Aureobasidium pullulans volatile organic compounds as alternative postharvest method to control brown rot of stone fruits

Research article

Aureobasidium pullulans volatile organic compounds as alternative postharvest method to control brown rot of stone fruits

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Abstract

Volatile compounds produced by L1 and L8 strains were assayed against mycelia and conidia growth of *Monilinia laxa*, *M. fructicola*, *M. polystroma*, and *M. fructigena* of stone fruits. Results showed that volatile metabolites inhibited significantly pathogens growth, in particular *M. fructigena* mycelium growth (70% by L1 and 50% by L8) and *M. fructicola* conidia germination (85% by L1 and 70% by L8) compared to the control. Moreover, the antagonistic activity was enhanced by the addition of asparagine (120 mg L^{-1}) in the culture media composition. Synthetic pure compounds were tested in vitro on pathogens mycelial and conidia growth and their EC50 values were estimated, confirming 2-phenethyl as the most active compound. For this reason, 2-phenethyl and VOCs of both yeast strains were assayed in vivo on cherry, peach, and apricot fruits. Regarding peach fruit, both treatments, yeasts and pure compounds, displayed the best inhibiting action against all the pathogens especially against *M. laxa* (100% by L1, 84% by L8 and 2-

phenethyl). ATR/IR spectroscopy analysis showed how VOCs produced by both strains increase the fruit waxes complexity reducing the pathogens attack so playing an essential role in the antagonistic activity of both yeast strains and on fruit structural composition.

Keywords: Stone fruits - *Monilinia* spp. – Metabolites – *Aureobasidium pullulans* – ATR Spectrometry

Introduction

Postharvest decays of fruit represent one of the major factor causing economic losses and significantly contribute to reduction of fruit value by deterioration of quality and nutrient composition (Mari et al, 2016). Commonly, postharvest decays are controlled by chemical fungicides, but nowadays consumers prefer fruit with no pesticide residues or obtained through organic agricultural systems. Besides this, the intense use of postharvest fungicides such as imazalil, thiabendazole, and sodium ortho-phenyl phenate, generally used against *Penicillium digitatum* and *Penicillium italicum*, developed resistant isolates causing problems in control management (Kinay et al., 2007).

Alternative defense strategies were investigated based on the use of natural secondary metabolites such as volatile organic compounds (VOCs) produced by plants, bacteria, yeasts, or fungi in a process defined biofumigation. The Biocontrol Agents (BCAs) can work as biofumigants, representing a particular application of biological control since they are not in direct contact with the pathogen and VOC production is their only action mechanism (Di Francesco et al., 2016). The volatile metabolites could be potentially employed with success as gaseous treatments in a biofumigation process, as in the case of *Muscodor albus* capable of controlling the major diseases of potato (Corcuff et al., 2011), lemon (Mercier and Smilanick, 2005), table grapes (Mlikota Gabler et al., 2006), and tomatoes (Freitas et al., 2005) when used as biofumigant during the postharvest phase.

53 Among BCAs used to control postharvest pathogens, *Aureobasidium pullulans* (Zhang et al., 2010;
54 Di Francesco et al., 2018) showed a high efficacy to control *Monilinia* spp. on stone fruits, *Botrytis*
55 *cinerea* and *Penicillium* spp. on pome and citrus fruits (Di Francesco et al., 2017a, 2015a), and also
56 in field to control *Phytopthora infestans* of tomato (Di Francesco et al., 2017), *Fusarium* spp. of
57 wheat (Wachowska and Glowacka, 2014) and *Neofusicoccum parvum* of woody plants (Rusin et al.,
58 2019). *Aureobasidium pullulans* strains L1 and L8 were known to produce VOCs, low-molecular
59 weight lipophilic compounds derived from a biosynthetic pathways, active against pome and citrus
60 fruit postharvest pathogens (Di Francesco et al., 2015a), with a scarce toxicity at low
61 concentrations, making them extremely attractive in postharvest diseases management (Mari et al,
62 2016).
63 Using the solid-phase microextraction (SPME) method, VOCs produced by the most of yeasts were
64 recognized mainly to belong to the alcohol (ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2-
65 phenylethanol) (Di Francesco et al., 2015a), to the esters (ethyl acetate, ethyl octanoate) (Fialho et
66 al., 2010) and aldehydes (2-methyl-2-hexenal and 2-isopropyl-5-methyl-2-hexenal) chemical groups
67 (Buzzini et al., 2003). The VOCs can also provide sensorial notes for the consumer, contributing to
68 the characteristic flavor and aroma in determinate foods (Sreekumar et al., 2009).
69 Compounds such as ethanol, acetaldehyde, and acetone are responsible for the pleasant or off-flavor
70 in foods (Salmerón et al., 2015; Kopsahelis et al., 2007). Furthermore, volatile metabolites can
71 influence fruit/food matrixes odor, taste, color, and texture. Recently, the ability of *A. pullulans* L1
72 and L8 strains to modify the fruit nutritional components as well as to inhibit the pathogens
73 development in peach and kiwi fruit was reported (Di Francesco et al., 2017a; Di Francesco et al.,
74 2017, 2018).
75 The objective of this study was i) evaluate the efficacy of the antifungal volatile compounds
76 produced by L1 and L8 strains against *Monilinia* spp. of stone fruits (cherry, peach, and apricot)
77 both in in vitro ii) and in vivo assays; iii) and evaluate their chemical effects on the structural
78 composition of fruits by ATR/IR spectroscopy, a fast and non-destructive analytical technique

79 already proven useful for the characterization of fruit chemical components (Szymanska-Chargot
80 and Zdunek, 2013).
81
82 **2. Materials and methods**
83 **2.1 Antagonists**
84 The strains L1 and L8, molecularly characterized by Di Francesco et al. (2018), were maintained on
85 nutrient yeast dextrose agar (NYDA: 8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose and
86 15 g of agar in 1 L of distilled water) at 4 °C until use. Two days before trials, each antagonist was
87 grown on NYDA at 25 °C, and the yeast cells were collected in sterile distilled water containing
88 0.05% (v/v) Tween 80 and quantified for a final concentration of 108 cell ml^{-1} by counting spore
89 suspension on hemocytometer cell.
90
91 **2.2 Pathogens**
92 *Monilinia laxa* (ML4), *M. fructicola* (MCL2), *M. polystroma* (MPC1), and *M. fructigena* (MCG5)
93 strains from the CRIOF-DISTAL collection (UniBo) and previously molecularly characterized
94 (Mari et al., 2012; Martini et al., 2014; Di Francesco et al., 2015b) were used. The pathogens were
95 grown and maintained on potato dextrose agar (PDA, 39 g L^{-1}, Oxoid, UK) at 25 °C for *M. laxa*
96 and *M. fructicola* and 20 °C for *M. polystroma* and *M. fructigena*.
97 Conidia suspensions of *Monilinia* species were prepared from 7 days old colonies grown on tomato
98 agar (250 mL tomato sauce, 15 g of agar technical (Oxoid, UK) in 1 L of distilled water) (Martini et
99 al., 2016) by scraping and suspending spores in sterile distilled water with 0.05% (v/v) of Tween 80
100 and adjusted to a final concentration relating to the experiments with a hemocytometer.
101
102 **2.3 Fruits**
103 Cherries cv "Sweet®", peaches cv "Red haven", and apricots cv "San Castrese" were harvested in
104 experimental orchards of Bologna University located in Altedo and Cadriano (Bologna, Italy). After

105 harvest, fruits with no visible wounds and rots, homogenous in size and quality (°Brix, hardness,
106 color), were disinfected by hypochlorite 0.1% (w/v) by immersion for 1 min, rinsed with tap water
107 and air dried at room temperature and after artificially inoculated.
108
109 **2.4 *In vitro* antifungal assays**
110 The antifungal effect exerted by the VOCs produced by L1 and L8 was assayed by the double Petri
111 dish assay (Rouissi et al., 2013; Di Francesco et al., 2015a). VOCs were tested against mycelium
112 growth and CFU of the *Monilinia* spp. cited above. For this purpose, NYDA plates amended or not
113 with asparagine (120 mg L^{-1}, Sigma Aldrich, USA) were inoculated by spreading 100 µL of
114 antagonist cell suspension (108 cell mL^{-1}), as reported in Di Francesco et al. 2017a; Di Francesco et
115 al., 2017. The lid of the plate was replaced, after 48 h of incubation at 25 °C, by a base plate of
116 MEA (Malt Extract Agar, 50 g L^{-1}, Oxoid, UK) inoculated with a mycelium plug (6 mm of
117 diameter) or with 100 µL of conidia suspension (10^3 conidia mL^{-1}) of each pathogen species. The
118 two base plates were sealed immediately with a double layer of Parafilm and incubated at 25 °C
119 with *M. laxa* and *M. fructicola* and at 20 °C with *M. polystroma* and *M. fructigena*, respectively for
120 6 and 2 days. The sample unit was represented by ten plates (replicates) for each pathogen, type of
121 inoculum (mycelium or conidia), with (treatments) or without (control) antagonist interaction. The
122 experiments were conducted twice.
123 The inhibition rate of mycelial growth and colony forming unit (CFU) was calculated using the
124 equation (Chen and Dai, 2012):
125 $$(\%) = \frac{d1-d2}{d1}$$
126 where (%) is the percent of inhibition of mycelial growth (mm of colony diameter) or CFU (n. of
127 colony); *d1* is the control value ; *d2* is treated value.
128
129 **2.5 Effect of pure VOCs on mycelium growth and CFU of *Monilini*a species**

130 Pure standards of 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol and phenethyl
131 alcohol (Sigma–Aldrich, St. Louis, MO), previously identified through HS-SPME-GCMS as the
132 main volatile compounds produced by L1 and L8 on NYDA plate (Di Francesco et al., 2015a), were
133 tested on Monilinia species mycelium and CFU growth. For this purpose, different aliquots of pure
134 compounds 25, 50 and 100 µL, were placed with a microsyringe on a filter paper (Whatmann No. 1,
135 90 mm diameter) positioned inside the cover of a MEA dish previously inoculated with 6 mm
136 pathogen mycelium plug or 100 µL of pathogen conidia suspension (10^3 conidia mL^{-1}). The
137 aliquots of pure compounds introduced in the Petri dishes corresponded to 2.25, 1.12 and 0.56 µL
138 mL^{-1} headspace, as described by Rouissi et al. (2013).
139 The dishes were quickly closed, sealed with Parafilm and incubated at 25 °C. The activity of each
140 pure compound against mycelial and colony growth was evaluated after 6 and 2 days of incubation
141 respectively. In the control, pure compounds were substituted by equivalent amounts of distilled
142 water. The sample unit was represented by 5 plates for each volatile compound concentration. EC_{50}
143 values were calculated as the headspace concentrations (µL/mL) that inhibited mycelial and CFU
144 growth by 50% compared with the control. The experiment was performed twice.
145
146 **2.6 *In vivo* assay: effect of VOCs on fungal pathogens in stone fruits**
147 Two different in vivo assays were conducted, the first to evaluate the antagonistic activity of VOCs
148 produced by L1 and L8 strains and second to test the efficacy of the pure compound phenethyl
149 alcohol in controlling *Monilinia* spp. of stone fruits. This particular VOC was chosen as the most
150 active on the pathogens.
151 For the antagonistic activity of L1 and L8 VOCs, cherries (15 fruits), peaches (5 fruits), and
152 apricots (8 fruits) were placed in sterile glass boxes (24 × 18 × 8 cm. L × W × H) with a thin layer
153 of NYDA (250 mL), inoculated 2 days before with 500 µL of a L1 and L8 suspension of 10^8 cell
154 mL^{-1}, positioned at the bottom and incubated at 25 °C. For phenethyl alcohol, six filters paper (90
155 mm diameter) were spread with 100 µL of the synthetic compound each and placed in the bottom of

156 the sterile glass boxes. Fruits were positioned on a sterile grid to separate them from the bottom
157 substrate and avoid the direct contact and possible contaminations. Each fruit was wounded (3 × 3 ×
158 3 mm) with a sterile needle and inoculated with 20 μL of suspension of each *Monilinia* specie (10^5
159 conidia mL^{-1}). The boxes were closed with plastic lid and sealed immediately with a double layer
160 Parafilm. The control consisted of inoculated fruit placed in boxes without yeast suspensions or the
161 synthetic compound. The boxes containing inoculated fruit were kept at 20 °C. The percentage of
162 rotten fruits (for cherries) and the lesion diameters (peach and apricot fruits) were measured after 5
163 days of incubation. The sample unit was represented by three boxes per each pathogen. The
164 experiment was conducted twice.
165
166 **2.7 ATR Spectroscopy**
167 Cherry, peach, and apricot fruits (5 for each sampling time) were exposed to VOCs produced by L1
168 and L8 strains for 24 h, 48 h, 72 h, and 96 h following the above cited in vivo assay methods (Di
169 Francesco et al., 2015a). Peel fruits were collected and stored at −80 °C in sterile plastic flask and
170 suddenly lyophilized by freeze-drying (FD-10 Freezing Dryer, Lab kits, H.K.) under vacuum (<20
171 Pa) at a temperature of −36 °C and freeze-dried for 7 days to avoid water spectroscopic
172 interferences. The control consisted in peel fruit tissues without yeasts VOCs exposition. ATR
173 spectra were recorded with a Bruker ALPHA series FT-IR spectrophotometer (Bruker, Ettlingen,
174 Germany) equipped with an apparatus for attenuated total reflectance (Diamond crystal). The
175 spectra were collected from 4000 to 400 cm^{-1} and averaged over 100 scans (resolution = 4 cm^{-1}): 4
176 spectra were measured for each sample for each sampling time.
177
178 **2.8 Data analysis**
179 Data were statistically handled by one-way analysis of variance (ANOVA). Statistical comparison
180 of means was carried out to reveal the differences between treatments using Tukey's HSD Test (α =
181 0.05).

182 All analyses were performed with Statgraphics software (version centurion 15.0). The experiments
183 were carried out in a completely randomized block design. The EC_{50} of each substance was
184 calculated using the probit analysis applied to the percentage of inhibition of mycelial and CFU
185 growth (Lesaffre and Molenberghs, 1991).
186
187 **3. Results**
188 **3.1 Effect of VOCs produced by L1 and L8 on mycelium and CFU growth of *Monilini*a species**
189 In order to assess the antifungal effect on mycelia growth and conidia germination due to the
190 metabolic volatile component, a double Petri dish assay system was set up to avoid any contact
191 between the L1 or L8 strains and pathogens.
192 The VOCs produced by both strains inhibited significantly the fungal mycelia growth, with some
193 differences between the pathogen species. The L1 strain volatile metabolites inhibited *M. laxa* and
194 *M. fructigena* mycelia more than L8 (40% and 75% for L1 and 20% and 50% for L8, respectively).
195 Both strains metabolites showed the same antagonistic inhibitory activity against *M. fructicola* and
196 *M. polystroma* (~40%). In particular, asparagine amended plate stimulated the antagonistic activity
197 of both strains mainly against *M. laxa* and *M. fructigena* showing a significant increase on the
198 mycelia growth inhibition with respect to no amended plate (>50% for *M. laxa* and >15% *for M.*
199 *fructigena* by L1 and >20% for both fungal species by L8). On the opposite, asparagine did not
200 increase *A. pullulans* strains antagonistic activity against *M. polystroma* (Fig. 1a).
201 Also considering CFU growth, VOCs produced by both strains inhibited significantly the fungus
202 species, specially *M. fructicola* displaying a reduction of 80% and 65% respectively by VOCs
203 produced by L1 and L8, independently from the presence or not of asparagine. Instead, the
204 asparagine presence showed a significant but relatively low increase in L1 and L8 inhibitory effect
205 against the remaining three species (Fig. 1b).
206
207 **3.2 Effect of synthetic volatile organic compound on mycelia and CFU of *Monilini*a spp.**

208 The pure VOCs 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol and 2-phenethyl
209 alcohol, previously identified as the main volatile compounds produced by L1 and L8 through HS-
210 SPME-GCMS (Di Francesco et al., 2015a), were tested for the inhibitory activity of mycelia and
211 CFUs growth of the *Monilinia* species. Results showed that phenethyl alcohol was the most
212 effective compound in mycelial growth inhibition, showing the total suppression of all *Monilinia*
213 (Table 1). The values for tested fungi CFU suppression ranged between 0.006 and 0.013 μL mL −1.
214 With respect to the other pure compounds, 1-propanol-2-methyl displayed the lowest antifungal
215 activity against *M. fructicola* and *polystroma*, or no antifungal activity against mycelial or CFU of
216 *M. fructigena* and *M. laxa*. For this compound, EC_{50} values ranging from 0.019 to 0.115 μL mL $^{-1}$
217 were obtained for the target pathogens.
218 In general, *M. fructigena* and *M. polystroma* resulted the most resistant pathogens, especially in
219 CFU growth, with the highest VOC EC_{50} values, while *M. laxa* and *M. fructicola* were the most
220 sensitive with low VOC EC_{50} values both for mycelia and CFU growth (Table 1).
221
222 **3.3 Effect of VOCs on *Monilinia* spp. in stone fruits: *in vivo* assay**
223 The VOCs produced by L1 and L8 and the most effective pure compound phenethyl alcohol were
224 tested against the target pathogens *in vivo* (Fig. 2). For cherry, results are reported as percentage of
225 disease incidence (a) due to the fruit small size, for peach and apricot as millimeters (mm) of the
226 lesion (b, c). With regard to cherry fruit, *M. laxa* was the specie more susceptible to phenethyl
227 alcohol and to the L1 and L8 VOCs, showing 90%, 90% and 75% of reduction of incidence,
228 respectively. When assayed against *M. polystroma*, the VOCs of both strains controlled better the
229 fungal incidence (78%) than the pure compound (~7%). The two strains and phenethyl alcohol
230 resulted less effective against *M. fructicola* and *M. fructigena*. Here, no significant difference ($P <$
231 0.05) was detected comparing L8 treatments with the control, and only a small but significant
232 reduction with L1 (~30%).

233 With respect to peach fruit both yeast and phenethyl alcohol treatments displayed an inhibitory
234 action against all the pathogens. The highest inhibition was apparent against *M. laxa* (100% for L1,
235 84% for L8 and 79% for phenethyl alcohol) and *M. fructicola* (91% by L1, 87% by L8 and 76% by
236 phenethyl alcohol), *M. polystroma* and *M. fructigena* were less inhibited. In the case of apricot, the
237 four *Monilinia* species were inhibited only by the application of L1 and L8 strains; L1 reduced the
238 lesion diameter resulting from the artificial inoculation of *M. laxa*, *fructicola*, *polystroma* and
239 *fructigena* by 100%, 100%, 63%, 51%, respectively and L8 by 22%, 34%, 61%, 59% respectively.
240 Phenethyl alcohol caused a growth reduction of 47% only in the case of *M. polystroma*.
241 In addition, the treatments slowed down the disease sporulation in all the infected fruits, as no
242 spores were observed on fruit symptoms after 5 days, when sporulation was clearly evident in
243 control fruits (Fig. 3).
244
245 **3.4 ATR/IR Spectroscopy**
246 ATR/IR spectra were measured in order to obtain a rapid and non-destructive analysis on the
247 surface chemical modification of fruit skin upon exposition of yeast VOCs. Indeed, VOCs can alter
248 the fruit surface structure as reported by other authors (Bonora et al., 2009; Fasoli et al., 2016). This
249 analytical technique measures the absorption of IR photons by chemical bonds vibrations. More in
250 details, chemical bonds can vibrate by changing the bond length (stretching vibrations, indicated by
251 the Greek letter ν), or by changing the bond angle (bending vibrations, indicated by the Greek letter
252 δ). The energy of vibrations (measured in cm^{-1}) is typical of each chemical functional group, thus
253 allowing a qualitative identification of chemical compounds. Fig. 4 shows the ATR/IR spectra of
254 control samples at the beginning of the experiment, with the attribution of the main spectral region
255 to the different biochemical compounds. These spectra highlighted some difference between the
256 three fruits independently of infection or yeast treatment: the apricot skin is the one containing the
257 lower amount of absorbed water (broad band at 3300 cm^{-1}), together with the highest content of
258 cuticle waxes, corresponding to intense ν CH bands at 2920 and 2850 cm^{-1} and ν C=O band at

259 1730-1720 cm^{-1} (Bertoluzza et al., 1994). Peach skin showed a more complex band profile in the
260 region between 1140 and 930 cm^{-1}, indicative of polysaccharides, with typical peaks attributed to
261 pectin vibrations at 990 and 920 cm^{-1} (Fasoli et al., 2016), while cherry showed intense peaks in the
262 900-760 cm^{-1} spectral region, attributed to ring deformation of pectin (Synytsya et al., 2003).
263 In order to understand the effects of L1 and L8 strains on fungal attack on fruit skin, the ATR/IR
264 spectra obtained on treated fruits were subtracted to control ones (i.e. without L1 or L8) for each
265 sampling time. The difference spectra were analyzed in order to assess which biochemical
266 compounds where affected by the presence of the strains and to which extent, in order to also
267 compare L1 with L8 (Fig. 4 and Table 2). Differences were found for several classes of biochemical
268 compounds: cellulose and hemicellulose, pectin, proteins, lipids and aromatic compounds. In
269 general, all those classes were affected by L1 and L8 presence to a different extent that depended
270 also by the fruit. The bands attributed to aromatic compounds (phenolics, flavonoids, anthocyanins
271 and lignin) showed an increase in the presence of L1 and L8 strains. Particularly, L1 induced a
272 higher increase of these compounds, mostly in cherry and apricot (mainly attributed to flavonoids
273 bands).
274 A more complex behavior was observed in lipids, since both strains showed to increase waxes and
275 lipids content in cherry and peach, while decreasing them in apricot (in particular L1 strain). The
276 intensity ratio between the bands at 3000 and 2850 cm^{-1}, respectively attributed to unsaturated and
277 saturated ν CH vibration, could be used to estimate the unsaturation degree of fruit skin lipids,
278 (Bertoluzza et al., 1994). This ratio was very low for all the three studied fruits. Nevertheless, it was
279 usually decreasing in cherry and peach, while increasing in apricot, in particular in L1-treated fruits.
280 Interestingly, these last fruits, i.e. apricots treated with L1, showed a general increase of protein
281 bands. Another interesting effect on fruit lipids measurable by IR spectroscopy is the degree of
282 peroxidation associated to the formation of free fatty acids. These come from the degradation of
283 palmitic and stearic acids (Bertoluzza et al., 1994) and cause an increase of the band at 1700 cm^{-1} (ν

284 C=O). From difference spectra, this band was found to decrease in apricot and peach, while
285 increasing in cherry.
286 The results on the polysaccharides content of fruit skin is more complicated, since many bands are
287 overlapping (Table 2): however, a particular behavior can be assessed by considering the main
288 diagnostic bands of hemicellulose and pectin. In fact, the intensity of the typical IR bands of
289 hemicellulose at 1130 and 1065 cm^{-1} (Bonora et al., 2009) are generally increasing, while the pectin
290 fraction at 1015 cm^{-1} is decreasing. The typical cellulose band at 1050 and 1030 cm^{-1}, attributed to
291 the degree of orientation of cellulose microfibrils (Fasoli et al., 2016), increased both in apricot and
292 peach, while decreased in cherry.
293
294 **4. Discussion**
295 Volatile metabolites produced by *A. pullulans* strains L1 and L8 were studied against some apple
296 and citrus fruit postharvest pathogens by Di Francesco et al. (2015a), as a part of their modes of
297 action showing a good efficacy. In the present study, these compounds were tested against the
298 principal stone fruit postharvest pathogens such as *M. laxa*, *M. fructicola*, *M. polystroma*, and *M.*
299 *fructigena*. The results of the antagonistic activity in in vitro assays (Petri dishes assay)
300 demonstrated that VOCs emitted by both strains were able to reduce mycelial and conidial growth
301 of *Monilinia* pathogens. In addition, in vitro assay results showed how both strains displayed a high
302 inhibitory activity (on average ~80%) against conidia germination of almost all tested pathogens,
303 except for *M. fructigena* (on average ~30%). This makes L1 and L8 A. pullulans strains promising
304 candidate as efficient alternative to agrochemicals in controlling postharvest diseases.
305 Previous works showed how VOCs production of other antagonists inhibited in vitro spore
306 germination and germ tube elongation of some postharvest pathogens such as *Botrytis cinerea*, *M.*
307 *laxa* and *M. fructicola* (Chen et al., 2008; Gotor-Vila et al., 2017), *Colletotrichum acutam*,
308 *Penicillium* spp. (Di Francesco et al., 2015a); such an inhibition was often supported by *in vivo*
309 results (Gotor-Vila et al., 2017). The antifungal activity of microorganisms, in particular the VOCs

310 production, can vary depending on the growth media composition, highlighting the importance of
311 the substrate on the antifungal volatiles production by microorganisms (Gotor-Vila et al., 2017);
312 Yánez-Mendizábal et al. (2012); Fiddaman and Rossall (1993), Fiddaman and Rossall (1994). Our
313 results showed how the yeast growth medium (NYDA) amended with asparagine can affect VOCs
314 production and effectiveness. This amino acid was previously showed as active amino acid involved
315 in nutrient competition between L1 and L8 strains and *M. laxa* (Di Francesco et al., 2017a; Di
316 Francesco et al., 2017). The asparagine presence increased the antifungal activity of both strains
317 especially against *M. laxa* both for mycelium growth (>50%) and conidia germination (>20%) (Fig.
318 1a and b), also showing a selective effect on *Monilinia* species. On the other hand, similar
319 experiments showed that *Bacillus amiloliquefaciens* CPA-8 grown on a media like TSA (Tryptone
320 Soya Agar) is more effective against *Monilinia* spp. and *Botrytis cinerea* with respect to a NA
321 (nutrient agar), both poor media and the produced VOCs were effective in the same way against the
322 tested pathogens (Gotor-Vila et al., 2017).
323 As showed by Di Francesco et al. (2015a), compounds as 2-phenyl, 1-butanol-3-methyl, 1-butanol-
324 2-methyl, and 1-propanol-2-methyl belonging to the group of alcohols and mainly produced by both
325 strains, are active against brown rot causal agents through in vitro and in vivo assays. Results
326 presented here confirmed 2-phenethyl as the most active compound, with 100% of inhibition on
327 mycelia growth and EC_{50} values ranging from 0.006 μL mL^{-1} to 0.013 μL mL^{-1} 1-propanol-2-
328 methyl was confirmed the least active compound with EC_{50} values ranging from 0.019 μL mL^{-1}
329 against *M. laxa* to 0.127 μL mL^{-1} against *M. fructigena* mycelia and respectively with no inhibition
330 rate for conidia germination. VOCs tested against *Monilinia* spp. have lower EC_{50} values and higher
331 efficiency than against *B. cinerea*, *C. acutatum*, and *Penicillium* spp., (Di Francesco et al., 2015a),
332 where 1-propanol-2-methyl was the least active VOC with the EC_{50} values over 0.8 μL mL^{-1}, while
333 the 2-phenethyl alcohol was the most active with EC_{50} values lower than 0.8 μL mL^{-1}.
334 Nevertheless, VOCs produced by microorganisms are commonly found at very low concentrations

335 and their effect is supposed to be due to synergic or additive action and not to a single component
336 activity (Mercier and Jimenez, 2004; Strobel et al., 2001).
337 L1 strain proved to have the best results in controlling brown rot disease caused by the four tested
338 pathogens, confirming previous results (Di Francesco et al., 2017; Rusin et al., 2019) obtained
339 against different pathogens and hosts.
340 Furthermore, both strains were able to reduce completely fungal sporulation on fruit surfaces and
341 reduce the brown rot lesion diameters after 5 days of incubation (Fig. 3), partially confirming in
342 vitro results. Peach resulted the most sensitive fruit to *Monilinia* spp. aggressiveness, especially to
343 *M. laxa*, *M. fructicola*, and *M. polystroma*. On the other hand, as it is known, *M. fructigena* is less
344 aggressive on stone fruits than on pome fruits (Jones and Aldwinckle, 1990). Our results confirmed
345 also the findings by Villarino et al. (2016), where isolates of *M. fructigena* exhibited a weaker
346 aggressiveness in peach fruit with respect to the other *Monilinia* spp.. Moreover, *M. polystroma*,
347 known to be a pathogen specialized in fruit infections (Van Leeuwen et al., 2002), displayed a great
348 aggressiveness on stone fruits, also showing the ability to produce a hyphal mantle of stroma on the
349 hosts cuticle (Poniatowska et al., 2012).
350 In the present study, we analyzed the influence of VOCs produced by L1 and L8 strains on the
351 chemical structural composition of stone fruits by using ATR/IR spectra registered directly on fruit
352 skin. The main findings of the spectroscopical analysis pointed out an influence of L1 and L8
353 strains on an increased production of aromatic compounds, such as unsaturated phenolics,
354 flavonoids and anthocyanins. Generally, difference spectra between treated and control fruits
355 showed an increase in the 1610-1480 cm^{-1} spectral region (Fig. 4 and Table 2): this enhancement
356 was more pronounced in the case of L1-treated fruits and less effective in peach, that is considered
357 to possess one of the lowest antioxidant activities between stone fruits (Park et al., 2015) and further
358 confirming the above mentioned sensibility of peach to *Monilinia* attack. The increase of this bands
359 following fungal attack, was previously described by Bonora et al. (2009) in kiwifruits affected by
360 elephantiasis, and thus represents a typical response of fruits to fungal decay. Therefore, we could

361 propose the monitoring of the 1610-1480 cm^{-1} spectral region by IR as a fast and useful method to
362 estimate fruit response to fungal attack. The biochemical mechanism related to the enhancement of
363 phenolic compounds productions by yeasts treated fruit was described by Hur et al. (2014): yeast-
364 released substances promote the synthesis of enzymes hydrolyzing β-glucosidic bonds (β-
365 glucosidases) of several phenolics that occurs as glyco-conjugates in fruits, leading to the release of
366 increased concentration of antioxidants.
367 ATR/IR spectroscopy showed the influence of L1 and L8 on the degree of unsaturation of lipids and
368 waxes (Bertoluzza et al., 1994): it decreased in cherry and peach, but increasing in apricot, denoting
369 a higher fluidity of this class of biochemical compounds in this last fruit. Moreover, a general
370 enhancement of protein IR bands was observed in apricot, in particular in L1 treated fruits: this
371 increase could further support a higher fluidity of cell membrane that, as a matter of fact, can be
372 obtained by either increasing the concentration of unsaturated lipids and by increasing the
373 concentration of membrane proteins. Since both biochemical compounds were reported to increase
374 in apricot fruits, it can be deduced that the increased membrane fluidity can be a mechanism
375 adopted by the fruit to protect from fungal attack. An increased protein content in yeast-treated
376 fruits has been previously reported by Hur et al. (2014). An increased membrane fluidity in fruits
377 has been reported by several authors (Bertoluzza et al., 1994; Aghdam and Bodbodak, 2013) as a
378 biochemical mechanism regulating chilling tolerance in fruits, increasing membrane integrity.
379 Therefore, a higher membrane fluidity could be regarded as an interesting consequence of yeast
380 application, enabling a better postharvest treatment of fruits. More in details, Aghdam and
381 Bodbodak (2013) reported that a treatment with phenolic compounds (i.e. salycilates and
382 jasmonates) enhanced both the antioxidant system activity and membrane integrity. Moreover, the
383 decrease of the band at 1700 cm^{-1}, previously described by Bertoluzza et al., (1994) to be an index
384 of the degree of peroxidation of fruit lipids, showed a decrease in both apricot and peach treated
385 with L1 and L8 strains, indicating a lower level of free saturated fatty acids (mainly stearic and

386 palmitic acids). Also, the decrease of the degree of peroxidation can be associated to an enhanced
387 membrane integrity as previously reported by Aghdam and Bodbodak (2013).
388 The general increase of IR bands associated to lipids and waxes in both cherry and peach, can be
389 associated to a thickening of the fruit cuticle: Yeats and Rose. (2013) indicated the presence of
390 pathogens as an environmental factor influencing cuticle biosynthesis and in particular wax
391 biosynthesis.
392 A more complex behavior was detected on the polysaccharides fraction, due to spectral overlapping
393 of bands coming from cellulose, hemicellulose and pectin. In general, an increase of the main
394 diagnostic bands of hemicellulose at 1130 and 1065 cm^{-1}, a fruit texture element (Bonora et al.,
395 2009), and a decrease of pectin band at 1015 cm^{-1} was observed, together with the increase of the
396 1050 and 1030 cm^{-1} bands of cellulose, that were an index of a higher degree of orientation of
397 cellulose microfibrils (Fasoli et al., 2016). Bacete et al. (2017) reported that modifications to the
398 cellulose and hemicellulose components of plants cell wall could explain an increased resistance to
399 pathogens in Arabidopsis thaliana. Unfortunately, IR spectra did not allow to have a clear picture on
400 the variation of the marker bands of pectin esterification (i.e. ν C=O band at 1740 cm^{-1}, ν CH_3CO at
401 1210 cm^{-1}, ν OCH_3 band at 990 cm^{-1}) or on the presence of free monosaccharides (i.e. glucose
402 bands at 920 and 775 cm^{-1}; fructose bands at 920, 885, 810 and 775 cm^{-1}; galactose bands at 956
403 and 756 cm^{-1}). Both the decrease of the degree of esterification and the presence of free
404 monosaccharides coming from the degradation of the pectic fraction were observed by Bonora et al.
405 (2009) as the consequences of fungal degradation on kiwifruits affected by elephantiasis. The
406 alteration of the modifications of pectins (mainly its acetylation and/or methyl esterification) of cell
407 wall has been recently pointed out as one of the main effects of fungal infections by Bacete et al.
408 (2017) on a model species (*Arabidopsis thaliana*).
409
410 **5. Conclusions**

411 In conclusion, we can assert that our study showed the capability of VOCs produced by *A. pullulans*
412 L1 and L8 strains to effectively reduce brown rot incidence caused by *Monilinia* spp. In addition,
413 we tried to better improve the knowledge about the VOCs production by L1 and L8 through the
414 addiction/modification of cultural medium with the objective to increase the efficacy of a future
415 bioformulate. VOCs produced by *A. pullulans* L1 and L8 notably increased the concentration of
416 membrane proteins, cuticle biosynthesis and wax biosynthesis, for this reason they may be applied
417 also with the purpose to increase the fruit mechanical defense structures. The study of the VOCs
418 influence on fruit structural composition is important to allow a most efficient use of L1 and L8
419 metabolites in future applications. Our results support the hypothesis that VOC metabolism is not
420 the only mechanism of action involved in the antagonists biological control function.
421

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425

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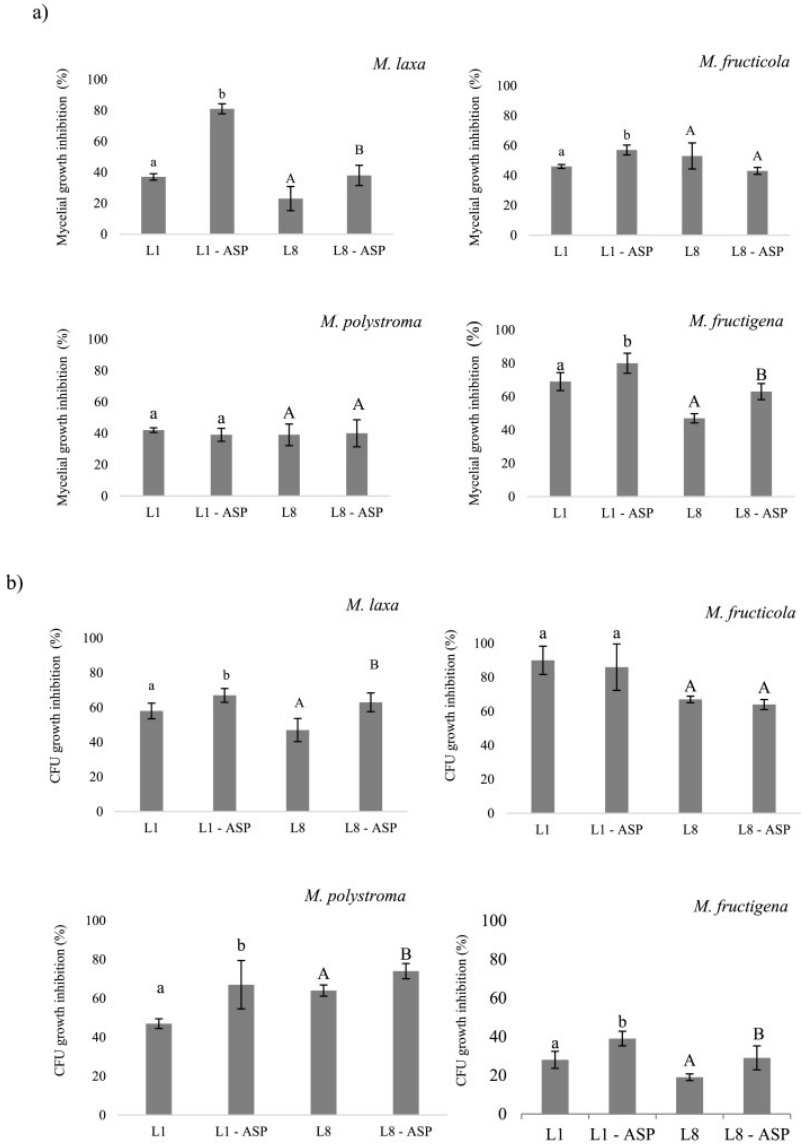
603
604 **Figures**
605 Figure 1. Effect of organic volatile compounds produced by two strains of *Aureobasidium pullulans* (L1 and
606 L8) on NYDA plate amended or not with asparagine (120 mg L^{-1}) on the mycelium growth (a) and CFU (b)
607 of *Monilinia* spp. Colony diameter (mm) and CFUs (n°) were measured after 5 and 2 days at 25 °C
608 respectively. Each value is the means of 10 plates (replicates) ± standard deviation. Within L1 strain (lower
609 case) and L8 (upper case) different letters represent significant differences among the strain to evaluate the
610 asparagine effect according to Tukey's HSD Test ($\alpha = 0.05$).



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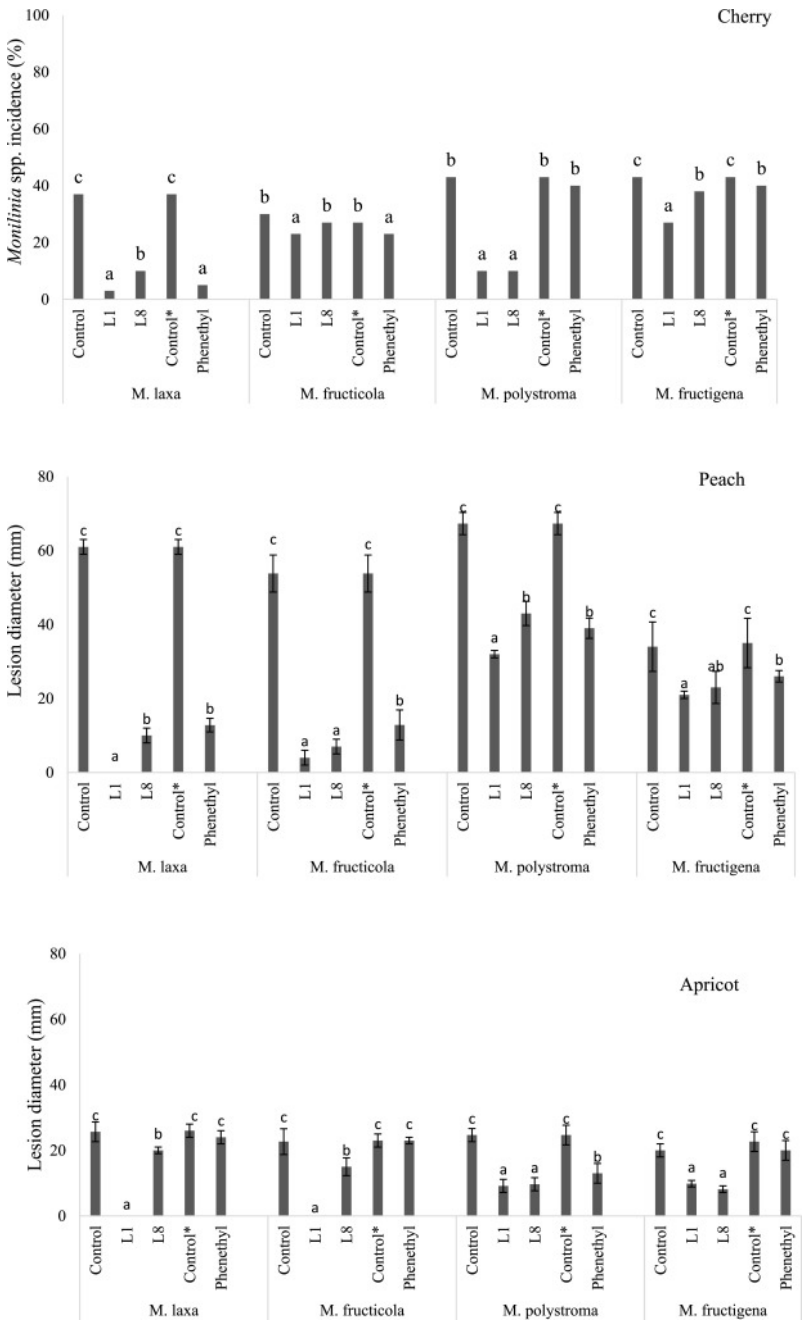
612

613 Figure 2. *In vivo* antagonistic effect of volatile compounds produced by L1 and L8 *Aureobasidium*
614 *pullulans* strains and the pure compound phenethyl alcohol on *Monilinia laxa*, *M. fructicola*, *M.*
615 *fructigena*, and *M. polystroma* in cherry, peach, and apricot fruits. Fruits were artificially inoculated
616 with conidia suspension (10^5 conidia mL^{-1}) of each *Monilinia* spp. and incubated for 5 day at 20 °C
617 and 85% RH. Control consisted of NYDA without L1 or L8. Control consisted of filters paper
618 spread with sterile water without phenethyl alcohol. Within the same stone fruit and *Monilinia* sp.
619 different letters represent significant differences among the treatments according to Tukey's HSD
620 Test ($\alpha = 0.05$).



621

622 Figure 3. Effect of volatile organic compounds (VOCs) produced by L1 strain on cherry, peach, and

623 apricot artificially inoculated with conidia suspensions of *M. laxa*, *M. fructicola*, *M. fructigena*, and

624 *M. polystroma*.

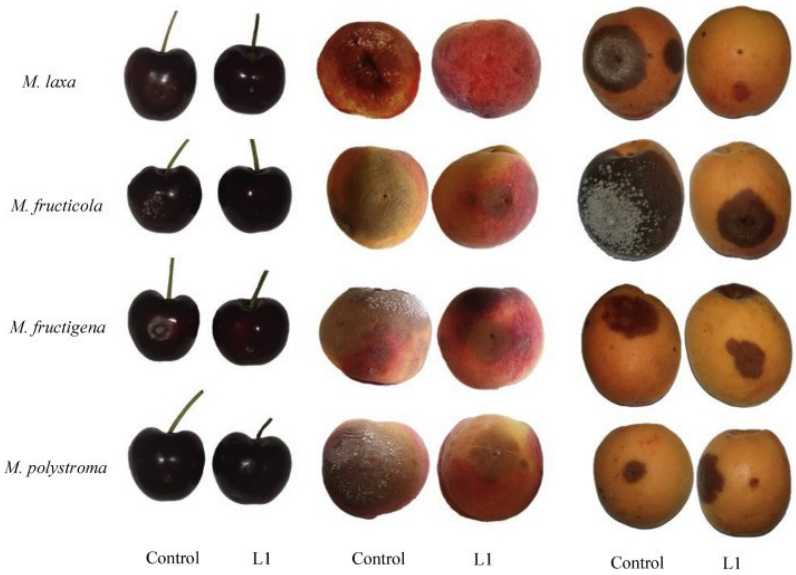
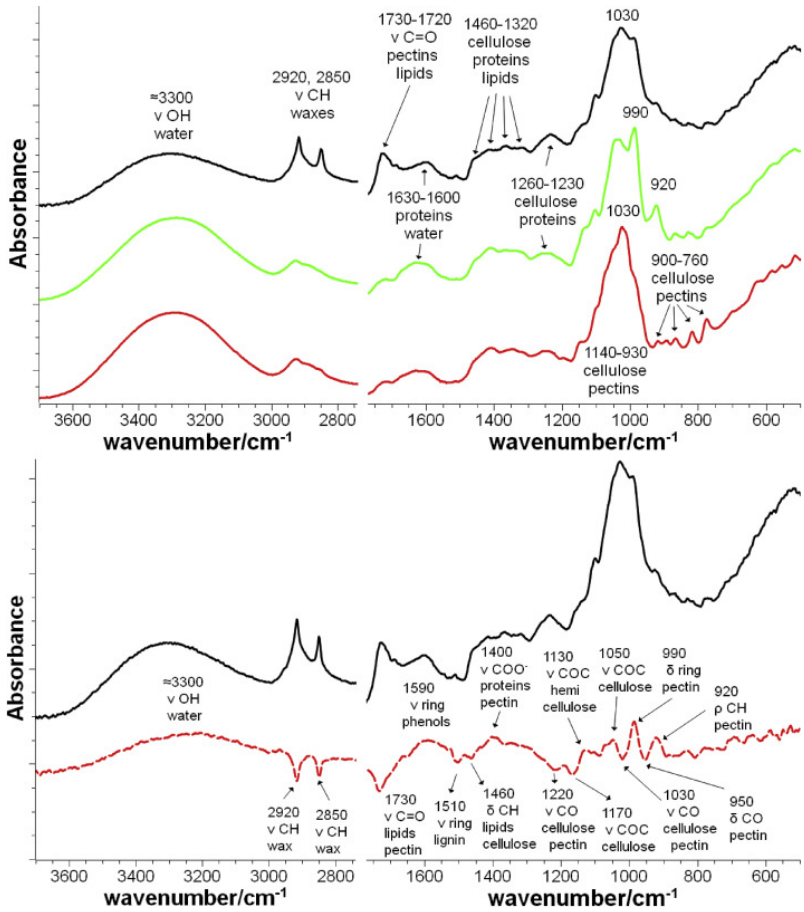


Figure 4. ATR/IR spectra of control fruits: apricot (black line), peach (green line) and cherry (red line), together with the attribution of the main spectral regions and ATR/IR spectra of control apricot at 96 h (black line) and the difference spectrum between L1 treated and control apricot at 96 h.



632

633 **Tables**

634 Table 1. Cadophora luteo-olivacea (Cad21) quantification (expressed as pg of C. luteo-olivacea
635 DNA/mg of kiwifruit tissue) on artificially wounded-inoculated kiwifruits stored for 4 months at 1
636 °C. Fruits (10 for each condition). were previously treated with sterile water (control) Pseudomonas
637 synxantha (117-2b) and Scholar® and successively inoculated with the pathogen conidial
638 suspension. Data reporting different letters are significantly different according to Tukey's test (α =
639 0.05).

640

641

642

Synthetic Compounds	*M. laxa*		*M. fructicola*		*M. polystroma*		*M. fructigena*	
	Ø	Cfu	Ø	Cfu	Ø	Cfu	Ø	Cfu
Phenethylalcohol	/	0.012	/	0.013	/	0.006	/	0.010
1-Propanol, 2-methyl	0.019	n.i.	0.021	0.022	0.019	0.115	0.127	n.i.
1-Butanol, 3-methyl	0.012	0.015	0.015	0.015	0.010	0.110	0.013	0.115
1-Butanol, 2-methyl	0.012	0.016	0.017	0.015	0.011	0.112	0.014	0.012

643 n.i.= no
644 mycelium or CFU growth inhibition observed
645 / = 100% inhibition at each dose concentration

646

647 Table 2. Summary of the results of the ATR/IR difference spectra between treated fruits (L1 and L8
648 strains) and control fruits for each sampling time. The + and - signs indicates an increase or a
649 decrease of the IR bands in the treated fruits respectively. Attribution of IR bands was performed
650 according to literature: Jamal et al. (2015), Oliveira et al. (2016),Kacurakova et al. (1999), Fasoli et
651 al., (2016), Grassino et al. (2016), Synytsya et al. (2003), Bertoluzza et al. (1994), Aghdam and
652 Bodbodak (2013).

CHERRY								APRICOT								PEACH									
CELLULOSE/HEMICELLULOSE																									
L1				**L8**				**L1**				**L8**				**L1**				**L8**				**Band**	**Attribution**
0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72		
-		+	+		+	+	+			+	+		-	+	+			+		+	+			1430	δ CH cellulose
-	+	+	+	-	+	+	+	+	+	+	+	-	-		+	-		+	+	-	-	+	+	1370	δ CH cellulose/hemicellulose
-	-	+	+	-	-	-	-	+	+	+	+	-	-		+	-	-	+	+	+	+	+	-	1342	δ CH hemicellulose
		+	+		+	+	+				+	-	-		+	-	-	+	+	-	-	+	+	1314	δ CH cellulose
			+					+	+	+		-	-		+	+		-	-	+				1295	δ CH cellulose
		+	+			-	-	+	+	+	+	+	-	-		+	-	-		-	-	-	+	1130	ν COC hemicellulose
-								+	+	+	+	+	+	+	+	+	+	+				+	+	1065	ν CO + ν CC hemicellulose
				-							+	+				+	+	+	+	+	+	+	+	1050	ν CO cellulose
	-	-	-	-	-	-	-	+	+	+	+	+	+									+	+	1030	ν COH cellulose
+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-				+	-			+	850	δ CH hemicellulose
-	-	-	+	-	-	-	+		+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	670	δ COH cellulose
-	-	-		-	-	-	-	+	+	+	+	+	+	-	-	+	+	-		+	+	-	-	590	ν CCO hemicellulose
	+	+	+	+	+	+	+	-	-	-	+	-	-	-	-	+	+	-	-		+	-	-	530	ν CCO hemicellulose
	-	-	-	+	-	-	-	-	-	-	+	+	+	-	-	+	+	-	-	+	+	-	-	510	δ skeletal cellulose

PECTIN AND MONOSACCHARIDES																									
0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72		
	+	+	+	+	+	+	+	-	-	-	-	-	-				+	+	-	-	-			1740	ν C=O esterified pectins
	+	+	+	+	+	+	+	+	+	+	+		-	+	+	-	-	-	-	+	-	-	-	1610	ν C=O carboxyl pectins
+	+	+	+	-	-	-	-	+	+	+	+	-	-		+		+	+	-	+	-	-	-	1400	ν COO^- pectins
		+	+	+				+	+	-	-				-	-						-	-	1210	ν CH_3CO pectins
-	-	+	+		+	+	+	+	+	+	+	-	+	+	+	-		+	+	-	-	+	+	1105	ν CO + ν CC pectins
	-	-	-	-	-	-	-	-	-	-	-	-	-			-	-	-		-	-	-	-	1015	ν CO + ν CC pectins
+	+	+						+	+	+	+	+		+	+		-	-	+		+	+	+	990	ν OCH_3 pectins
	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+		-	+	+	956	ν CCO + ν CCH galactose
+	+	+	+	+	+	+	+	+	+	+	+		-	-	+	+	+	+	+	-	-	+	+	920	ν CCO + ν CCH fructose and glucose
	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-				-	885	δ CCH + ν CC + δ CCH fructose
-	-	+	+	+	+	+	+	+	+	-	-	+		-	-	-	+			-			-	810	ν CC fructose
-	-	-	-	-	-	-	-				+	+	+	+	+	-	+			+	+	-	-	775	δ CCO + δ CCH fructose and glucose
	+	+	+	+	+	+	+				+	+		-	-		-			-				756	δ CCO + δ CCH galactose
	-	-	+	-	-	-	-				+	+	-	-		+	-	-	-			+	+	702	δ COH pectins
-	-	-	+	-		+	+	-	-	+	+			+	+			-		+	+			685	δ ring pectin
-	-	-		-	-	-	+			+	+		-	+	+	+		-	-		+	-	-	650	δ ring pectin
-	-	-	+		-	-	-	+	+	+	+		-	-	+	-	-	-	+			-	-	630	δ CC pectin
LIPIDS																									
0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72		
								+	+	+	+			+	+	-	-	-	-	-				3000	ν CH unsaturated lipids
	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+			+	+			2920	ν CH saturated lipids
	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+		2850	ν CH saturated lipids
	+	+	+	+	+	+	+	-	-	-	-	-	-				+	+	-	-	-			1735	ν C=O waxes
-	+	+	+		+	+	+			-	-	-	-				-	-	-	-	-			1700	ν C=O free fatty acids
-	+	+	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+		-	1462	δ CH waxes
-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-		+	+			1170	ν_s COC waxes
-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	720	ρ CH_2 waxes
PROTEINS																									
0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72		
	-	+		-	-	-	-	+	+	+	+				+		+	-	-		+	-	-	3270	ν NH

-	-	-		-	-	-	-	+	+	+	+	+	-	-	-	-	-				-		-	-	2875	ν CH_3
-	-	-	-	-	-	-	-	-		+	+	+	+	-	-	+		-	-	+	+	-	-	1630	Amide 1
-	-	-	-		+	+	+	+	+	+	+				+			+	+	-	-	+	+	1540	Amide 2
+	+	+	+	-	-	-	-	+	+	+	+	-	-		+		+	+	-	+	-	-	-	1400	ν COO^-
	+	+	+	+	+	+	+	-	-	-		-	-		-	-	+	+	+		-	+	+	1240	Amide 3
AROMATIC COMPOUNDS																										
0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72		
+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+			+	+	-	-	1610	ν C=O α,β phenolics and flavonoids
																									ν C=C lignin
+	+	+	+		+	+	+	+	+	+	+				+	+	+				+	+		1580	ν C=C phenolics, anthocyanins, flavonoids
		+	+			+	+	+	+	+	+	-	-		+		+	+	+	-	-	+	+	1565	ν C=C + ν C=O flavonoids
	+	+	+			+	+	+	+	+	+	+	+	+	+									1530	ν C=C + ν C=O flavonoids
+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-									1510	Ring def. lignin
+	+	+	+	+	+	+	+			+	+	+	+	+	+		-	-	-	+			-	1480	Ring def. flavonoids
	+	+	+	+	+	+	+		-	-	-							-	-	+	+	+	+	1270	ν CO lignin
			+						-	+	+		-	-	+				-		+	+		1190	ν aromatic lignin