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# Substantial Equivalence of Transgenic Lemon Fruit Showing Postharvest Fungal Pathogens Resistance

Vera Muccilli,[†,∇] Alessandro Vitale,[‡,∇] Ling Sheng,[§] Alessandra Gentile,[‡,§,*] Nunzio Cardullo,[†] Corrado Tringali,[†] Cinzia Oliveri,[‡] Rosa La Rosa,[‡] Mario Di Guardo,[‡] Stefano La Malfa,[‡] Ziniu Deng,[§] Gaetano Distefano[‡]

[†] Department of Chemical Sciences, University of Catania, 95125-Catania, Italy

[‡] Department of Agriculture, Food and Environment, University of Catania, 95123-Catania, Italy

[§] College of Horticulture and Landscape, Hunan Agricultural University, 410128-Changsha, China

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ABSTRACT

The development of GM crops speeds up the obtainment of novel varieties with improved agronomic characteristics. However, risk evaluation of the use of GMs is mandatory before their release in the market. In this paper, an untargeted and comprehensive NMR-based metabolomic study was carried out on peel and flesh of a transgenic lemon clone (E23) expressing chit42 gene and exhibiting an increased tolerance to some pathogenic fungi, and on its wild type. Results highlighted a substantial equivalence of the metabolomics profile of the transgenic clone compared to the wild type. In addition, an enhanced response of the E23 clone towards fungal pathogens affecting post-harvest management in lemon was evidenced. These results confirm the potential of genetic engineering for the punctual modification of specific agronomic traits without altering the whole pattern of metabolites and open new perspectives for a more sustainable and effective management of specific postharvest diseases in citrus.

KEYWORDS: *chitinase gene (chit42), Citrus, NMR analysis, PCA, Botrytis cinerea, Penicillium italicum, Trichoderma sp.*

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28 **INTRODUCTION**

29 For its wide spectrum of application and cost-effectiveness, genetic engineering for plant disease
30 resistance has become popular for the set-up of novel and superior cultivars. Valuable example of
31 genetically modified (GM) organisms showing resistance/tolerance to pathogen(s) can be found in
32 nearly every cultivated species. In many cases, resistance/tolerance against fungal pathogens in GM
33 crops has been achieved through the use of a wide spectra of cell wall degrading enzymes
34 (chitinase, glucanase, etc.).[1]

35 Among these, endo-chitinases genes such as *chit*42 and *chit*33 from the mycoparasitic
36 *Trichoderma harzianum* were successfully transferred into several horticulture crops to increase
37 tolerance/resistance against a wide range of plant pathogenic fungi.[2,3]

38 Even though no transformed Citrus species have been so far released in the market, genetic
39 engineering has been widely used for rootstocks and varieties breeding.[4,5] In lemon, GM clone
40 (E23) of 'Femminello siracusano' expressing the *chit*42 gene was constituted; these plants showed
41 an increased *in vitro* and *in vivo* tolerance to several pathogenic fungi.[6] In addition, leaf tissue of
42 E23 in presence of artificial infections of gray mold, revealed a significant reduction of disease
43 incidence compared to the wild type, showing a proof of concept of the direct correlation between
44 resistance and transgene expression.[7] Fruit loss due to postharvest decay has direct repercussion on
45 the marketability of the fresh and transformed product. In Citrus, many causal agents belonging to
46 *Penicillium, Geotrichum, Colletotrichum, Alternaria, Diaporthe* and *Botrytis* may rapidly colonize
47 fruit inducing modification in color, shape and consistency leading eventually to fruit rot.[8,9]
48 Moreover, some fungi do not show a specific timing for the infection, being these pathogens able to
49 damage fruits both prior the harvest and during postharvest storage, handling and packaging
50 operations. To control fungal infections, citrus growers are forced to a widespread use of chemicals,
51 thus implying a high risk of fungicide-resistant strains occurrence.[10-12]

52 Preliminary data on lemon fruits collected from the transgenic line E23 exhibited a significant
53 delay on postharvest decay symptoms appearance.[13]

54 GM plants showing increased tolerance/resistance to pathogens provide a product requiring a
55 lower amount of chemicals to control pathogens infection. However, the use of GM plants is
56 hampered by a general adversion against the adoption of biotechnology in agro-food sector by large
57 sectors of the public opinion. In turn this also affects political choices on the acceptance of GM
58 crops and legislation rules concerning their use. To this extent, the ascertainment of the substantial
59 equivalence (SE) principle between a GM variety and its wild type counterpart is of pivotal
60 interest.[14]

61 The SE is based on the assumption that a comparison between a non-GM and a GM crop should
62 lead to any significant difference on key nutrients and toxicants.[15,16]

63 Several strategies have been developed to identify compositional changes in GM food crops, to
64 this extent metabolomics is considered one of the most effective approach.[17] Among the different
65 analytical platforms used for investigating the metabolite profiles, the Nuclear Magnetic Resonance
66 (NMR) technique provides qualitative and quantitative data for many metabolites and it is often
67 indicated as the 'truly universal detector'.[18] NMR analysis was successfully used for comparative
68 metabolomics studies for several GM and non-GM food crops.[16]

69 The present study is aimed (i) to establish whether there is a SE between the fruits of E23 lemon
70 and its non-GM counterpart trough an untargeted and comprehensive metabolomic approach and
71 (ii) to evaluate the response of the GM and not-GM fruits affected by either blue or gray decays,
72 both fungal diseases causing severe fruit loss during post-harvest storage.

73

## 74 MATERIALS AND METHODS

75 **Plant Material.** Six adult plants of transgenic lemon clone E23 and of the wild type (WT)
76 (*Citrus limon* L. Burm cv 'Femminello siracusano') were grown in a confined greenhouse under
77 standard conditions at the National Center for Citrus Improvement, Changsha; Horticulture and
78 Landscape College Hunan Provincial Key Laboratory of Crop Germplasm Innovation and
79 Utilization, Hunan Agricultural University. Leaf tissue for protein extraction was obtained from

80 fully expanded leaves of spring flush randomly picked from three plants of each clone. Fruits for 81 protein expression assays, NMR and pathogenic analysis were harvested at full ripening stage and 82 stored at 20 °C and 90% relative humidity (RH) for 24 h.

83 ***Trichoderma harzianum* Chitinase Synthesis in Transgenic Lemon Fruits by SDS-PAGE.** 84 Extracellular proteins were extracted from the leaves and fruit peel of transgenic lemon E23 and the 85 wild type. SDS-Prestained protein ladder (Bio-Rad, Hercules, CA, USA) was used as molecular 86 weight marker. About 10 μg of extracellular foliar and peel proteins were used for sodium dodecyl 87 sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Gentile et al. (1993)[19] 88 and the gel was stained with Coomassie Brilliant Blue.

89 **NMR Samples Preparation.** NMR spectroscopy was performed on extracts obtained from 90 freeze-dried flavedo and albedo (peel; PE) and flesh (FL) samples. Sample preparation was 91 performed according to Kim et al. (2010).[20] Samples were treated with liquid nitrogen and ground 92 using a pestle and a mortar. The extraction was performed on 250 mg of peel or flesh treated with 93 0.75 ml of $CD_3OD$ and 0.75 ml of $KH_2PO_4$ buffer in $D_2O$ (pH 6.0) containing 0.1% (wt/wt) TSP-$d_4$. 94 Samples were shaken by Vortex for 1 min at room temperature and subsequently sonicated at 180 95 W for 20 min. Crude extracts were centrifuged at room temperature for 15 min using a microtube 96 centrifuge (9,000 g, in order to obtain a clear supernatant). Supernatants, containing the polar 97 metabolites from the crude extracts, were transferred to a 1.5 ml Eppendorf tube and stored for less 98 than 48h at 0-4 °C. The supernatant samples (each 800 μl) were transferred to 5 mm NMR tube and 99 subjected to NMR analyses. Each biological sample (three for each tissue of both the transgenic 100 clone E23 and the WT fruits) was independently analyzed with three technical replicates.

101 **NMR Analysis and Data Processing.** NMR spectra were run on a Varian Unity Inova 102 spectrometer (Milan, Italy) operating at 499.86 ($^1H$) and 125.70 MHz ($^{13}C$) and equipped with a 103 gradient-enhanced, reverse-detection probe. Chemical shifts (δ) were recorded in parts per million 104 (ppm).

105 $^{1}H$ NMR spectra were recorded setting spectral width at 6009 Hz, a pulse angle of 60°, a
106 relaxation delay of 5 s and a total of 128 transient. Acquired spectra were manually phased followed
107 by baseline correction and calibration performed by setting TSP-$d_4$ peak at 0.00 ppm. Water pre-sat
108 was applied during 2 relaxation delay and 8 transients.

109 $^{13}C$ NMR spectra were recorded setting a total of 12000 transients, and calibration was
110 performed by setting $^{13}C$ $CD_3OD$ peak at 49.00 ppm.

111 Two-dimensional NMR experiments (2D-NMR) were conducted to support compounds
112 identification. The g-COSY spectra were recorded with the gradient selected sequences with 256
113 transients and 951 data points covering 6345 × 6345 Hz, recorded with 8 scans per each increment.

114 The g-HSQCAD experiments were performed with matched adiabatic sweeps for coherence
115 transfer, corresponding to a central $^{13}C-^{1}H$ J-value of 146 Hz, with 256 scans for each increment
116 with a relaxation delay of 2 s. Spectral width was set at 25141 × 6009 Hz.

117 The g-HMBCAD experiments were optimized for a long-range $^{13}C-^{1}H$ coupling constant of 8.0
118 Hz and recorded with 16 scans of each increment and a total of 400 transient, setting a spectral
119 width of 30166 × 6009 Hz.

120 The J-resolved pulse sequence (JRES) spectra were acquired with 200 transient and 8 scans per
121 each increment. Spectral width was set at 64 x 6009 Hz.

122 All NMR experiments were performed using software supplied by the manufacturer and
123 acquired at constant temperature (300 K).

124 **NMR Data Processing and PCA Statistical Analysis.** NMR spectra were aligned with Origin
125 2019 (v.9.6) and converted in ASCII files. The chemometric analysis was performed constructing
126 buckets via AMIX software (version 3.8, Bruker) using a single rectangular shape with a width of
127 0.03 ppm, an integration mode of the sum of the intensities scaled to the total intensity on the
128 samples, and Pareto scaling of the variables. Statistical analyses were performed using the
129 integration values from the signals with high loadings in PCA relative to the TSP-$d_4$ signal in the $^{1}H$

130 NMR spectra. The integration values were derived using the 'prcomp' function of the stat R
131 package and results were displayed using the 'factoextra' R package.[21]
132 The extent of the changes for discriminant metabolites was calculated as relative areas of non-
133 overlapping signals. Student's t-test was applied to each metabolite to assess the differences
134 between the means of E23 and WT fruits. A p value threshold of 0.05 was used to indicate
135 statistical significance.
136 **Pathogens**. *Penicillium italicum* was originally isolated from lemon fruits with blue mold
137 symptoms while *Botrytis cinerea* was previously recovered from infected lemon flowers (blossom
138 blight) directly from gray sporulating tissues. Both pathogen colonies were subcultured in 9-cm
139 Petri dishes containing potato dextrose agar (PDA, Oxoid, Basingstoke, UK) and incubated in a
140 growth chamber at 25 °C and two monosporic *P. italicum* and *B. cinerea* strains were used
141 respectively for artificial inoculations.
142 ***In Vivo* Evaluation of Transgenic Line on Fungal Decays.** Two experiments were carried out
143 in a growth cabinet (Qingdao Tlead International Co., Ltd.Shandong, China) to evaluate the
144 performance of E23 clone and WT in reducing artificial decays caused by *B. cinerea* (Experiment I)
145 and *P. italicum* (Experiment II). The development of symptoms was evaluated both in absence of
146 fungicide treatments and through the application of the imazalil fungicide (44.6% a.i., Fungazil™
147 500 EC at label rate of 2 mL/L).
148 In both experiments, treatments were arranged in a randomized complete block design with three
149 replicates (each formed by 9 lemon fruits) and consisted of (i) untreated and inoculated WT, (ii)
150 untreated and inoculated E23, (iii) fungicide-treated and inoculated WT and (iv) fungicide-treated
151 and inoculated E23. Prior to artificial inoculation, the fruit surfaces were disinfected using a 2%
152 sodium hypochlorite (NaOCl) solution for 2 min. Disinfected fruits were rinsed twice with sterile
153 distilled water, dried and artificially wounded at 4 points in the equatorial fruit region. Two hours
154 prior to inoculation, both WT and E23 lemon fruits (iii and iv) were sprayed with a fungicide
155 solution containing imazalil at label rate for 25 sec whereas corresponding untreated fruits (i and ii)

156 were treated with tap water. Following treatment, an amount of 10 µl of conidial suspension (about
157 $1 \times 10^6$ conidia/ml either for *B. cinerea* or *P. italicum*), obtained from 7 to 10-day-old monosporic
158 fungal cultures were applied at each wound, while control fruits were treated with the same amount
159 of SDW.[13] After the inoculation, fruits were placed on plastic packaging trays and incubated at 20
160 °C and 90-95% RH (to mimic standard packaging conditions) for 20 days. Each experiment was
161 performed twice. During the incubation period, the development of typical symptoms was assessed
162 at 0, 4, 7 and 15 days after inoculation (DAI). Disease incidence was expressed as the percentage of
163 inoculation wounds (%) showing gray (*B. cinerea*) and blue (*P. italicum*) decay symptoms whereas
164 disease severity was referred to the average lesion diameter (mm).

165 Data obtained from both experiments were subjected to analysis of variance (ANOVA) using
166 Statistica 10 software package (Statsoft Inc., Tulsa, OK) with factorial treatment structure and
167 interactions. Initial analyses of decay incidence and severity were conducted by calculating F and
168 the correspondent p values associated with the main source of variation (treatment, evaluation time
169 and targeted fungus) and whether there are significant interactions among factors. The *post-hoc*
170 comparisons among treatments within each time point were achieved by means of Fisher's least
171 significant difference test at $\alpha = 0.05$ and related to the significant differences between the
172 incidence and severity means. All data were presented as the mean ± standard error of the means
173 (SEM).

174

175 **RESULTS AND DISCUSSION**

176 ***Trichoderma harzianum* chitinase synthesis in transgenic lemon fruits.** Extracellular proteins
177 were extracted from the leaves and fruit peel of transgenic lemon clone E23 and WT to verify the
178 synthesis of the protein *chit*22. Gel electrophoresis bands at 42 KD corresponding to protein *chit*22
179 were clearly identified in fruit peel as well as in leaves of transgenic clones compared to control
180 confirming the results previously reported from Gentile and colleagues (2007)[6] about transgenic
181 protein expression in leaves (Figure S1).

182 **Identification of Metabolites in PE and FL Extracts by 1D and 2D NMR.** The metabolic
183 profiles of transgenic lemon clone E23 and WT peel and flesh were determined by $^{1}H$ and $^{13}C$ NMR
184 analysis. Representative $^{1}H$ NMR spectra of clone E23 and WT tissue extracts are reported in
185 Figure 1. The corresponding $^{13}C$ NMR spectra are reported in Supporting Information (Figures S2,
186 S7, S12 and S13). The identification of main metabolites was normally based on comparison of $^{1}H$
187 and $^{13}C$ chemical shifts with literature data; when useful, these assignments were also corroborated
188 by a careful analysis of two-dimensional NMR spectra including JRES, gCOSY, gHSQC and
189 gHMBC experiments. The assignments are summarized in Table 1. Representative 2D spectra are
190 reported in Supporting Information (Figures S3-S6, S8-S11).

191 Widespread metabolites such as carbohydrates, amino acids and organic acids were detected;
192 overall, 34 compounds were identified among which 20 were common to both tissues.

193 In Figure 2 the $^{1}H$ NMR assignments for a representative WTPE extract are reported. The NMR
194 spectra of peel extracts showed three signals in the region of carbohydrate anomeric protons easily
195 assigned to β-glucose (**1**) (4.58 ppm, 1H, d, J = 7.9 Hz, H-1), α-glucose (**2**) (5.18 ppm, 1H, d, J =
196 3.7 Hz, H-1) and α-glucose moiety of sucrose (**3**) (5.40, 1H, d, J = 3.8 Hz, H-1 G). The
197 corresponding carbon resonances were found at 97.2 (1-CH of **1**), 93.3 (1-CH of **2**) and 93.2 ppm
198 (1-CH of glucose of **3**) in the $^{13}C$ NMR spectrum. Furthermore, several intense overlapping signals
199 between 3.0 and 4.0 ppm in the $^{1}H$ NMR spectrum and between 80 and 60 ppm in the $^{13}C$ NMR
200 spectrum attributable to CH and $CH_2$ signals of these sugars were observed. Usually, the severe
201 overlapped carbohydrate signals are not useful for identification; nevertheless, in this work, these
202 resonances were assigned through careful analysis of 2D NMR spectra, namely gCOSY, gHSQC
203 and gHMBC assisted by comparison with literature data.[22–24]

204 Low-intensity signals were recorded for the following minor constituents, namely the amino
205 acids alanine (**6**), valine (**7**), proline (**8**), aspartic acid (**9**), asparagine (**10**), glutamic acid (**11**),
206 glutamine (**12**), lysine (**13**), threonine (**14**), γ-aminobutyric acid (**15**) and stachydrine (**16**); the
207 organic acids formic (**17**), and malic (**18**) acids; the isoprenoids α-pinene (**21**), β-pinene (**22**),

208 limonene (**23**), γ-terpinene (**24**), geranial (**25**) and neral (**26**); choline (**30**), scyllo-inositol (**31**) and
209 myo-inositol (**32**). 2D NMR analyses corroborated most of these identifications. In addition,
210 through comparison of literature data[25] and COSY correlation (6.48 and 7.58 ppm for **28**), two
211 groups of protons were tentatively assigned to distinct 3-furyl moieties of two unidentified
212 limonoids (**27** and **28**), members of these well-known nortriterpenoids are commonly found in
213 *Citrus* spp.

214 The same analysis was performed on the flesh extract of both E23 and WT clones. In Figure 3
215 the $^{1}$H NMR assignments for a representative WTFL extract are reported. The $^{1}$H NMR spectra
216 showed two very prominent doublets at 2.81 (2H, d, $J$ = 15.8 Hz) and 2.95 ppm (2H, d, $J$ = 15.8
217 Hz) corresponding to the diastereotopic methylene protons of citric acid (**19**); this is a well-known,
218 abundant constituent of lemon flesh, and its identification was corroborated by the intense carbon
219 signal in the $^{13}$C NMR spectrum at 44.2 ppm ($CH_2$), correlated with the diasterotopic protons in the
220 HSQC spectrum. As a confirmation, key HMBC correlations for these signals were observed with
221 other carbons resonating at 74.2 (2-COH), 174.3 (4-COOH) and 177.7 ppm (1-COOH). Some of the
222 above-cited widespread metabolites, namely carbohydrates **1** – **3,** amino acids **6** – **16** and polyols **31**
223 and **32** were identified in FL as well (Table 1). The signals of aspartic acid (**9**), asparagine (**10**) and
224 glutamine (**12**) were shifted downfield in $^{1}$H NMR spectra, and upfield in $^{13}$C NMR spectra, with
225 respect to those observed for the same compounds in PE extracts; this is due to the lower pH caused
226 by the high amount of citric acid, a behavior previously reported.[25,26] Two further minor
227 constituents were identified in FL extracts: lactic acid (**20**) and trigonelline (**33**). Lactic acid was
228 identified on the basis of the $^{1}$H NMR doublet signal at 1.40 ppm (d, $J$ = 6.9 Hz, 3-$CH_3$),
229 attributable to the methyl group; this showed a HSQC correlation with the $^{13}$C NMR methyl
230 resonance at 26.2 ppm; the assignment respectively of the methine carbon at 65.6 ppm (2-CHOH)
231 and the carboxylic acid function at 176.0 ppm (1-COOH) were based on HMBC correlations with
232 the methyl signal at 1.40 ppm. The alkaloid **33** showed well defined proton signals for the pyridine
233 ring at 8.11 (1H, bt, H-5), 8.86 (1H, d, $J$ = 6.8, Hz, H-6), 8.90 (1H, d, $J$ = 7.4 Hz, H-4) and 9.19

234 ppm (1H, s, H-2); in addition, carbon resonances, confirmed through HMBC correlations, were
235 observed at 155.8 (2-CH), 150.2 (6-CH), 140.6 (4-CH) and 127.5 ppm (5-CH).
236 It is worth noting that eleven metabolites (**4**, **5**, **21** – **28** and **34**) are present exclusively in PE and
237 three (**19**, **20** and **33**) exclusively in FL; these data are in agreement with previous literature data.[24]
238 **Multivariate Analysis by PCA in Peel and Flesh of Lemon Fruits**. An unsupervised approach
239 was applied to highlight the differences in metabolic profile of E23 and WT lemon fruit tissues
240 according to the data obtained from the $^{1}H$ NMR spectra analysis. Results were visualized as score
241 plots (Figures 4A and 4C), in which each point represented a technical replicate, and loading plots
242 (Figures 4B and 4D), showing buckets responsible for the discrimination observed in the
243 corresponding score plot. A unit variance scaling method was applied for PCA of both clone E23
244 and WT lemon fruits to give similar weights to major and minor metabolites.
245 In particular, the model obtained for PE showed that the first two principal components (Dim1
246 and Dim2) explained 98.1% of the total variance (77.5% and 20.6% respectively) (Figure 4A).
247 The inspection of the score plot depicted in Figure 4A showed the presence of three separate
248 clusters: one, grouping all E23 samples, was characterized by both Dim1 and Dim2 negative values
249 while the other two clusters grouped one (WT_BR1) and two fruits (WT_BR2 and WT_BR3) of the
250 WT respectively. In this analysis WT lemons showed a much greater metabolic variability
251 compared to E23, implying that neither Dim1 (Welch Two Sample t-test, $t = -2.62$, p value = 0.11)
252 or Dim2 (Welch Two Sample t-test, $t = -1.51$, p value = 0.23) could efficiently discriminate
253 between WT and E23. The inspection of the loading plot of the first two dimensions provided
254 insights on the buckets determining the highest variation among the sample in analysis: α-glucose
255 (**2**) and sucrose (**3**).
256 The multivariate analysis of the FL reported in Figure 4C showed a similar pattern with respect
257 to what previously observed for PE: the first two dimensions explained a cumulative variability
258 equal to 94.1% (74.3% for Dim1 and 19.8% for Dim2). WT and E23 replicates showed Dim1
259 positive and negative values respectively; Dim1 allowed a separation between the two groups

260 (Welch Two Sample t-test, t = -5.41, p value = 0.006) while Dim2 was not significantly associated
261 to differences between the two groups (Welch Two Sample t-test, t = 1.12, p value = 0.327) but it
262 provided information on the within-group variability. The inspection of loading plot highlighted
263 that the meaningful components are related to α-glucose (**2**), sucrose (**3**) and citric acid (**19**) buckets
264 (Figure 4D).

265 Figure 5 shows the relative proportion of the metabolites responsible for the separation of WT
266 and E23 samples in the loading plots. The quantitative analysis of sucrose (**2**), α-glucose (**3**) and
267 citric acid (**19**) content was performed by $^{1}$H NMR spectroscopy using TSP-$d_4$ as internal standard
268 and measuring peak area of selected, non-overlapping signals.

269 The data in Figures 5A and 5B show that WTPE had a lower α-glucose content (p value =
270 0.0008) and a higher sucrose content (p value = 0.00093) compared to E23. Analogously, the data
271 reported in Figures 5C, 5D and 5E showed a higher content of citric acid in the E23FL (p value =
272 0.0026) and a lower content of both α-glucose (p value = 0.002) and sucrose (p value 0.0025)
273 compared to WT.

274 Taking into account that WT and E23 were harvested at the same day and fruits did not show
275 visible defect, the detected differences in α-glucose and sucrose content may be imputable to
276 environmental factors such as light exposition that could have affected fruit ripening process at one
277 specific stage. In fact, the different concentration of these metabolites is associated to maturation
278 and to post-harvest stress.[27] It is known that the organoleptic properties of fruits can be related to
279 the abundance of sugars and organic acids, which are responsible for characteristic flavor of a
280 particular fruit and can be also used as indicators of fruit maturity.[28] The slight changes in the
281 abundance of few primary metabolites (α-glucose, sucrose and citric acid) among WT and E23are
282 not significant to create a health risk.

283 ***In Vivo* Effects of Transgenic Line on Fruit Decays**. *P. italicum*, is the causal agent of blue
284 mold. This fungus represents a serious threat affecting lemon fruit quality during postharvest
285 inducing firstly a soft water-soaked area on the peel, followed by the development of a circular

286 colony of white mold that finally turns in blue color. *B. cinerea* (teleomorph *Botryotinia*
287 *fuckeliana*), causes a wide spectrum of symptoms in lemon ranging from flower fall and rind fruit
288 distortion to destructive pre- and postharvest fruit rot (gray mold).[29-31]

289 Fruits treated with fungicide developed neither blue nor gray mold up to 7 days after inoculation
290 (DAI). Moreover, fungicide treated E23 fruits did not show symptoms referable to gray mold decay
291 up to last evaluation (15 DAI) while they were slightly affected by blue mold at the same time
292 evaluation. Otherwise, untreated WT developed decay amounts for both fungal pathogens starting
293 from the first evaluation at 4 DAI (Figure S14).

294 Since all first (treatment × evaluation time; treatment × target; target × evaluation time) and
295 second order (treatment × evaluation time × target) interactions among factors were always
296 significant on both decay incidence and severity parameters (Table 2), the results for each
297 experiment were presented individually (Figure 6).

298 The decay progressions for both fungal pathogens along time showed always an increasing trend
299 but with dissimilar patterns. The incidence and severity values for both fungal decays were always
300 significantly higher in untreated WT in comparison to the remaining treatments (Figure 6).

301 In experiment I (*B. cinerea*), decay amounts were relatively low up 7 DAI and they reached
302 maximum levels at 15 DAI (incidence of 100% and maximum severity in untreated WT). At this
303 evaluation, both disease parameters recorded in each treatment differed significantly among them.
304 The most effective in reducing botrytis decay was the fungicide-treated E23 (iv) immediately
305 followed by untreated E23 (ii), that was at this time better than fungicide-treated WT (iii).

306 Otherwise, blue mold decay levels detected in experiment II (*P. italicum*) were averagely higher
307 than those observed for gray mold in the previous experiment. In detail, high decay amounts were
308 already observed starting from 4 DAI evaluation and increased at the following evaluations. As in
309 the previous experiment, treatments differed significantly among them in reducing decay amount
310 and the fungicide-treated E23 revealed the most effective in reducing strongly blue mold decay.

311 Although to a lesser and increasing extent, WT + fungicide (iii) and untreated E23 (ii) were also
312 effective in reducing *P. italicum* infections.
313 Comprehensively, it is noteworthy that untreated E23 showed better performances in reducing *B.*
314 *cinerea* decay than WT + fungicide whereas this latter combination revealed more effective than
315 E23 alone in reducing *P. italicum* decay.
316 **A Sustainable Approach to Contain Post-Harvest Loss.** Lemon management requires a large
317 number of fungicide applications both in the field and during postharvest to reduce both fruit loss
318 and to maintain an acceptable fruit quality level. For such reason, the setting-up of more sustainable
319 strategies for the design of novel varieties showing increased resistance/tolerance is a fundamental
320 step. In lemon, breeding approaches are hampered by the long juvenile phase and the
321 heterozygosity of the genome making both traditional breeding and marker-assisted selection a long
322 and expensive process. The unraveling of the genetic basis underlying resistance to a pathogen can
323 allow the transfer of the causative gene (and its resistance function) from one genotype to another
324 through biotechnological tools such as the transgenic approach. The development of GM varieties
325 speed up the breeding process and, in the case of resistant GM plants, reduce the phytosanitary risks
326 and the negative repercussion of fungicide to other plants and human health. However, an
327 evaluation of all the aspects related to the risk valuation of the use of GM varieties must be carried
328 out before their release also to fit the request of safety assessment coming from consumers.
329 In this context, an NMR-based metabolomic study was carried out to evaluate the substantial
330 equivalence of polar WT and clone E23 lemon fruits extracts. NMR profiling provided a rapid,
331 unbiased and reliable method for the assessment of possible differences in all the extracted
332 metabolites. The analysis focused onto the metabolomic profiles of peel and flesh of fruits from a
333 transgenic lemon clone and its WT. The results highlighted the occurrence of a few metabolites
334 showing a different concentration in the two samples in analysis. Such differences were referred as
335 the relative concentration of α-glucose (**2**) and sucrose (**3**; both for PE and FL) and citric acid (**19**,
336 only for FL). To the best of our knowledge, these metabolites are not involved in down or upstream

337 biochemical pathways involving chitinase, but such differences are likely due to the ripening stage
338 of the fruits at harvest, or environmental conditions such as light exposure or the occurrence of
339 stress.

340 The present study also confirmed the different behavior of the GM clone and sheds light on the
341 different responses of the transgenic clone (E23) to two of the most widespread pathogens affecting
342 post-harvest management in lemon. Our findings suggested that transgenic clone was strongly
343 effective against *B. cinerea* decay as reported in our paper, where transgenic line E23 provided
344 alone higher efficacy than WT treated with fungicide. Comprehensively, E23 line showed slightly
345 lower performances against to *P. italicum* decay compared to that observed for *B. cinerea*. For
346 example, WT treated with fungicide showed better effectiveness against blue mold compared to
347 E23 alone. Nevertheless, the lowest decay levels were always detected for E23 treated with
348 fungicide. Moreover, transgenic clone can optimize the possibility for the citrus growers to limit
349 losses due to specific postharvest diseases as well as improving the consumers' product safety. The
350 latter aspect appears of particular importance also in consideration of the wide use of the lemon skin
351 for different purposes in human consumption, essential oil extraction and livestock feeding. The
352 aspects of biosafety of modified horticultural crops has gained popularity for the importance paid to
353 the absence of pesticide residues in food matrices.[32] As suggested by our findings the perfect
354 success of these strategies depend also on target pathogen and further research should be performed
355 to confirm our findings against postharvest diseases.

356 Despite the long-time debated aspects on the adoption of GM line on large scale, this paper
357 provides evidence on the possibility to produce fruit with high quality standard minimizing (or even
358 avoiding) the use of chemicals and open new perspectives for a more sustainable and effective
359 management of specific postharvest diseases in citrus.

360

361 **ABBREVIATIONS USED**

362 BR biological replicate; DAI days after inoculation; Dim dimension; E23FL, E23 clone flesh;
363 E23PE, E23 clone peel; FL, flesh; gCOSY, gradient correlation spectroscopy; gHMBC, gradient
364 heteronuclear multiple-bond correlation; gHSQC, gradient heteronuclear single-quantum
365 coherence; GM, genetically modified; JRES, homonuclear J-resolved spectroscopy; NMR, nuclear
366 magnetic resonance; PCA, principal component analysis; PE, peel; TSP-$d_4$, 2,2,3,3-d4-3-
367 (trimethylsilyl)-propionic acid sodium salt; WT, wild-type; WTFL, wild type flesh; WTPE, wild-
368 type peel.
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371 **ASSOCIATED CONTENT**
372 ***S Supporting Information**$^{13}$C NMR and 2D NMR spectra of WT and E23 flesh and peel extracts.
373 **AUTHOR INFORMATION**
374 ∇These authors contributed equally to this work.
375 **Corresponding Authors**
376 *Mailing Address gentilea@unict.it. Tel:+39 095 4783430
377 **ORCID**
378 Name corresponding authors: 0000-0002-4135-5620
379
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473 **Figure and table captions**

474 **Figure 1.** Representative $^{1}$H NMR water-presaturated spectrum (500 MHz, $CD_3OD$:$D_2O$) of a
475 WTPE extract (A), a WTFL extract (B), a E23PE extract (C), a E23FL extract (D).

476 **Figure 2.** Partial $^{1}$H NMR water-presaturated spectrum (500 MHz, $CD_3OD$:$D_2O$ 1:1) of a WTPE
477 extract: A) region between 5.50 and 3.10 ppm; B) region between 3.05 and 0.60 ppm.

478 **Figure 3.** Partial $^{1}$H NMR water-presaturated spectrum (500 MHz, $CD_3OD$:$D_2O$ 1:1) of a WTFL
479 extract: A) region between 5.50 and 3.10 ppm; B) region between 3.15 and 0.85 ppm.

480 **Figure 5.** Relative ratio (obtained by integration from signal of the compounds in the 1HNMR
481 spectra normalized by TMSP-$d_4$ signal) of sucrose (A) and α-glucose (B) in the extracts from peel;
482 citric acid (C), α-glucose (D) and sucrose (E)in the extracts from flesh. Data are displayed as a
483 mean of relative ratio ± standard deviation ($P < 0.05$).

484 **Figure 6.** Time progression of gray mold (experiment I) and blue mold (experiment II) decay
485 amounts in different treated E23 and WT lemon fruit following artificial inoculation. Incidence was
486 expressed as a percentage of infected fruit while severity as average size (mm) of decayed sites.
487 DAI = days after inoculation.

488 [x] Averages of 3 replicates each formed by 9 fruit. Vertical bars represent the standard error of the
489 means (SEM).
490 [y] Values followed same letter within each evaluation time are not significantly different according
491 to Fisher's least significance differences test ($\alpha = 0.05$).
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513 **Table 1. ^{1}H and ^{13}C NMR Assignments of the Compounds Identified in the Peel (PE) and**
514 **Flesh (FL) extracts.**

| compound | $\delta$ [1]H | multiplicity ($J$ Hz) | $\delta$ [13]C | assignment | tissue |
|---|---|---|---|---|---|
| | | Carbohydrates | | | |
| β-glucose (**1**) | 4.58 | d ($J$ =7.9) | 97.2 | 1-CH | PE - FL |
| | 3.20 | dd ($J$ = 9.3, 7.9) | 75.4 | 2-CH | PE - FL |
| | 3.42 | dd ($J$ = 9.3, 4.4) | 77.2 | 3-CH | PE - FL |
| | 3.39 | m | 70.4 | 4-CH | PE - FL |
| | 3.47 | m | 77.1 | 5-CH | PE - FL |
| | 3.72 | dd ($J$ = 12.2, 4.8) | 62.0 | 6a-$CH_2$ | PE - FL |
| | 3.87 | dd ( $J$= 12.2, 2.0) | | 6b-$CH_2$ | PE - FL |
| α-glucose (**2**) | 5.18 | d ($J$ = 3.7) | 93.3 | 1-CH | PE - FL |
| | 3.45 | dd ($J$ = 3.7, 2.4) | 71.0 | 2-CH | PE - FL |
| | 3.68 | m | 74.1 | 3-CH | PE - FL |
| | 3.41 | m | | 4-CH | PE |
| | 3.83 | m | 72.3 | 5-CH | PE - FL |
| | 3.75 | m | 61.9 | 6a-$CH_2$ | PE - FL |
| | 3.77 | m | | 6b-$CH_2$ | |
| sucrose (**3**) | 5.40 | d ($J$ = 3.8) | 93.2 | 1-CH G | PE - FL |
| | 3.52 | dd ($J$ = 9.9, 3.8) | 72.8 | 2-CH G | PE - FL |
| | 3.76 | m | 73.5 | 3- CH G | PE - FL |
| | 3.45 | m | 70.0 | 4-CH G | PE - FL |
| | 3.82 | m | 73.4 | 5-CH G | PE - FL |
| | 3.78 | m | 61.4 | 6-$CH_2$ G | PE - FL |
| | 3.65 | s | 62.7 | 1'-$CH_2$ F | PE - FL |
| | | | 104.8 | 2' C F | PE - FL |
| | 4.17 | d ($J$ = 8.6) | 78.9 | 3'-CH F | PE - FL |
| | 4.01 | t ($J$ = 8.6) | 75.8 | 4'-CH F | PE - FL |
| | 3.86 | m | 82.7 | 5'-CH F | PE - FL |
| | 3.79 | d ($J$ = 4.9) | 63.7 | 6'-$CH_2$ F | PE - FL |
| α-galacturonic acid (**4**) | 5.35 | d ($J$ = 3.5) | 92.2 | 1-CH | PE |
| α-glucuronic acid (**5**) | 5.14 | d ($J$ = 3.5) | 94.3 | 1-CH | PE |
| | | Amino acids | | | |
| alanine (**6**) | 3.79 (3.94 FL) | bs | 51.0 | α-CH | PE - FL |
| | 1.48 (1.51 FL) | d ($J$ = 7.2) | 15.6 (16.7 FL) | β-$CH_3$ | PE - FL |
| valine (**7**) | 2.28 | m | | β-CH | PE - FL |
| | 1.03 | d ($J$ = 6.7) | 17.5 | γ-$CH_3$ | PE - FL |
| | 1.06 | d ($J$ = 7.0) | 18.5 | γ'-$CH_3$ | PE - FL |
| proline (**8**) | 4.12 | dd ($J$ = 7.0, 3.4) | 69.9 | α-CH | PE - FL |
| | 2.34 | m | 29.1 | β-$CH_2$ | PE - FL |
| | 2.08 | m | | β'-$CH_2$ | PE - FL |
| | 2.15 | | | γ-$CH_2$ | PE - FL |
| | 1.96 | | | γ'-$CH_2$ | PE - FL |
| | 3.42 | m | | δ-$CH_2$ | PE - FL |
| | 3.35 | m | | δ'-$CH_2$ | PE - FL |
| aspartic acid (**9**) | 4.33 (4.52 FL) | dd ($J$ = 7.7; 4.4) | 63.9 (68.6 FL) | α-CH | PE - FL |
| | 2.59 (2.76 FL) | dd ($J$ = 16.1; 7.7) | 39.7 (38.6 FL) | β-CH | PE - FL |
| | 2.79 (2.87 FL) | dd ($J$ = 16.2; 4.4) | | β'-CH | PE - FL |
| | | | 177.3 | COOH | FL |
| | | | 175.1 | COOH | FL |
| asparigine (**10**) | 3.97 (4.05 FL) | bdd | 50.76 | α-CH | PE - FL |
| | 2.85 (3.00 FL) | dd ($J$ = 16.4; 3.8) | 33.9 (33.7 FL) | ß-CH | PE - FL |
| | 2.95 (3.08 FL) | m | | ß'-CH | PE - FL |
| | | | 170.3 | CO-$NH_2$ | FL |

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|---|---|---|---|---|---|
| | | | 173.9 | COOH | FL |
| glutamic acid (**11**) | 2.09 | m | 28.1 | ß-$CH_2$ | PE - FL |
| | 2.35 | m | 35.9 | γ-$CH_2$ | PE - FL |
| glutamine (**12**) | 3.86 | m | 55.6 | α-CH | PE - FL |
| | 2.14 (2.17 FL) | m | 26.4 (24.8 FL) | β-$CH_2$ | PE - FL |
| | 2.45 (2.56 FL) | m | 33.3 (30.6 FL) | ɣ-$CH_2$ | PE - FL |
| lysine (**13**) | 3.74 | t ($J$ = 5.5) | | α-CH | PE - FL |
| | 1.75 | m | | γ-$CH_2$ | PE - FL |
| | 3.01 | t ($J$ = 7.6) | | ε-$CH_2$ | PE - FL |
| threonine (**14**) | 3.81 | m | 54.8 | α-CH | PE - FL |
| | 4.26 | m | 64.7 | β-CH | PE - FL |
| | 1.34 | d ($J$ = 6.6) | 19.6 | γ-$CH_3$ | PE - FL |
| γ-aminobutyric acid | 2.37 | t ($J$ = 7.2) | 31.1 | α-$CH_2$ | PE - FL |
| (GABA, **15**) | 1.90 | m | 24.7 | β-$CH_2$ | PE - FL |
| | 3.01 | t ($J$ = 7.2) | 39.7 | ɣ-$CH_2$ | PE - FL |
| stachydrine (**16**) | 3.30 | s | 52.6 | $CH_3$ | PE – FL |
| | 3.12 | s | 46.5 | $CH_3$ | PE - FL |
| | | | Organic acids | | |
| formic acid (**17**) | 8.43 | s | | | PE - FL |
| malic acid (**18**) | 4.33 | m | 72.1 | α-CH | PE - FL |
| | 2.56 | m | 39.2 | β-CH | PE - FL |
| | 2.76 | m | | β'-CH | PE - FL |
| citric acid (**19**) | 2.95 | d ($J$ = 15.8) H-3a | 44.2 | 3a-$CH_2$ | FL |
| | 2.81 | d ($J$ = 15.8) H-3b | | 3b-$CH_2$ | FL |
| | | | 174.3 | 4-*C*OOH | FL |
| | | | 177.7 | 1-*C*OOH | FL |
| | | | 74.2 | 2-*C*OH | FL |
| lactic acid (**20**) | 1.40 | d ($J$ = 6.9) | 26.2 | 3-$CH_3$ | FL |
| | 4.35 | m | 65.6 | 2-*C*HOH | FL |
| | | | 176.0 | 1-*C*OOH | FL |
| | | | Isoprenoids | | |
| α-pinene (**21**) | 0.81 | s | 18.7 | $CH_3$ | PE |
| | 1.13, 2.26 | | | CH-$CH_2$-CH | PE |
| β-pinene (**22**) | 4.49 | s | | $CH_2$= | PE |
| | 1.95 | m | | *CH*-$CH_2$ | PE |
| | 2.36 | m | | *CH*-C | PE |
| | 1.36, 2.29 | m | 25.8 | CH-$CH_2$-CH | PE |
| | 2.45, 2.14 | m | | $CH_2$-C= | PE |
| limonene (**23**) | 2.04 | m | 40.4 | CH | PE |
| | 1.59 | s | | $CH_3$-C=$CH_2$ | PE |
| | 1.66 | s | | $CH_3$-C=$CH_2$ | PE |
| γ- terpinene (**24**) | 0.95 | d ($J$ = 7.8) | 23.8 | $CH_3$-CH | PE |
| | 2.08 | m | 35.7 | CH | PE |
| geranial (**25**) | 9.87 | s | 189.0 | CHO | PE |
| | 2.01 | s | | $CH_3$-CH | PE |
| | 1.55 | s | | $CH_3$-C($CH_3$) | PE |
| | 1.66 | s | | $CH_3$-C($CH_3$) | PE |
| | 5.76 | s | | CHO-CH= | PE |
| neral (**26**) | 9.69 | s | 186.6 | CHO | PE |
| | 1.85 | s | | $CH_3$-CH | PE |
| | 1.53 | s | | $CH_3$-C($CH_3$) | PE |
| | 1.66 | s | | $CH_3$-C($CH_3$) | PE |
| | 5.70 | s | | CHO-CH= | PE |

| | | | | | |
|---|---|---|---|---|---|
| 3-furyl group (**27**) | 7.78 | s | | =CH-O | PE |
| | 7.51 | d | | CH=C*H*-O | PE |
| | 6.68 | d | | C*H*=CH-O | PE |
| 3-furyl group (**28**) | 7.58 | s | | =CH-O | PE |
| | 7.38 | d | | CH=C*H*-O | PE |
| | 6.48 | d | | C*H*=CH-O | PE |
| | | | Other compounds | | |
| ethanol (**29**) | 1.17 | t ($J$ = 6.9) | 16.4 | $CH_3$ | PE - FL |
| | 3.67 | m | | $CH_2$ | PE - FL |
| choline (**30**) | 3.21 | s | 54.8 | $CH_3$ | PE - FL |
| scyllo-inositol (**31**) | 3.30 (3.31 FL) | s | 73.9 | CH | PE - FL |
| myo-inositol (**32**) | 3.6 | t ($J$ = 9.4) | 72.3 | 4/6-CH | PE – FL |
| | 3.24 | t ($J$ = 9.4) | 74.2 | 5-CH | PE - FL |
| trigonelline (**33**) | 9.19 | s | 155.8 | 2-CH | FL |
| | 8.90 | d ($J$ = 7.4) | 140.6 | 4-CH | FL |
| | 8.86 | d ($J$ = 6.8) | 150.2 | 6-CH | FL |
| | 8.11 | bt | 127.5 | 5-CH | FL |
| | 4.42 | s | | $N^+CH_3$ | FL |
| fatty acid derivatives(**34**) | 0.91 | bs | 14.5 | $CH_3$ | PE |
| | 2.03 | m | 36.9 | $CH_2$-CH=CH | PE |
| | 2.75 | m | | CH=CH-$CH_2$ | PE |

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518 **Table 2. ANOVA effects of factors and their interactions on fruit decays caused by *Botrytis***
519 ***cinerea* and *Penicillium italicum* over time (0, 4, 7, 15 days after inoculation) in two**
520 **experiments**.

| | | *decay incidence* | | *decay severity* | |
|---|---|---|---|---|---|
| **source of variation** | ***df*** | ***F*** | ***P value*** | ***F*** | ***P value*** |
| treatment | 3 | 408.420 | < 0.0001 | 319.621 | < 0.0001 |
| time | 2 | 88.765 | < 0.0001 | 343.479 | < 0.0001 |
| target | 1 | 298.194 | < 0.0001 | 135.763 | < 0.0001 |
| treatment × time | 6 | 14.130 | < 0.0001 | 105.850 | < 0.0001 |
| treatment × target | 3 | 68.563 | < 0.0001 | 35.543 | < 0.0001 |
| time × target | 2 | 11.780 | < 0.0001 | 21.049 | < 0.0001 |
| treatment × time × target | 6 | 23.445 | < 0.0001 | 13.556 | < 0.0001 |

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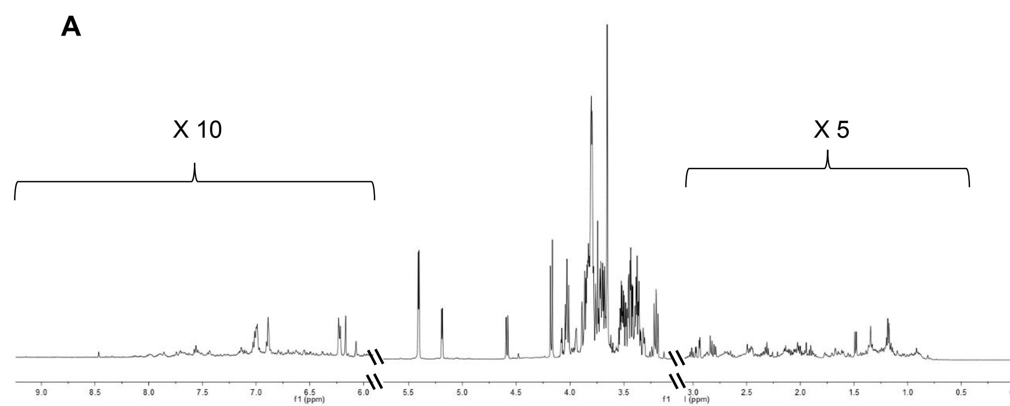
A
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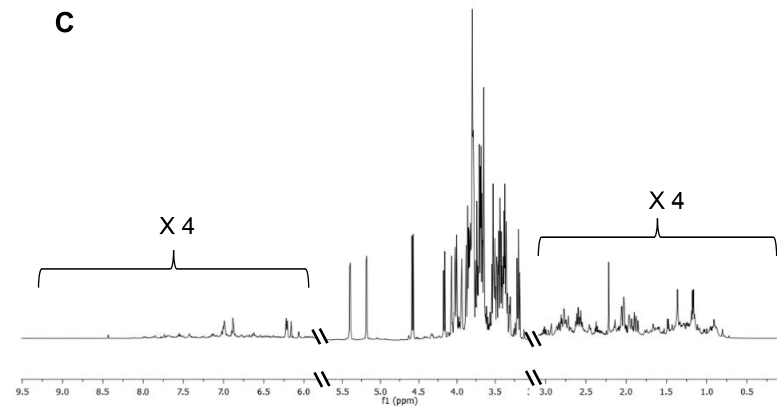
C
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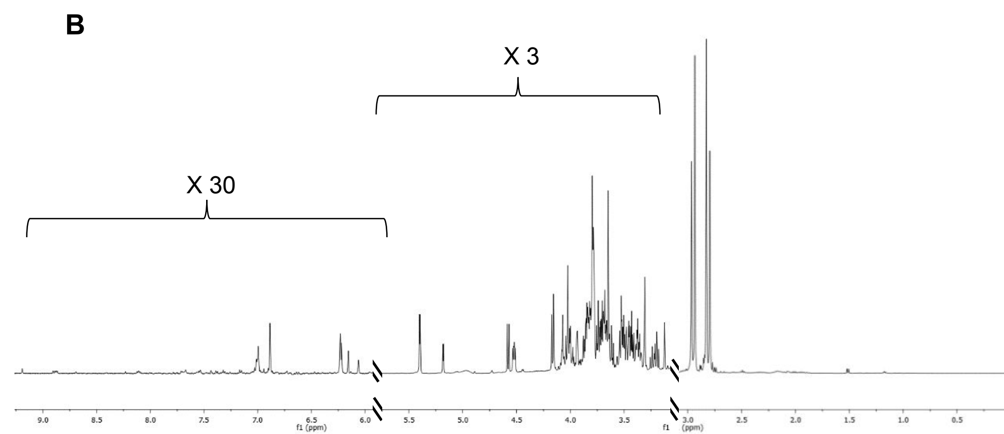
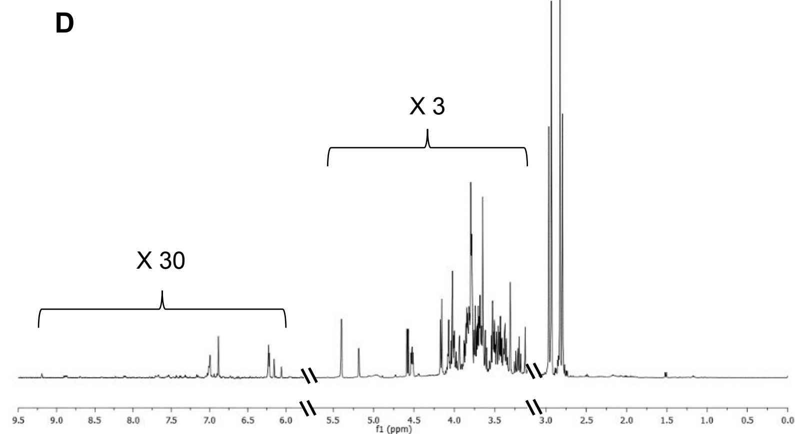
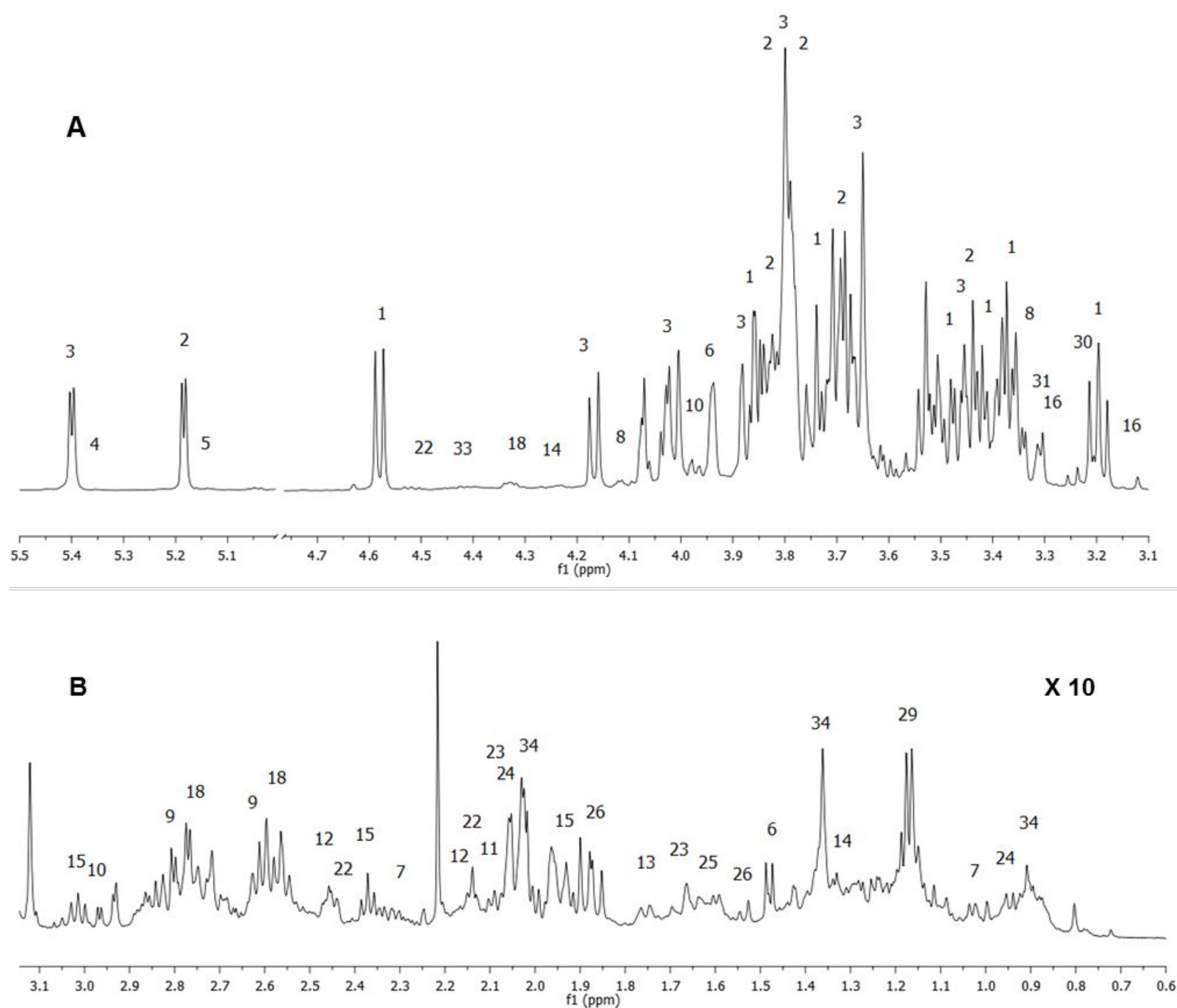

B
X 30
X 3
f1 (ppm)


D
X 30
X 3
f1 (ppm)

527 **Figure 1.** Representative $^{1}H$ NMR water-presaturated spectrum (500 MHz, $CD_3OD:D_2O$) of a WTPE extract (A), a WTFL extract (B), a E23PE

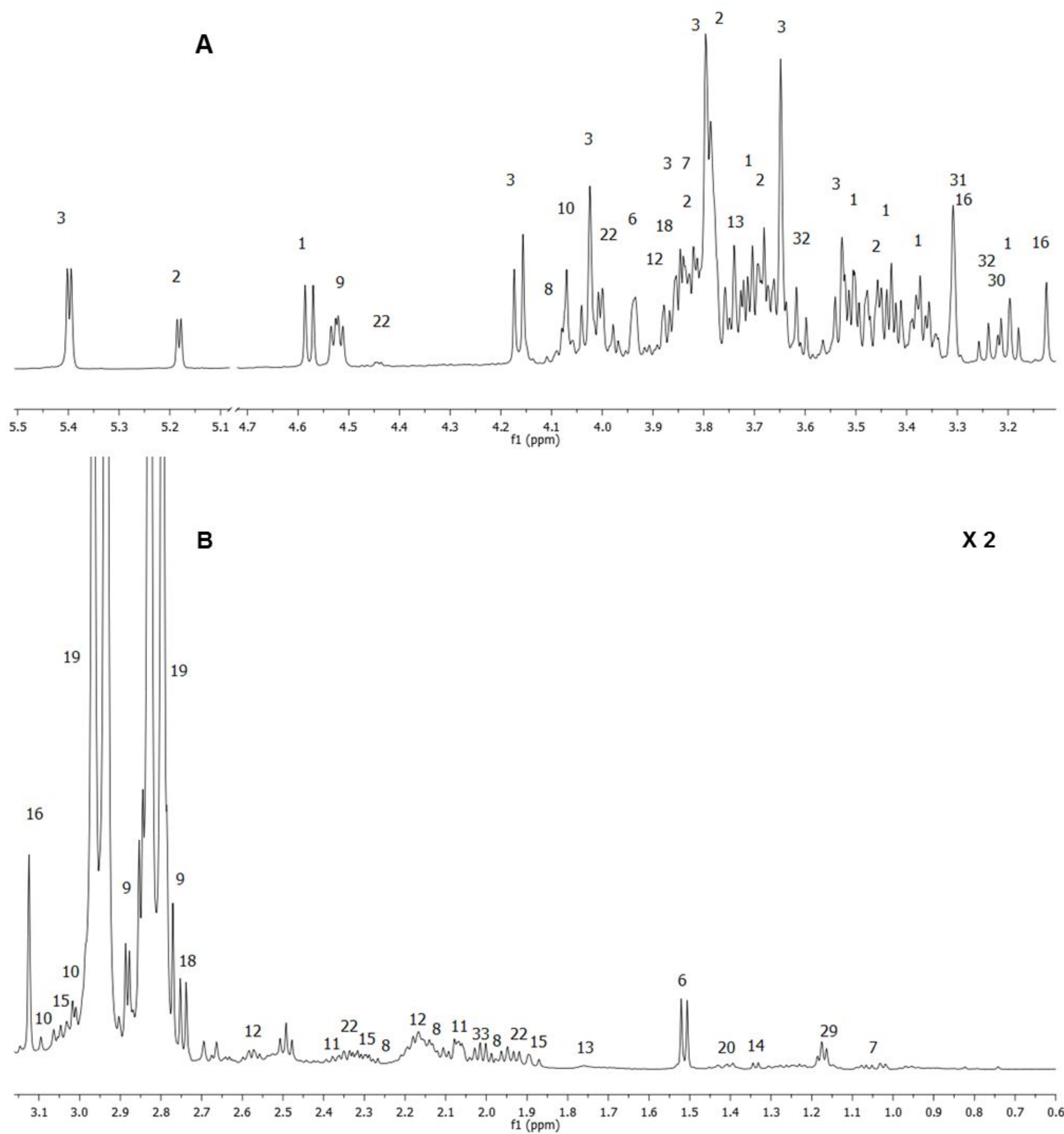
528 extract (C), a E23FL extract (D).

529



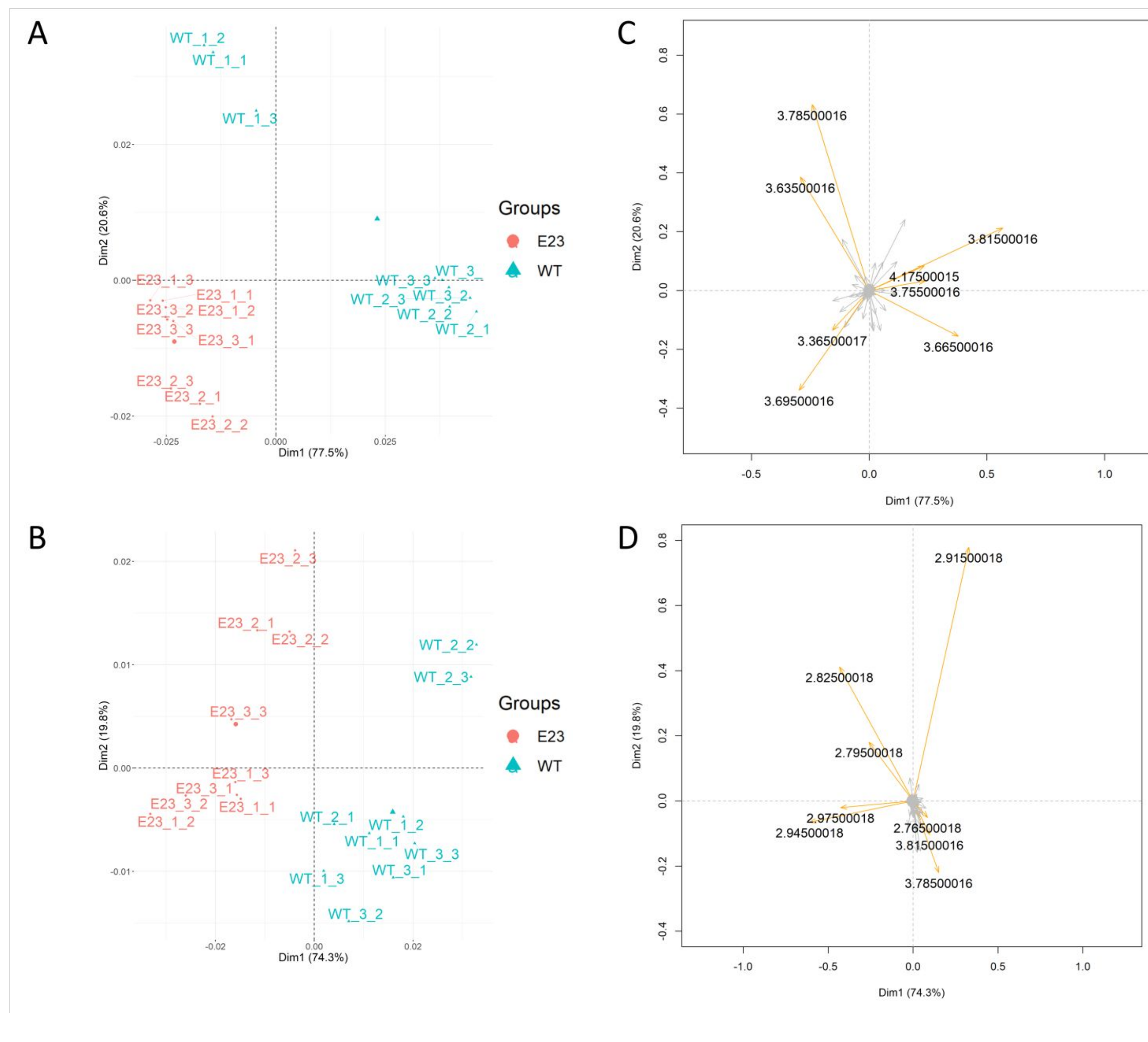

530

531 **Figure 2.** Partial ^{1}H NMR water-presaturated spectrum (500 MHz, $CD_3OD:D_2O$ 1:1) of a WTPE
532 extract: A) region between 5.50 and 3.10 ppm; B) region between 3.05 and 0.60 ppm.




533

534 **Figure 3.** Partial $^1$H NMR water-presaturated spectrum (500 MHz, $CD_3OD:D_2O$ 1:1) of a WTFL
535 extract: a) region between 5.50 and 3.10 ppm; B) region between 3.15 and 0.85 ppm.

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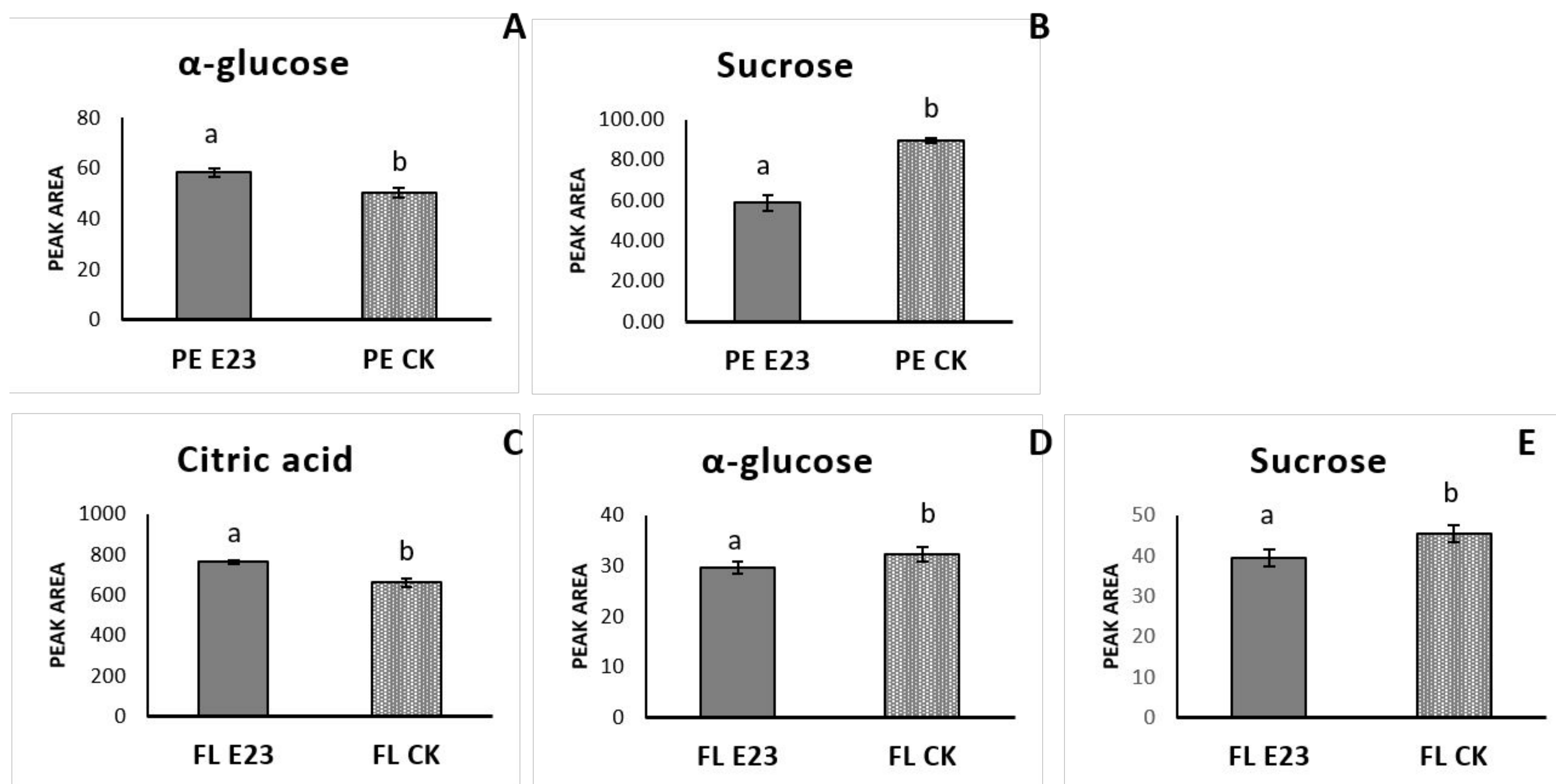
537

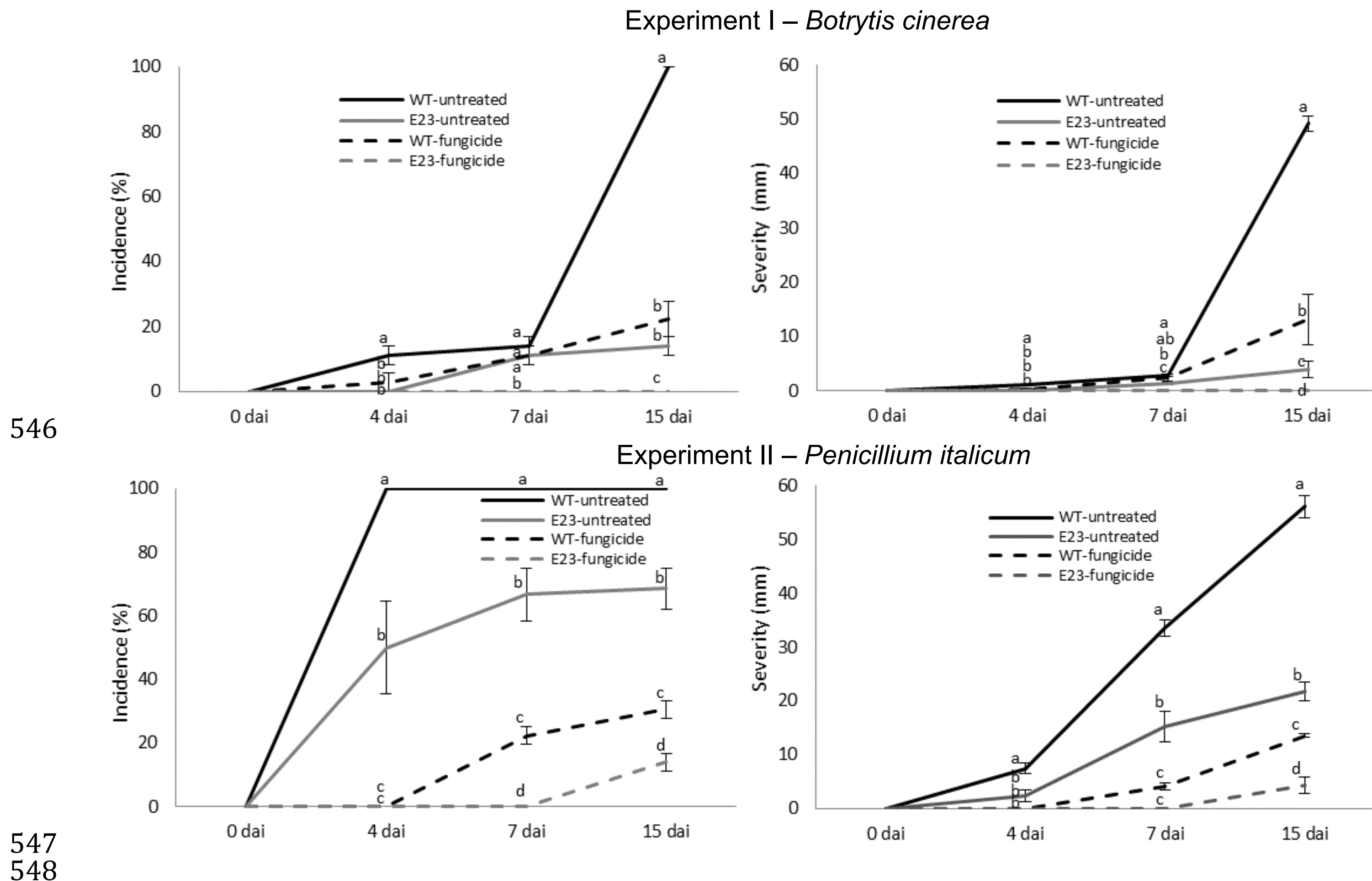
538 **Figure 4.** PCA results for the metabolites identified by $^{1}H$ NMR spectra from polar extracts of peel (A-C) and flesh (B-D). Score plots (A and B)
539 and loading plots (C and D).

540




541

542 **Figure 5.** Relative ratio (obtained by integration from signal of the compounds in the ^{1}HNMR spectra normalized by TMSP-$d_4$ signal) of sucrose
543 (A) and α-glucose (B) in the extracts from peel; citric acid (C), α-glucose (D) and sucrose (E)in the extracts from flesh. Data are displayed as a mean
544 of relative ratio ± standard deviation ($P < 0.05$). Different letters on bars indicate significant differences ($P < 0.05$).
545

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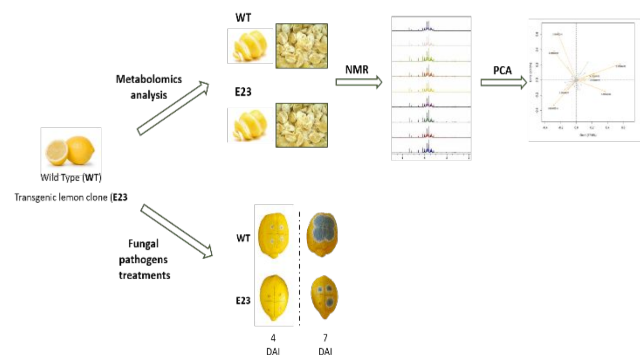
547
548

549 **Figure 6.** Time progression of gray mold (experiment I) and blue mold (experiment II) decay amounts in different treated E23 and WT lemon fruit
550 following artificial inoculation. Incidence was expressed as a percentage of infected fruit while severity as average size (mm) of decayed sites. DAI
551 = days after inoculation.
552 [x] Averages of 3 replicates each formed by 9 fruit. Vertical bars represent the standard error of the means (SEM).
553 [y] Values followed same letter within each evaluation time are not significantly different according to Fisher's least significance differences test ($\alpha$ =
554 0.05).
555

**TOC**




## Supporting Information

WTFL extract: representative $^{13}$C NMR spectrum; gCOSY spectrum; JRES spectrum; gHSQC spectrum; gHMBC spectrum.

WTPE extract: representative $^{13}$C NMR spectrum; gCOSY spectrum; JRES spectrum; gHSQC spectrum; gHMBC spectrum.