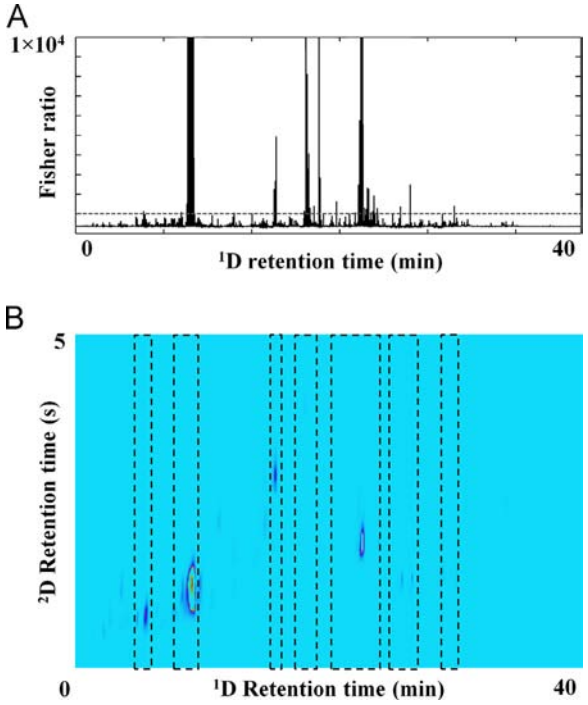

Fig. 2. Results from Fisher ratio analysis: (A) sum of weighted 1D Fisher ratios with cutoff value of 1×10^3; (B) weighted sum of 2D Fisher ratios plot showing sections of the chromatogram selected for multivariate data analysis

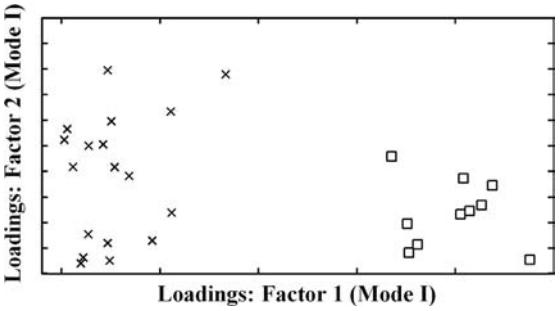

Fig. 3. Results of two-component PARAFAC modeling (loadings values of the first modes of Factor 1 and Factor 2 plotted one against other); □ = clones resistant to *Eucalyptus* rust and × = clones susceptible to the same disease.

contained in the Modes II and III (^{2}D and ^{1}D chromatographic profiles, respectively), illustrated in Fig. 4(A) and (B), were evaluated. More specifically, the loadings of Modes II and III of Factor 1 were inspected and matched to the chromatographic peaks found in the *Eucalyptus* samples. It was observed that a peak found at the retention coordinates of 8.83 min × 1.2 s displayed the highest weight value in the loadings of Factor 1, Fig. 4(A) and (B), that are related to the resistance/susceptibility. Furthermore, additional peaks found in the retention coordinates of 24.33–25.42 min × 0.8–1.6 s also contributed, although less significantly, to the clustering of the samples observed in Fig. 3.

Peak identification of the chromatographic regions designed to be relevant for the differentiation between susceptible and non-susceptible clones was performed by matching the experimental mass spectra against spectra databases combined with LTPRI inspection. Table 1 shows the tentative identification of relevant analytes found in the volatile and semi-volatile fraction of these *Eucalyptus* leaves. It was observed that both eucalyptol and α-terpinyl acetate could be successfully used for the detection of plant clones resistant of susceptible to rust disease. In addition, the information contained in the Mode IV of Factor 1, Fig. 4(C), was successfully matched with the mass spectra of eucalyptol, found in NIST mass spectra library.

Furthermore, the results obtained with the proposed method were compared to the standard method, rating scale, to evaluate the presence of erroneous attributions of the samples: susceptible and resistant. It was readily observed that these results were consistent with those determined by standard method, the rating scale. Remarkably, considering the time-frame required by the

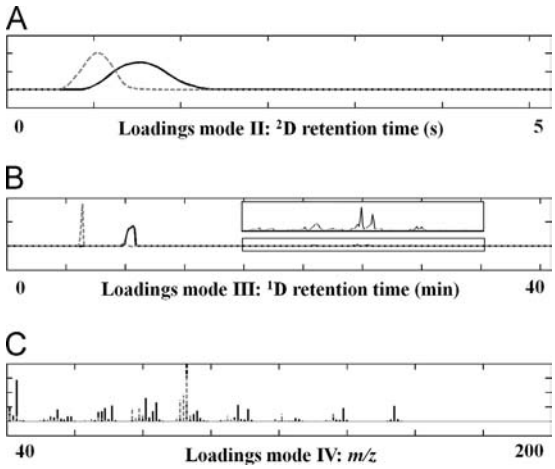

Fig. 4. Loadings of two-component PARAFAC modeling: (A) ^{2}D chromatographic profile (Mode II), (B) ^{1}D chromatographic profile (Mode III) and (C) mass spectra (Mode IV). The loadings of Factor 1 and 2 are illustrated by a black continuous line (–) and a gray dashed line (–) respectively. In (B) is also shown an expansion of the loadings graph of Factor 1 (Mode III).

Table 1
Tentative identification of the analytes commonly found in the leafs sampled from hybrids from *Eucalyptus grandis* and *Eucalyptus urophylla* by HS-SPME and GC × GC-qMS.

Compound name	Formula	Similarity	LTPRI exp.	LTPRI lit.	CAS
α-Pinene[a]	$C_{10}H_{16}$	96	946	948	80-56-8
Myrcene	$C_{10}H_{16}$	92	996	991	123-35-3
3-Hexen-1-ol	$C_6H_{12}O$	95	1010	1008	928-96-1
Eucalyptol[a,b]	$C_{10}H_{18}O$	92	1036	1032	470-82-6
γ-Terpinene	$C_{10}H_{16}$	91	1064	1058	99-85-4
Terpinolene	$C_{10}H_{16}$	94	1091	1086	586-62-9
Linalool[a]	$C_{10}H_{18}O$	94	1104	1101	78-70-6
Fenchone	$C_{10}H_{18}O$	91	1123	1123	1195-79-5
(Z)-neo-allo-ocimene	$C_{10}H_{16}$	92	1130	1128	7216-56-0
α-Campholenal	$C_{10}H_{16}O$	90	1129	1126	4501-58-0
(E)-Pinocarveol	$C_{10}H_{16}O$	89	1144	1141	547-61-5
Borneol	$C_{10}H_{18}O$	88	1175	1173	507-70-0
Terpinen-4-ol	$C_{10}H_{18}O$	91	1184	1180	562-74-3
Neral	$C_{10}H_{16}O$	85	1239	1238	5392-40-5
β-Phenethyl acetate	$C_{10}H_{12}O_2$	89	1254	1259	103-45-7
Bornyl acetate	$C_{12}H_{20}O_2$	92	1283	1285	76-49-3
Benzyl isobutyrate	$C_{11}H_{14}O_2$	94	1295	1294	103-28-6
Geranyl formate	$C_{11}H_{18}O_2$	92	1297	1300	105-86-2
α-Cubebene	$C_{15}H_{24}$	97	1344	1344	17699-14-8
α-Terpinyl acetate[b]	$C_{12}H_{20}O_2$	93	1350	1349	80-26-2
Geranyl acetate	$C_{12}H_{20}O_2$	93	1378	1380	105-87-3
β-Elemene	$C_{15}H_{24}$	91	1387	1390	338880-83-0
α-Gurjunene	$C_{15}H_{24}$	88	1404	1406	489-40-7
chenopodene	$C_{15}H_{24}$	86	1418	1422	156372-03-1
α-Humulene	$C_{15}H_{24}$	88	1454	1454	6753-98-6
α-Farnesene	$C_{15}H_{24}$	87	1456	1458	502-61-4
δ-Cadinene	$C_{15}H_{24}$	89	1467	1469	483-76-1
Viridiflorene	$C_{15}H_{24}$	91	1487	1491	21747-46-6
Bicyclogermacrene	$C_{15}H_{24}$	92	1491	1497	24703-35-3
Geranyl isobutyrate	$C_{14}H_{24}O_2$	93	1505	1507	2345-26-8

[a] Confirmation with analytical standards.
[b] Assigned as resistance biomarkers.

conventional method, rating scale, the current method is potentially interesting for the selection of resistant clones because it can be performed in just a few hours after sample collection.

4. Conclusions

Frequently, the inspection of the metabolome in plants is a particularly challenging task, because the measured chromatographic profile can be easily influenced by many other sources that can cause stress to the plant. The combination of SPME and GC × GC-qMS combined with multivariate data analysis, such as PARAFAC and Fisher ratio variable selection, as shown here, can be successfully employed for the determination of resistance biomarkers in *Eucalyptus* hybrids against rust disease. The proposed method allowed the recognition of BVOC patterns of several clones of *E. grandis* × *E. urophylla*, which, ultimately, lead to the differentiation of resistant and susceptible samples. A major contributor to these good results was the proper selection of relevant regions of the four-way raw data tensor, which was made by Fisher ratio analysis. Thus, the combination of a pre-selection strategy such as Fisher ratio analysis prior to multivariate modeling, through PARAFAC or other similar chemometric tool, can be a powerful alternative to mine metabolomic information from GC × GC–MS data.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.08.033.

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