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# Fungal transformation and reduction of phytotoxicity of grape pomace waste

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**Abbreviations**

ATR – Attenuated Total Reflectance; BSTFA – N, O-Bistrifluoroacetamide; FTIR – Fourier Transform Infrared spectra; GC×GC-MS – comprehensive two dimensional gas chromatograph-quadrupole mass spectrometer; GP – grape pomace; SF – solid fraction; SSF – solid-state fermentation; TRSC – total reducing sugar content; UGP – uninoculated grape pomace; $WSF_d$ – dry basis water-soluble fraction; $WSF_w$ – wet basis water-soluble fraction.

1 **Abstract**

2 Grape pomace (GP) from *Vitis labrusca*, the main byproduct from "American table wine"

3 production, is recalcitrant to degradation, and its accumulation is a serious problem with negative

4 environmental impacts. In this work, transformation of grape pomace using a steam pretreatment

5 followed by incubation of GP during a 90-day period with six different fungi were evaluated.

6 Several fungi tested reduced the phytotoxicity of water-soluble fraction ($WSF_d$) from steam-

7 pretreated GP after 90 days' incubation to lettuce and tomato seeds. *U. botrytis* caused the largest

8 effective phytotoxicity reduction of $WSF_d$ (used in the concentration range of 10-1.25% p/v) and

9 was the only fungus causing the removal of monoaromatic compounds. Therefore, this procedure

10 with *U. botrytis* effectively reduces the availability of phytotoxic monoaromatic compounds in

11 GP, which opens a way for the development of guidelines for the management of these wastes

12 and their potential use as organic amendments in agricultural soil.

13 **Keywords:** grape pomace; *Vitis labrusca*; fungi; waste revalorisation; *Ulocladium botrytis*.

**1. Introduction**

15 Winemaking using *Vitis* grapes generates large quantities of waste byproducts including

16 grape pomace (GP) that, if inadequately managed, causes environmental and economic

17 problems. GP, the main solid winemaking residue, is composed of seeds, skins, so is rich in

18 lignified fibre and soluble compounds such as phenolics and sugars (Guerra-Rivas et al., 2017).

19 Alternative solutions to exploit and add value to GP are of interest to the scientific and

20 industrial communities because they would produce economic, social and environmental

21 advantages (Jara-Palacios et al., 2016). GP has potential as a raw resource to obtain value-added

22 products: spirits, grape seed oil, antibacterials, anthocyanins, organic acids (tartaric and citric),

23  ethanol, butanol, lignin, cellulose and hemicellulose as sources of fermentable sugars and a broad

24  spectrum of polyphenols and other tannins with strong antioxidant activity and health promoting

25  properties (Rockenbach et al., 2011; Prozil et al., 2012; Pervin et al., 2014; García-Lomillo and

26  González-San José, 2017; Karpe et al., 2017). GP has been evaluated as a substrate for

27  production of medicinal mushrooms (Zervakis et al., 2013), microbial protein (Sotiropoulou et

28  al., 2017) and as a biosorbent for the removal of pollutants, heavy metal ions, xenobiotics and

29  mycotoxins (Belayachi et al., 2015; Avantaggiato et al., 2014). However, GP is mainly used for

30  soil conditioning, as a bulking agent in composting processes or as low-cost raw material for

31  animal feed (Spigno et al., 2008). Other applications for GP could be as fertiliser, support for

32  immobilization of yeast in winemaking (Genisheva et al., 2012), or as an additive to culture

33  medium for fungi to enhance production of biotechnologically important enzymes (e.g., laccase

34  and several hydrolases) (Lorenzo et al., 2002; Botella et al., 2007).

35  Although these alternative uses for GP should minimise its significant environmental

36  impact (Federici et al, 2009; García-Lomillo and González-San José, 2017), pre-processing steps

37  are needed to avoid limitations associated with this byproduct. GP has a high water retention

38  capacity (between 55 and 75 %), a feature that limits its chemical stability and enhances the

39  deterioration of GP by various pests and pathogenic microorganisms that cause GP spoilage

40  (Spigno et al., 2013; García-Lomillo and González-San José, 2017). The presence of lignin in

41  GP, which is very resistant to degradation, limits the accessibility to polysaccharides (Rouches et

42  al, 2016). Additionaly, hydroxilated and metoxilated monoaromatic compounds originating from

43  lignin are known for their strong phytotoxic effects and antibacterial activity (Di Gioia et al.,

44  2002).

45    Pre-processing should reduce oxidation and microbial action that can spoil GP, re-balance

46    the unfavourable C:N ratio in order to shorten its decomposition time in soil, break down

47    linkages between polysaccharides and lignin and reduce the concentrations of monoaromatic

48    compounds (Pedroza et al., 2012, Di Gioia et al., 2002, Rouches et al, 2016). Pre-treatment

49    methodologies to prevent deterioration of fresh GP include dehydration, sonication, thermal or

50    chemical processes and biological pre-treatment using fungi (Ratti, 2001; Singh and Heldman,

51    2009; Pedroza et al., 2012; Rouches et al, 2016). Although they can reduce GP's weight and

52    volume, which improves its handling and storage, physical, structural and chemical changes

53    occur (Karpe et al., 2017). Pre-treatment processes affect the availability and reactivity of GP

54    components such as volatiles, polyphenols and chemical groups, so the properties of final

55    materials obtained must be analysed. Clearly, pretreatment influences the future purpose of

56    pretreated GP, and any additional chemical and biotechnological processes needed prior to its

57    complete valorisation. The goal is complete exploitation of the byproduct, to remarkably

58    improve the environmental and economic sustainability of winemaking.

59    Although wine is mainly produced from *Vitis vinifera*, other *Vitis* species are utilised in

60    regions unfavourable for *V. vinifera* cultivation. In Brazil and regions neighbouring Rio de La

61    Plata, Argentina *V. Labrusca* is the dominant cultivar (De Castilhos et al., 2012; Velarde et al.,

62    2013). To date, GP in these regions and others often accumulates without any pretreatment over

63    long periods of time, leading to deleterious environmental impacts and phytosanitary risks. Other

64    problems related to the management of GP include its generation in large amounts during a short

65    period (February and March annually), low pH and high contents of phytotoxic and antibacterial

66    phenolics that resist biological degradation. Therefore, technological procedures allowing the

67    profitable and environmentally sound use of GP must be explored. Although most studies about

3

68 revalorisation of GP and its fungal transformation are on *V. vinifera* cultivars, relatively few are

69 on *V. labrusca* (Ribeiro et al., 2015; Rockenbach et al., 2011; Haas et al., 2017).

70 The aim of this work was to study the effect of combined thermal and biological pre-

71 treatment on the overall chemical composition of *V. labrusca* GP. In the present study heat

72 treatment of GP, followed by inoculation with six different fungi was analysed based on GP

73 weight loss, FT-IR spectroscopy, and GCxGC-MS analysis. Finally, the phytotoxicity of a dry

74 basis water-soluble fraction ($WSF_d$) was analysed on lettuce and tomato seeds.

75 **2. Materials and methods**

76 *2.1. Grape pomace material, pretreatment with steam and production of wet basis water-soluble*

77 *fraction ($WSF_w$)*

78 Fresh GP, which consisted of pressed grape residue (mixture of seeds and skins), was

79 collected from a local manufacturer (Cooperativa de la Costa de Berisso, Berisso, Argentina) that

80 produces *V. labrusca* wine from the Isabella cultivar (Velarde et al., 2013). GP was obtained

81 using a composite random sampling method (Dick et al., 1996) during the harvest season on

82 March 15, 2015. The material collected from different piles of pressed grape residues was pooled

83 to form a composite sample of GP. It was dried in an oven with forced air circulation at 60 °C for

84 48 h, fractionated in autoclavable bags, treated by autoclaving at 121 ºC for 30 min and then

85 stored at room temperature and protected from light until use.

86 A wet basis water-soluble fraction ($WSF_w$) from the steam-treated GP in a 1:10 (w/v)

87 proportion was obtained according to Inalbón et al. (2015) and then analysed for pH (3.55 ±

88 0.02), phenols (12.62 ± 0.96 mg/100 mL) according to the Folin-Ciocalteau method (Saparrat et

89 al., 2010a), chromophores (optical density at 395 nm; Aloui et al., 2007; 0.27 ± 0.02), $NH_4^+$-N

90    (4.09 ± 0.10 mg/100 mL) according to the Saparrat et al. (2010b) method and reducing sugars

91    (0.96 ± 0.05 mM) by the Somogy-Nelson method (Somogyi, 1945).

92    *2.2. Fungal isolates*

93    Six fungi, previously isolated from various sources including decaying wood, soil and

94    leaf litter, which belong to different ecophysiological groups were used: white rots, *Coriolopsis*

95    *rigida* (Berk. & Mont.) Murrill LPSC (Culture Collection of the Instituto Spegazzini,

96    Universidad Nacional de La Plata, La Plata, Argentina) 232 (Saparrat et al. 2002b), *Peniophora*

97    *albobadia* (Schwein.) Boidin LPSC 285 (Saparrat et al., 2008) and *Pycnoporus sanguineus* (L.)

98    Murrill LPSC 163 (Saparrat et al., 2000); a brown rot fungus, *Gloeophyllum sepiarium* (Wulf.:

99    Fr.) P. Karst. LPSC 735 (Murace et al., 2016); a soil fungus, *Trichoderma harzianum* Rifai

100   FALH (Facultad Ciencias Agrarias y Forestales, UNLP) 18 (Stocco et al., 2015) and a litter

101   degrading fungus, *Ulocladium botrytis* G. Preuss LPSC 813 (Saparrat et al., 2008). These strains

102   were selected due to their ability to grow on different lignocellulosic materials. Stock cultures

103   were kept at 4 °C on 2 % (w v$^{-1}$) agar-malt extract slants.

104   *2.3. Fungal treatment of steam-pretreated grape pomace under solid-state fermentation (SSF)*

105   *conditions, its fractions and analytical determinations*

106   Fungal growth on steam-pretreated GP under SSF conditions was evaluated in

107   autoclavable bags containing 30 g of sterile dry material at a humidity level adjusted to 70 %.

108   Cultures grown on 2 % malt extract agar for 7 days were used for inoculation. Each bag was

109   inoculated axenically with 6 mL of a 0.15 % (mass vol$^{-1}$) mycelial suspension (Inalbon et al.,

110   2015). Inoculated bags were incubated at 28 ± 1.5 ºC in the dark for 90 days in a humidified

111   chamber, as previously reported (Saparrat et al, 2010b). Sterile uninoculated grape pomace

112    (UGP) incubated under the same conditions for the same period of time was used as control.

113    Each treatment was prepared in triplicate. Ninety days after inoculation, a $WSF_w$ was obtained

114    from an aliquot of both uninoculated and fungal-inoculated steam-pretreated GP as described.

115    Then, all the solid content (i.e., solid fraction; SF) of each bag was dried at 60 °C to a constant

116    mass, and degradation was measured as a percentage of GP weight reduction (percentage

117    reduction of inoculated substrate in relation to the uninoculated one. Each dried SF sample was

118    ground in an agate mill, and sieved through a <1 mm screen (Saparrat et al, 2010b). The resultant

119    material was kept at 25 °C and stored without exposure to atmospheric moisture until its use for

120    chemical analysis and the phytotoxicity assay. The pH, phenols, chromophores, $NH_4^+$-N and

121    total reducing sugar content (TRSC) of the $WSF_w$ from control and inoculated steam-pretreated

122    GP were determined.

123    *2.4. Preparation of methanol extracts of SF*

124        To prepare GP methanol extracts, 30 mg of steam-pretreated GP SF was extracted with

125    methanol (150 µL) in an ultrasonic bath for 20 min. After sonication, the samples were filtered

126    through Whatman filter No. 4, and supernatants were evaporated in a stream of nitrogen to

127    dryness. The extracts were stored at room temperature for further analyses (Devrnja et al., 2017).

128    *2.5. Structural instrumental analysis*

129    *2.5.1. Chromatographic analysis*

130        The methanol extracts were derivatised with BSTFA for 45 min at 60 °C and analysed

131    using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) comprehensive two dimensional gas

132    chromatograph-quadrupole mass spectrometer (GC×GC-MS) with ZX2 thermal modulation

133    system (Zoex Corp.). A RtxR-1 (RESTEK, CrossbondR 100% dimethyl polysiloxane, 30 m, 0.25

134 mm ID, df=0.25 μm) and a BPX50 (SGE Analytical Science, 1 m, 0.1 mm ID, df=0.1 μm)

135 columns were connected through the GC×GC modulator as the first and second capillary

136 columns, respectively. The oven was programmed at an initial temperature of 40 °C for 5 min,

137 than ramped at 5.2 °C min$^{-1}$ to 300 °C. The modulation period was 9 s. The GC×GC-MS data

138 were analysed using Chrome Square 2.1 software, and MS spectra were compared with the MS

139 libraries NIST 11, NIST 11s, and Wiley 8.

140 *2.5.2. FT-IR measurement*

141       Fourier Transform Infrared (FTIR) spectra were recorded using a Thermo Scientific

142 Nicolet 6700 FT-IR Spectrophotometer, using attenuated total reflectance (ATR) technique from

143 Smart accessory with diamond crystal (Smart Orbit, Thermo Scientific, Madison, WI, USA).

144 Spectral data were collected in the mid-IR range (1800–600 cm$^{-1}$) with 64 scans and resolution

145 of 4 cm$^{-1}$ for all methanol extracts of: uninoculated GP (UGP) before incubation (0 days); UGP

146 after 90 days' incubation, and; GP inoculated with fungi after 90 days from inoculation.

147 Methanol solutions were applied on the ATR crystal using a capillary. Solvent was evaporated

148 by a nitrogen stream in order to obtain thin ATR films. The spectra were fitted using OMNIC

149 software (Version 7.0, Thermo Scientific, USA). A curve-fitting procedure was used to

150 decompose each original spectrum to its Voigt curve constituents that could be assigned to

151 certain structural features. The contribution of each curve was assessed by integrating the area

152 under the curve, and then normalising it to the total area of all spectra. Vibrational bands were

153 assigned based on literature data (Pretsch et al., 2000).

154 *2.5.3. Elemental analysis*

155    To assess the content of carbon, hydrogen, nitrogen, oxygen and sulphur, the steam-

156    pretreated GP SF powders were analysed using a Vario EL III CHNS/O Elemental Analyzer.

157    *2.6. Germinability assays*

158    The effect of a $WSF_d$ obtained from each steam-pretreated GP SF powder from both

159    uninoculated and fungal-inoculated GP after 90 days' incubation was determined on germination

160    of lettuce (*Lactuca sativa*) and tomato (*Lycopersicon esculentum*) seeds according to the method

161    described by Zucconi et al. (1981). Each $WSF_d$ at 10% (p/v), obtained according to Gopinathan

162    and Thirumurthy (2012), was filtered through a 0.2 μm pore membrane before use. The seeds

163    were surface-sterilised using 1% sodium hypochlorite (NaClO) solution at for 1 minute and

164    washed 6-7 times with sterile distilled water to remove traces of NaClO. In sterile Petri plates,

165    sterile filter paper was kept soaked in $WSF_d$, and sterile distilled water soaked filter paper was

166    used as a control. Twenty (lettuce) or fifteen (tomato) seeds were kept in each Petri plate and the

167    experiment was conducted in triplicate. These experiments were performed on undiluted and

168    variably diluted (1:2, 1:4, 1:8 in water) $WSF_d$. Seed germination was observed for seven days.

169    The experiment was conducted in the dark at 25 °C. The number of germinated seeds was

170    counted and radial growth measured. The germination index (GI) was calculated according to the

171    formula GI = (G/Go) x (L/Lo) x  100, where G and Go are the number of grown seeds in sample

172    and control and L and Lo are the average sum of root lengths in sample and control, respectively.

173    *2.7. Statistical analysis*

174    Mean and standard deviation were calculated from data obtained for each treatment.

175    Results were analysed by a one-way ANOVA and means of all variables were contrasted by

176    Tukey's test (Statistix 8.0).

8

## 3. Results

*3.1. Physical and chemical properties of steam-treated grape pomace*

We monitored several physical and chemical properties of steam-treated GP inoculated with fungi after 90 days' incubation and its corresponding $WSF_w$. Only some slight differences were found in ammonium and phenolic levels of $WSF_w$ obtained from UGP after 90 days' incubation compared to those in $WSF_w$ from UGP at the beginning of the incubation. The six fungal strains examined were able to grow and colonise steam-pretreated GP, using it as the only substrate source in SSF conditions and reducing its weight and modifying several physico-chemical features (**Table 1**). The greatest weight loss was caused by *U. botrytis* (up to 32% after 90 days), which degraded the GP much faster than the other fungal species. Substrate weight loss caused by *C. rigida*, *G. sepiarium*, *Pe. albobadia*, *Py. sanguineus* and *T. harzianum* was between 18 and 26% at the end of the incubation period, although differences were not always statistically significant. The chemical composition of the $WSF_w$ extracted from UGP and GP treated with each fungus differed widely. The pH of all fungi-inoculated steam-pretreated GP increased slightly compared to the UGP control after 90 days. The most prominent pH increase was measured in the steam-pretreated GP inoculated with *U. botrytis*. All the fungi examined increased the $NH_4^+$-N content in $WSF_w$, except *U. botrytis*. While lower TRSC was found in the $WSF_w$ from material inoculated with *Pe. albobadia*, followed by those inoculated with *C. rigida* and *T. harzianum*, *G. sepiarium* and *Py. sanguineus* slightly increased the TRSC. Regarding the phenolics in $WSF_w$, apart from in the steam-pretreated GP inoculated with *G. sepiarium*, there was a drastic decrease in all other inoculated, steam-pretreated GP. The highest phenol reduction was caused by *Pe. albobadia*. The level of chromophores in $WSF_w$ was only increased by *U. botrytis* compared to the UGP.

9

200 **Table 1.** Steam-pretreated *Vitis labrusca* GP inoculated with fungi selected and uninoculated grape pomace after 90

201 days of incubation: weight loss and physico-chemical parameters in the $WSF_w$[a].

| Parameter | UGP | | LPSC 232 | | LPSC 285 | | LPSC 163 | | LPSC 735 | | FALH 18 | | LPSC 813 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GP mass loss (%) | - | | 18.04 ± 2.84 | d | 20.03 ± 1.57 | cd | 21.75 ± 1.32 | c | 25.63 ± 2.83 | b | 22.42 ± 1.93 | c | 32.05 ±1.72 | a |
| pH | 3.63 ± 0.05 | e | 4.87 ± 0.12 | b | 4.01 ± 0.02 | d | 4.17 ± 0.12 | cd | 4.37 ± 0.07 | c | 4.25 ± 0.49 | cd | 8.41 ± 0.09 | a |
| $NH_4^+$-N (mg/100 ml) | 6.97 ± 0.40 | d | 15.24 ± 1.01 | a | 14.23 ± 1.39 | a | 15.10 ± 1.50 | a | 12.34 ± 1.77 | b | 9.44 ± 0.96 | c | 2.76 ± 0.75 | e |
| Phenols (mg/100 ml) | 11.91 ± 0.40 | b | 2.56 ± 0.54 | e | 1.04 ± 0.06 | f | 4.30 ± 0.81 | d | 13.44 ± 1.01 | a | 6.93 ± 0.75 | c | 7.77 ± 0.35 | c |
| TRSC (mM) | 0.95 ± 0.02 | b | 0.58 ± 0.06 | d | 0.38 ± 0.03 | e | 1.08 ± 0.03 | a | 1.09 ± 0.10 | a | 0.75 ± 0.03 | c | 0.90 ± 0.10 | b |
| Abs. $_{395\,nm}$ | 0.15 ± 0.02 | c | 0.22 ± 0.04 | bc | 0.15 ± 0.01 | c | 0.17 ± 0.01 | c | 0.47 ± 0.06 | b | 0.19 ± 0.03 | bc | 1.67 ± 0.52 | a |

202 [a]means ± SD of four replicates; data followed by the same letter are not significantly different (Tukey's test $p <$

203 0.05).

204

205 *3.2. Instrumental analysis of grape pomace methanol extracts*

206 Elemental analysis showed that control samples of steam treated GP at the beginning of

207 the experiment and after 90 days (UGP 0 and UGP 90) had very similar elemental composition

208 (1.9% of N, 39.2-39.4 % of C, 4.9-5 % of H), which might suggest that the overall elemental

209 composition in controls had not changed over time. Samples inoculated with fungi had between

210 2.5-3.8 % of N, 36.5-44.1 % of C and 4.6-5.8 % of H. The highest percent of all elements was in

211 the sample inoculated with LPSC 813, which could be due to the highest fungal growth. In all

212 samples, sulphur was below the limit of detection.

213 The presence of monoaromatics in uninoculated and inoculated GP was determined using

214 comprehensive two dimensional gas chromatography. Results (**Fig. 1A-B**; **Table 2**) showed

215 several monoaromatics were present in steam-pretreated GP at the beginning of the experiment.

216    However, in uninoculated steam-pretreated GP after 90 days' incubation, additional

217    monoaromatics appeared (isovanillic, protocatechoic, cinnamic). All steam-pretreated GPs

218    inoculated with fungi had several other monoaromatics present in addition to those found in the

219    UGP after 90 days' incubation, and the list of compounds present in each is given below (**Table**

220    **2**; **Fig. 1C-H**). The exception was steam-pretreated GP inoculated with *U. botrytis* (**Fig. 1F**),

221    which did not contain any monoaromatics.
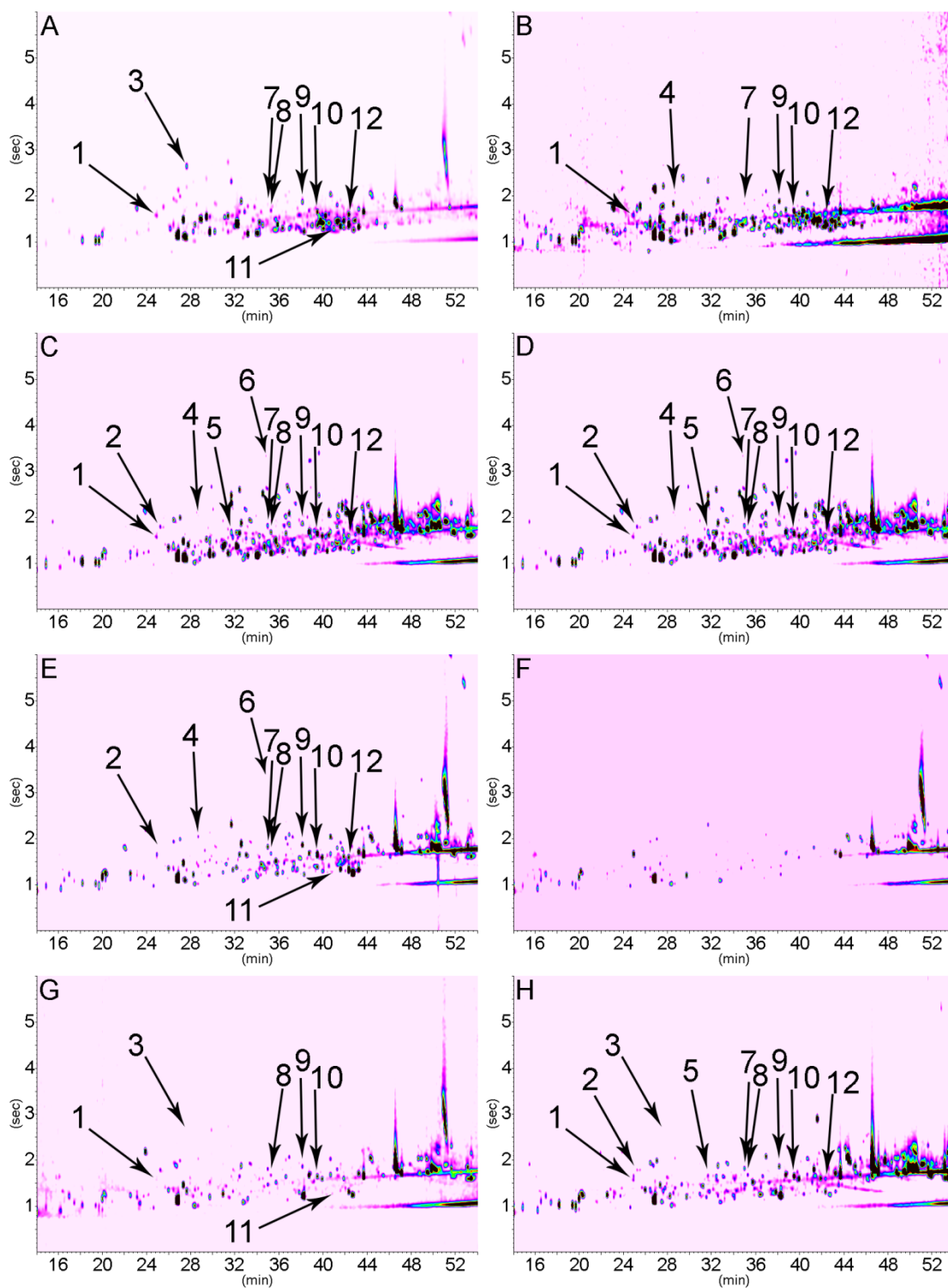
225    *Peniophora albobadia* LPSC 285 after 90 days' incubation; **(D)** steam-treated grape pomace inoculated with

226    *Pycnoporus sanguineus* LPSC 163 after 90 days' incubation; **(E)** steam-treated grape pomace inoculated with

227    *Trichoderma harzianum* FALH 18 after 90 days' incubation; **(F)** steam-treated grape pomace inoculated with

228    *Ulocladium botrytis* LPSC 813 after 90 days' incubation; **(G)** steam-treated grape pomace inoculated with

229    *Coriolopsis rigida* LPSC 232 after 90 days' incubation; **(H)** steam-treated grape pomace inoculated with

230    *Gloeophyllum sepiarium* LPSC 735 after 90 days' incubation (the numbers in the chromatogram correspond to the

231    number of compound in the **Table 2**).

232    **Table 2.** Compounds identified by GCxGC-MS analysis, detected (+) and not detected (-) in each sample from both

233    uninoculated (control, UGP) and fungal-inoculated grape pomace after 90 days' incubation[a].

| No. | Compound | tR | 2tR | 0 days UGP | 90 days | | | | | | |
|-----|----------|-----|-----|------------|---------|---------|---------|---------|---------|---------|---------|
| | | | | | UGP | LPSC 232 | LPSC 285 | LPSC 163 | LPSC 735 | FALH 18 | LPSC 813 |
| 1 | 2-Phenylethanol, TMS | 24.944 | 0.81 | + | + | + | + | + | + | - | - |
| 2 | Benzoic acid, TMS | 25.248 | 1.89 | - | - | - | + | - | + | + | - |
| 3 | Benzeneacetic acid, TMS | 26.749 | 2.64 | + | - | + | - | + | + | - | - |
| 4 | 4-Hydroxybenzaldehyde, TMS | 28.702 | 1.5 | - | + | - | + | + | - | + | - |
| 5 | Mandelic acid, 2TMS | 31.695 | 1.32 | - | - | - | + | - | + | - | - |
| 6 | Benzophenone | 34.873 | 1.38 | - | - | - | + | - | - | + | - |
| 7 | Salicylic acid, 2TMS | 34.998 | 1.26 | + | + | - | + | + | + | + | - |
| 8 | 4-Hydroxybenzoic acid, 2TMS | 35.295 | 1.41 | + | - | + | + | + | + | + | - |
| 9 | Isovanillic acid, 2TMS | 38.148 | 3.24 | + | + | + | + | + | + | + | - |
| 10 | Protocatechoic acid, 3TMS | 39.495 | 1.41 | + | + | + | + | + | + | + | - |
| 11 | Cinnamic acid, 2TMS | 41.599 | 0.99 | + | - | + | - | - | - | + | - |
| 12 | Gallic acid, 4TMS | 42.344 | 0.96 | + | + | - | + | + | + | + | - |

234    [a]The GC×GC-MS chromatograms of steam-treated GP inoculated with other fungi is given in **Fig. 1**.

235

236    In order to monitor the changes in structure of GP, in addition to GC×GC-MS, methanol

237    extracts of UGP and inoculated GP were analysed using FTIR. **Fig. 2** shows that steam-

238    pretreated GP inoculated with *U. botrytis* had a spectrum that differed from those of the controls

13

239    (UGP at the beginning of the experiment and after 90 days). The bands at 1740 cm$^{-1}$ and 1712

240    cm$^{-1}$ were attributed to the stretching vibrations of the acid carbonyl groups, and bands from

241    1690 cm$^{-1}$ belong to vibrations of the fatty acid carbonyl groups. Numerous bands attributed to

242    aromatic C-H and O-H in plane deformation vibrations of phenols were present from 1460 to

243    1020 cm$^{-1}$. An additional, wide peak at 1563 cm$^{-1}$ was present in steam-pretreated GP inoculated

244    with *U. botrytis* after 90 days; that peak corresponded to amide I and amide II of methanol

245    soluble proteins from fungus (Pretsch et al., 2000; Naumann, 2009; Lecellier et al., 2015). The

246    content of soluble phenols and absorbance at 395 nm, which depend on the presence of aromatic

247    groups (such as tyrosine, tryptophan, phenylalanine), were higher in steam-pretreated GP

248    inoculated with *U. botrytis* than in the controls. This, together with the appearance of the band at

249    1563 cm$^{-1}$, could be due to increased soluble protein content resulting from fungal growth in this

250    treatment. The significant reductions of a carbonyl band at 1741 cm$^{-1}$ and a C-O anti-

251    symmetrically stretched band at 1036 cm$^{-1}$ in steam-pretreated GP with *U. botrytis* after 90 days'

252    incubation were caused by consumption of these compounds by the fungus.
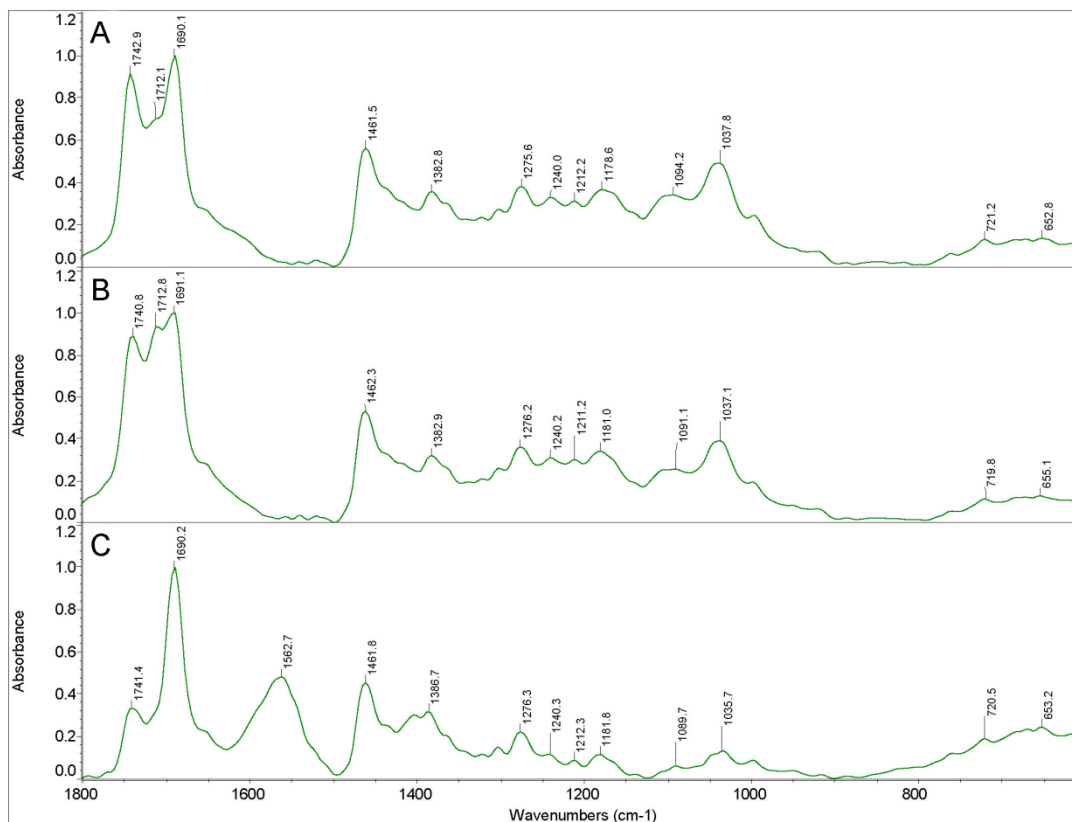
253

254    **Figure 2.** FTIR spectra of: (A) Uninoculated grape pomace (UGP) at the beginning of the experiment (0 days), (B)

255    UGP after 90 days of incubation, (C) Grape pomace inoculated with *Ulocladium botrytis* LPSC 813 after 90 days of

256    incubation.

257    *3.3. Reduction of phytotoxicity*

258         To assess the potential of each fungus to detoxify steam-pretreated GP, we tested the

259    effect of a WSF$_d$ obtained from dried GP SF powder from both uninoculated and fungal-

260    inoculated material after 90 days' incubation on lettuce and tomato germination. As shown in

261    **Table 3**, steam-pretreated UGP (10 % p/v) and GP inoculated with *G. sepiarium* (10 to 5 % p/v)

262    inhibited the germination of lettuce. However, inoculation with other fungi reduced the

263    phytotoxicity of GP on lettuce seed compared to that of the steam-pretreated UGP. The greatest

264    reduction of phytotoxicity was achieved by *U. botrytis* on steam-pretreated GP (at a

265    concentration of 1.25 % p/v) followed by *Pe. albobadia* (at a concentration of 2.5 % p/v). The

266    same five fungi (i.e., all except *G. sepiarium*) also reduced the phytotoxicity of steam-pretreated

267    GP on tomato seed, although some differences in the germination indices were found compared

268    to those measured on lettuce seed.

269    **Table 3.** Germination index (%) of lettuce and tomato seed in the presence of $WSF_d$ obtained from powder samples

270    from both uninoculated (control) and fungal-inoculated grape pomace after 90 days' incubation[a].

| $WSF_d$ (%, p/v) | Control | *C. rigida* LPSC 232 | *Pe. albobadia* LPSC 285 | *Py. sanguineus* LPSC 163 | *G. sepiarium* LPSC 735 | *T. harzianum* FALH 18 | *U. botrytis* LPSC 813 |
|---|---|---|---|---|---|---|---|
| **Lettuce** | | | | | | | |
| 10 | 0.0 ± 0.0 c | 11.9 ± 3.4 a | 18.1 ± 3.3 a | 2.8 ± 0.9 b | 0.0 ± 0.0 c | 2.2 ± 1.4 b | 5.6 ± 1.6 b |
| 5 | 9.4 ± 0.7 d | 16.4 ± 1.9 c | 33.8 ± 3.7 b | 18.0 ± 4.7 c | 0.0 ± 0.0 e | 27.0 ± 1.3 b | 56.5 ± 2.3 a |
| 2.5 | 25.3 ± 3.5 bc | 31.3 ± 0.7 b | 62.3 ± 6.6 a | 25.9 ± 2.4 bc | 16.7 ± 1.4 c | 36.1 ± 8.8 b | 69.1 ± 2.7 a |
| 1.25 | 45.8 ± 3.3 bc | 53.4 ± 11.0 b | 56.8 ± 9.6 ab | 50.4 ± 20.5 bc | 23.6 ± 2.9 c | 50.4 ± 5.2 bc | 83.1 ± 7.8 a |
| **Tomato** | | | | | | | |
| 10 | 3.3 ± 0.9 ef | 15.4 ± 2.3 a | 17.4 ± 4.9 a | 10.3 ± 2.0 bc | 0.9 ± 0.3 f | 7.9 ± 1.8 cd | 4.3 ± 0.2 de |
| 5 | 13.0 ± 2.1 b | 20.6 ± 2.7 ab | 32.5 ± 13.1 a | 18.3 ± 0.5 ab | 11.6 ± 1.1 b | 25.2 ± 9.9 ab | 33.3 ± 3.8 a |
| 2.5 | 34.3 ± 3.7 cd | 44.3 ± 7.1 bc | 54.1 ± 1.6 ab | 46.8 ± 7.1 abc | 26.3 ± 4.0 d | 42.3 ± 6.5 bc | 60.4 ± 5.3 a |
| 1.25 | 41.9 ± 1.2 cd | 65.1 ± 7.0 bcd | 76.3 ± 13.4 ab | 69.2 ± 4.5 bc | 36.8 ± 2.5 d | 58.2 ± 7.0 bcd | 90.1 ± 8.8 a |

271    [a]Data are means of three replicates ± SD (standard deviation). Row values followed by the same letter are not

272    significantly different as determined by one way ANOVA followed by Tukey's test ($p < 0.05$).

273

274    **4. Discussion**

275    Among organisms involved in revalorising organic wastes, fungi play a great role due to

276    their ability to degrade severely recalcitrant plant polymers and various toxic compounds, such

277    as phenolic compounds, as well as recycle nitrogen more efficiently than bacteria (Karpe et al.,

278    2017; Hodge et al., 2000). Six fungi, including wood-rotting Basidiomycota and two

279    Ascomycota, were inoculated in steam-treated GP from *V. labrusca* under SSF axenic

280    conditions. When compared, these fungi differently degraded GP and its components such as

281    free phenolic compounds. This could be due, at least partly, to their ability to detoxify inhibitory

282    compounds present in GP and/or to synthesise different enzymes to degrade lignocellulose.

283    However, after 90 days' incubation, *U. botrytis* caused the highest mass loss of GP and the

284    highest reduction of GP phytotoxicity compared with other fungi used. Also, it was the only

285    strain that degraded the monoaromatics found in steam-treated GP. Previous reports showed the

286    outstanding ability of *U. botrytis* LPSC 813 to attack *Scutia buxifolia* leaf-litter using its

287    cellulolytic enzyme complex and extracellular enzymes with peroxidase (EC 1.11.1.7) activity,

288    which might be considered ecologically advantageous in the colonisation of a broad spectrum of

289    aromatic-rich substrates, which might also possibly extend to the ones from GP (Saparrat et al.,

290    2008, 2010b). Extracellular oxidative enzymes in another non ligninolytic fungus (*Ciliochorella*

291    *buxifolia*) growing on *Scutia buxifolia* leaf-litter have been reported to be involved in

292    mechanisms of detoxification of phenolics (Troncozo et al. 2015). So, this latter information

293    hypothesizes that the peroxidase activity of *U. botrytis* LPSC 813 might play a key role in

294    detoxification reactions of GP phenolics. However, we did not evaluate any enzyme activity in

295    this study.

296    $WSF_w$ indirectly reflects alteration of the insoluble lignocellulose macromolecular

297    fractions (Dorado et al., 1999). Variations in sugar levels of GP transformed by our six fungi,

17

298  including those that generated reducing substances in $WSF_w$ after 90 days' incubation, could be

299  due to the relationship between the activity of their carbohydrate depolymerising enzymes and

300  their efficiency to absorb the resultant soluble sugars. However, biomass and chemical

301  composition were not monitored over the incubation period, and the different fungi likely have

302  different ecological strategies to obtain nutrients. Although fungi belonging to different

303  ecophysiological groups have different substrate decomposition strategies, lignocellulose

304  decomposition can vary among fungi belonging to the same ecophysiological group (Presley and

305  Schilling, 2017). *U. botrytis* LPSC 813 is a litter fungus with little ligninolytic activity (Saparrat

306  et al., 2008), however we speculate it might have, a yet unknown, strategy to colonise GP, giving

307  it greater ability to degrade GP than the other fungi examined and enabling it to degrade

308  monoaromatics.

309  Levels of colour and free phenols can be used as indicators of organic matter quality in

310  $WSF_w$ (Dorado et al., 1999, Saparrat et al., 2008). We found *U. botrytis*-treated GP produced a

311  strongly coloured $WSF_w$, likely due to chromophoric pigments derived from fungal

312  transformation of phenols. While our white rot fungi and ascomycetes severely reduced the free

313  phenol levels in GP, *G. sepiarium* was unable to do so. This might have some relation with

314  fungal ability to detoxify GP compounds, since *G. sepiarium* did not reduce the phytotoxicity of

315  the steam-pretreated GP when it was evaluated on lettuce and tomato seed. However, free

316  phenols cannot be uniquely responsible for the phytotoxicity of GP, as *Py. sanguineus* decreased

317  phenol levels, but this did not result in increased seed germinability. The presence of heavy

318  metals in GP and its wastewater could be another plausible cause of phytotoxicity. Although we

319  didn't analyse the content of metals in GP, Speltini et al (2011) have reported that inferior levels

320  of Cu, Pb and Cd than the maximum limit allowed by the Instituto Nacional de Vitivinicultura

321 (Argentina) are present in water samples taken from a creek that passes through vineyards in a

322 region neighbouring Rio de La Plata, Argentina (the Arroyo Sarandí basin).

323    In our study, monoaromatic compounds were the main biotransformation products of

324 lignin and related compounds. Monoaromatics were found in the uninoculated GP at the

325 beginning of the experiment (day 0), but also in the uninoculated GP after 90 days. This was

326 probably due to heat and spontaneous oxidation by molecular oxygen and its reactive species

327 (Flores et al., 2016). Removal of monoaromatics from GP by *U. botrytis* could involve two

328 possible mechanisms. Firstly, the fungus could use these compounds as sources of carbon and

329 energy (Prenafeta-Boldú et al., 2006). Secondly, *U. botrytis* might detoxify monoaromatics by

330 using its extracellular oxidative enzymes (Saparrat et al., 2008) to polymerise them. Since *U.*

331 *botrytis* is a melanin-producing fungus (Bell and Wheeler, 1986), it could consequently

332 immobilize these polymerised products in its own cell walls. The removal of monoaromatics

333 from GP by *U. botrytis* LPSC 813 could be the basis for the reduced phytotoxicity of this waste

334 towards tomato and lettuce seeds.

335    Alkalinisation of GP by *U. botrytis* can facilitate the availability of nutrients to plants, so

336 this fungal transformation could improve the ecological management and utilisation of the

337 lignocellulosic byproduct as an effective fertilizer or soil amendment. Although ammonia has

338 been considered as a key player in environmental alkalinisation by several fungi, it is not a well-

339 understood phenomenon (Vylkova, 2017). In the current study, *U. botrytis*-treated GP had a

340 lower concentration of ammonia compared to UGP or GP treated with the other five fungi. In the

341 GP treated with these five remaining fungi ammonium contents in $WSF_w$ increased compared to

342 the UGP, although they were only related to the low pH increments measured. The higher

343 ammonium levels in $WSF_w$ from these fungal cultures compared to the control could indicate

344 these fungi mineralise organic N in the GP. Nitrogen in GP is mainly incorporated in proteins (6-

345 15% dry matter in GP) (García-Lomillo and Gonzalez-San José, 2017). Therefore, the chemical

346 mechanism behind the rise in pH by *U. botrytis*-treated GP might be, at least in part, independent

347 of fungal nitrogen metabolism and/or it could be due to a combination of several processes.

348 Potential alkalinisation generating processes involved are the secretion of carbon compounds, the

349 increase in availability of ions such as $Ca^{2+}$, or the lack of nutrient availability forcing the fungus

350 to consume organic acids as nutrient sources (Danhof et al., 2016). Therefore, our results suggest

351 that *U. botrytis* is an alkalinisation bioagent for the treatment of GP. However, none of these

352 mechanisms were tested during the transformation of GP by *U. botrytis*, so further study is

353 needed.

354 **5. Conclusions**

355 Steam pre-treatment of GP followed by inoculation with six fungi results in considerable

356 alteration of the GP and its $WSF_w$ after 90 days' incubation. The combination of a thermal

357 treatment followed by incubation with *U. botrytis* reduces the phytotoxic monoaromatic

358 compounds in GP and opens the way for guidelines on management of these wastes and their

359 potential use as organic amendments in agricultural soils. The mechanisms of removal of

360 monoaromatics by *U. botrytis* LPSC 813 likely involve detoxification and/or degradation

361 reactions that should be further studied.

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371 **Author Contributions**

372      M.I.T. and M.C.N.S. were responsible for conception and design of research. M.I.T.,

373 M.L., V.P.B, P.A.B. and M.C.N.S. performed experiments. M.I.T., M.L., V.P.B., B.A., P.A.B.

374 and M.C.N.S. analyzed data. M.L., V.P.B, B.A. and M.C.N.S. prepared the figures. M.C.N.S.

375 wrote the first draft of the manuscript. V.P.B. and M.C.N.S. wrote the final version of the

376 manuscript.

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**Table 1.** Steam-pretreated *Vitis labrusca* GP inoculated with fungi selected and uninoculated grape pomace after 90 days of incubation: weight loss and physico-chemical parameters in the $WSF_w$[a].

| Parameter | UGP | | LPSC 232 | | LPSC 285 | | LPSC 163 | | LPSC 735 | | FALH 18 | | LPSC 813 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GP mass loss (%) | - | | $18.04 \pm 2.84$ | d | $20.03 \pm 1.57$ | cd | $21.75 \pm 1.32$ | c | $25.63 \pm 2.83$ | b | $22.42 \pm 1.93$ | c | $32.05 \pm 1.72$ | a |
| pH | $3.63 \pm 0.05$ | e | $4.87 \pm 0.12$ | b | $4.01 \pm 0.02$ | d | $4.17 \pm 0.12$ | cd | $4.37 \pm 0.07$ | c | $4.25 \pm 0.49$ | cd | $8.41 \pm 0.09$ | a |
| $NH_4^+$-N (mg/100 ml) | $6.97 \pm 0.40$ | d | $15.24 \pm 1.01$ | a | $14.23 \pm 1.39$ | a | $15.10 \pm 1.50$ | a | $12.34 \pm 1.77$ | b | $9.44 \pm 0.96$ | c | $2.76 \pm 0.75$ | e |
| Phenols (mg/100 ml) | $11.91 \pm 0.40$ | b | $2.56 \pm 0.54$ | e | $1.04 \pm 0.06$ | f | $4.30 \pm 0.81$ | d | $13.44 \pm 1.01$ | a | $6.93 \pm 0.75$ | c | $7.77 \pm 0.35$ | c |
| TRSC (mM) | $0.95 \pm 0.02$ | b | $0.58 \pm 0.06$ | d | $0.38 \pm 0.03$ | e | $1.08 \pm 0.03$ | a | $1.09 \pm 0.10$ | a | $0.75 \pm 0.03$ | c | $0.90 \pm 0.10$ | b |
| Abs. $_{395 nm}$ | $0.15 \pm 0.02$ | c | $0.22 \pm 0.04$ | bc | $0.15 \pm 0.01$ | c | $0.17 \pm 0.01$ | c | $0.47 \pm 0.06$ | b | $0.19 \pm 0.03$ | bc | $1.67 \pm 0.52$ | a |

[a]means ± SD of four replicates; data followed by the same letter are not significantly different (Tukey's test $p < 0.05$).

**Table 2**. Compounds identified by GCxGC-MS analysis, detected (+) and not detected (-) in each sample from both uninoculated (control, UGP) and fungal-inoculated grape pomace after 90 days' incubation[a].

| No. | Compound | tR | 2tR | 0 days UGP | 90 days | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | UGP | LPSC 232 | LPSC 285 | LPSC 163 | LPSC 735 | FALH 18 | LPSC 813 |
| 1 | 2-Phenylethanol, TMS | 24.944 | 0.81 | + | + | + | + | + | + | - | - |
| 2 | Benzoic acid, TMS | 25.248 | 1.89 | - | - | - | + | - | + | + | - |
| 3 | Benzeneacetic acid, TMS | 26.749 | 2.64 | + | - | + | - | + | + | - | - |
| 4 | 4-Hydroxybenzaldehyde, TMS | 28.702 | 1.5 | - | + | - | + | + | - | + | - |
| 5 | Mandelic acid, 2TMS | 31.695 | 1.32 | - | - | - | + | - | + | - | - |
| 6 | Benzophenone | 34.873 | 1.38 | - | - | - | + | - | - | + | - |
| 7 | Salicylic acid, 2TMS | 34.998 | 1.26 | + | + | - | + | + | + | + | - |
| 8 | 4-Hydroxybenzoic acid, 2TMS | 35.295 | 1.41 | + | - | + | + | + | + | + | - |
| 9 | Isovanillic acid, 2TMS | 38.148 | 3.24 | + | + | + | + | + | + | + | - |
| 10 | Protocatechoic acid, 3TMS | 39.495 | 1.41 | + | + | + | + | + | + | + | - |
| 11 | Cinnamic acid, 2TMS | 41.599 | 0.99 | + | - | + | - | - | - | + | - |
| 12 | Gallic acid, 4TMS | 42.344 | 0.96 | + | + | - | + | + | + | + | - |

[a]The GC×GC-MS chromatograms of steam-treated GP inoculated with other fungi is given in **Fig. 1**.

**Table 3.** Germination index (%) of lettuce and tomato seed in the presence of $WSF_d$ obtained from powder samples from both uninoculated (control) and fungal-inoculated grape pomace after 90 days' incubation[a].

| $WSF_d$ (%, p/v) | Control | *C. rigida* LPSC 232 | *Pe. albobadia* LPSC 285 | *Py. sanguineus* LPSC 163 | *G. sepiarium* LPSC 735 | *T. harzianum* FALH 18 | *U. botrytis* LPSC 813 |
|---|---|---|---|---|---|---|---|
| **Lettuce** | | | | | | | |
| 10 | 0.0 ± 0.0 c | 11.9 ± 3.4 a | 18.1 ± 3.3 a | 2.8 ± 0.9 b | 0.0 ± 0.0 c | 2.2 ± 1.4 b | 5.6 ± 1.6 b |
| 5 | 9.4 ± 0.7 d | 16.4 ± 1.9 c | 33.8 ± 3.7 b | 18.0 ± 4.7 c | 0.0 ± 0.0 e | 27.0 ± 1.3 b | 56.5 ± 2.3 a |
| 2.5 | 25.3 ± 3.5 bc | 31.3 ± 0.7 b | 62.3 ± 6.6 a | 25.9 ± 2.4 bc | 16.7 ± 1.4 c | 36.1 ± 8.8 b | 69.1 ± 2.7 a |
| 1.25 | 45.8 ± 3.3 bc | 53.4 ± 11.0 b | 56.8 ± 9.6 ab | 50.4 ± 20.5 bc | 23.6 ± 2.9 c | 50.4 ± 5.2 bc | 83.1 ± 7.8 a |
| **Tomato** | | | | | | | |
| 10 | 3.3 ± 0.9 ef | 15.4 ± 2.3 ab | 17.4 ± 4.9 a | 10.3 ± 2.0 bc | 0.9 ± 0.3 f | 7.9 ± 1.8 cd | 4.3 ± 0.2 de |
| 5 | 13.0 ± 2.1 b | 20.6 ± 2.7 ab | 32.5 ± 13.1 a | 18.3 ± 0.5 ab | 11.6 ± 1.1 b | 25.2 ± 9.9 ab | 33.3 ± 3.8 a |
| 2.5 | 34.3 ± 3.7 cd | 44.3 ± 7.1 bc | 54.1 ± 1.6 ab | 46.8 ± 7.1 abc | 26.3 ± 4.0 d | 42.3 ± 6.5 bc | 60.4 ± 5.3 a |
| 1.25 | 41.9 ± 1.2 cd | 65.1 ± 7.0 bcd | 76.3 ± 13.4 ab | 69.2 ± 4.5 bc | 36.8 ± 2.5 d | 58.2 ± 7.0 bcd | 90.1 ± 8.8 a |

[a]Data are means of three replicates ± SD (standard deviation). Row values followed by the same letter are not significantly different as determined by one way ANOVA followed by Tukey's test ($p < 0.05$).

**Highlights**

- Grape pomace is a source of phytotoxic monoaromatic compounds

- *Ulocladium botrytis* LPSC 813 can grow on steam-pretreated grape pomace

- *Ulocladium botrytis* LPSC 813 removes phytotoxic monoaromatic compounds

- *Ulocladium botrytis* LPSC 813 causes effective phytotoxicity reduction of grape pomace

- Phytotoxicity reduction was confirmed on lettuce and tomato seeds