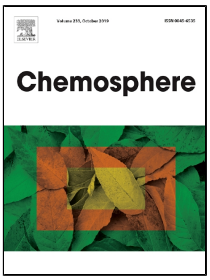

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PII:	S0045-6535(19)31682-0
DOI:	https://doi.org/10.1016/j.chemosphere.2019.124458
Article Number:	124458
Reference:	CHEM 124458
To appear in:	*Chemosphere*
Received Date:	15 January 2019
Accepted Date:	25 July 2019

Please cite this article as: María I. Troncozo, Marija Lješević, Vladimir P. Beškoski, Boban Anđelković, Pedro A. Balatti, Mario C.N. Saparrat, Fungal transformation and reduction of phytotoxicity of grape pomace waste, *Chemosphere* (2019), https://doi.org/10.1016/j.chemosphere.2019.124458

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.

Abstract

Grape pomace (GP) from *Vitis labrusca*, the main byproduct from "American table wine" production, is recalcitrant to degradation, and its accumulation is a serious problem with negative environmental impacts. In this work, transformation of grape pomace using a steam pretreatment followed by incubation of GP during a 90-day period with six different fungi were evaluated. Several fungi tested reduced the phytotoxicity of water-soluble fraction (WSF_d) from steam-pretreated GP after 90 days' incubation to lettuce and tomato seeds. *U. botrytis* caused the largest effective phytotoxicity reduction of WSF_d (used in the concentration range of 10-1.25% p/v) and was the only fungus causing the removal of monoaromatic compounds. Therefore, this procedure with *U. botrytis* effectively reduces the availability of phytotoxic monoaromatic compounds in GP, which opens a way for the development of guidelines for the management of these wastes and their potential use as organic amendments in agricultural soil.

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

1. Introduction

Winemaking using *Vitis* grapes generates large quantities of waste byproducts including grape pomace (GP) that, if inadequately managed, causes environmental and economic problems. GP, the main solid winemaking residue, is composed of seeds, skins, so is rich in lignified fibre and soluble compounds such as phenolics and sugars (Guerra-Rivas et al., 2017).

Alternative solutions to exploit and add value to GP are of interest to the scientific and industrial communities because they would produce economic, social and environmental advantages (Jara-Palacios et al., 2016). GP has potential as a raw resource to obtain value-added products: spirits, grape seed oil, antibacterials, anthocyanins, organic acids (tartaric and citric),

ethanol, butanol, lignin, cellulose and hemicellulose as sources of fermentable sugars and a broad spectrum of polyphenols and other tannins with strong antioxidant activity and health promoting properties (Rockenbach et al., 2011; Prozil et al., 2012; Pervin et al., 2014; García-Lomillo and González-San José, 2017; Karpe et al., 2017). GP has been evaluated as a substrate for production of medicinal mushrooms (Zervakis et al., 2013), microbial protein (Sotiropoulou et al., 2017) and as a biosorbent for the removal of pollutants, heavy metal ions, xenobiotics and mycotoxins (Belayachi et al., 2015; Avantaggiato et al., 2014). However, GP is mainly used for soil conditioning, as a bulking agent in composting processes or as low-cost raw material for animal feed (Spigno et al., 2008). Other applications for GP could be as fertiliser, support for immobilization of yeast in winemaking (Genisheva et al., 2012), or as an additive to culture medium for fungi to enhance production of biotechnologically important enzymes (e.g., laccase and several hydrolases) (Lorenzo et al., 2002; Botella et al., 2007).

Although these alternative uses for GP should minimise its significant environmental impact (Federici et al, 2009; García-Lomillo and González-San José, 2017), pre-processing steps are needed to avoid limitations associated with this byproduct. GP has a high water retention capacity (between 55 and 75 %), a feature that limits its chemical stability and enhances the deterioration of GP by various pests and pathogenic microorganisms that cause GP spoilage (Spigno et al., 2013; García-Lomillo and González-San José, 2017). The presence of lignin in GP, which is very resistant to degradation, limits the accessibility to polysaccharides (Rouches et al, 2016). Additionaly, hydroxilated and metoxilated monoaromatic compounds originating from lignin are known for their strong phytotoxic effects and antibacterial activity (Di Gioia et al., 2002).

Pre-processing should reduce oxidation and microbial action that can spoil GP, re-balance the unfavourable C:N ratio in order to shorten its decomposition time in soil, break down linkages between polysaccharides and lignin and reduce the concentrations of monoaromatic compounds (Pedroza et al., 2012, Di Gioia et al., 2002, Rouches et al, 2016). Pre-treatment methodologies to prevent deterioration of fresh GP include dehydration, sonication, thermal or chemical processes and biological pre-treatment using fungi (Ratti, 2001; Singh and Heldman, 2009; Pedroza et al., 2012; Rouches et al, 2016). Although they can reduce GP's weight and volume, which improves its handling and storage, physical, structural and chemical changes occur (Karpe et al., 2017). Pre-treatment processes affect the availability and reactivity of GP components such as volatiles, polyphenols and chemical groups, so the properties of final materials obtained must be analysed. Clearly, pretreatment influences the future purpose of pretreated GP, and any additional chemical and biotechnological processes needed prior to its complete valorisation. The goal is complete exploitation of the byproduct, to remarkably improve the environmental and economic sustainability of winemaking.

Although wine is mainly produced from *Vitis vinifera*, other *Vitis* species are utilised in regions unfavourable for *V. vinifera* cultivation. In Brazil and regions neighbouring Rio de La Plata, Argentina *V. Labrusca* is the dominant cultivar (De Castilhos et al., 2012; Velarde et al., 2013). To date, GP in these regions and others often accumulates without any pretreatment over long periods of time, leading to deleterious environmental impacts and phytosanitary risks. Other problems related to the management of GP include its generation in large amounts during a short period (February and March annually), low pH and high contents of phytotoxic and antibacterial phenolics that resist biological degradation. Therefore, technological procedures allowing the profitable and environmentally sound use of GP must be explored. Although most studies about

revalorisation of GP and its fungal transformation are on *V. vinifera* cultivars, relatively few are on *V. labrusca* (Ribeiro et al., 2015; Rockenbach et al., 2011; Haas et al., 2017).

The aim of this work was to study the effect of combined thermal and biological pre-treatment on the overall chemical composition of *V. labrusca* GP. In the present study heat treatment of GP, followed by inoculation with six different fungi was analysed based on GP weight loss, FT-IR spectroscopy, and GCxGC-MS analysis. Finally, the phytotoxicity of a dry basis water-soluble fraction (WSF_d) was analysed on lettuce and tomato seeds.

2. Materials and methods

2.1. Grape pomace material, pretreatment with steam and production of wet basis water-soluble fraction (WSF_w)

Fresh GP, which consisted of pressed grape residue (mixture of seeds and skins), was collected from a local manufacturer (Cooperativa de la Costa de Berisso, Berisso, Argentina) that produces *V. labrusca* wine from the Isabella cultivar (Velarde et al., 2013). GP was obtained using a composite random sampling method (Dick et al., 1996) during the harvest season on March 15, 2015. The material collected from different piles of pressed grape residues was pooled to form a composite sample of GP. It was dried in an oven with forced air circulation at 60 °C for 48 h, fractionated in autoclavable bags, treated by autoclaving at 121 ºC for 30 min and then stored at room temperature and protected from light until use.

A wet basis water-soluble fraction (WSF_w) from the steam-treated GP in a 1:10 (w/v) proportion was obtained according to Inalbón et al. (2015) and then analysed for pH (3.55 ± 0.02), phenols (12.62 ± 0.96 mg/100 mL) according to the Folin-Ciocalteau method (Saparrat et al., 2010a), chromophores (optical density at 395 nm; Aloui et al., 2007; 0.27 ± 0.02), NH_4^+-N

(4.09 ± 0.10 mg/100 mL) according to the Saparrat et al. (2010b) method and reducing sugars (0.96 ± 0.05 mM) by the Somogy-Nelson method (Somogyi, 1945).

2.2. Fungal isolates

Six fungi, previously isolated from various sources including decaying wood, soil and leaf litter, which belong to different ecophysiological groups were used: white rots, *Coriolopsis rigida* (Berk. & Mont.) Murrill LPSC (Culture Collection of the Instituto Spegazzini, Universidad Nacional de La Plata, La Plata, Argentina) 232 (Saparrat et al. 2002b), *Peniophora albobadia* (Schwein.) Boidin LPSC 285 (Saparrat et al., 2008) and *Pycnoporus sanguineus* (L.) Murrill LPSC 163 (Saparrat et al., 2000); a brown rot fungus, *Gloeophyllum sepiarium* (Wulf.: Fr.) P. Karst. LPSC 735 (Murace et al., 2016); a soil fungus, *Trichoderma harzianum* Rifai FALH (Facultad Ciencias Agrarias y Forestales, UNLP) 18 (Stocco et al., 2015) and a litter degrading fungus, *Ulocladium botrytis* G. Preuss LPSC 813 (Saparrat et al., 2008). These strains were selected due to their ability to grow on different lignocellulosic materials. Stock cultures were kept at 4 °C on 2 % (w v^{-1}) agar-malt extract slants.

2.3. Fungal treatment of steam-pretreated grape pomace under solid-state fermentation (SSF) conditions, its fractions and analytical determinations

Fungal growth on steam-pretreated GP under SSF conditions was evaluated in autoclavable bags containing 30 g of sterile dry material at a humidity level adjusted to 70 %. Cultures grown on 2 % malt extract agar for 7 days were used for inoculation. Each bag was inoculated axenically with 6 mL of a 0.15 % (mass vol^{-1}) mycelial suspension (Inalbon et al., 2015). Inoculated bags were incubated at 28 ± 1.5 ºC in the dark for 90 days in a humidified chamber, as previously reported (Saparrat et al, 2010b). Sterile uninoculated grape pomace

(UGP) incubated under the same conditions for the same period of time was used as control. Each treatment was prepared in triplicate. Ninety days after inoculation, a WSF_w was obtained from an aliquot of both uninoculated and fungal-inoculated steam-pretreated GP as described. Then, all the solid content (i.e., solid fraction; SF) of each bag was dried at 60 °C to a constant mass, and degradation was measured as a percentage of GP weight reduction (percentage reduction of inoculated substrate in relation to the uninoculated one. Each dried SF sample was ground in an agate mill, and sieved through a <1 mm screen (Saparrat et al, 2010b). The resultant material was kept at 25 °C and stored without exposure to atmospheric moisture until its use for chemical analysis and the phytotoxicity assay. The pH, phenols, chromophores, NH_4^+-N and total reducing sugar content (TRSC) of the WSF_w from control and inoculated steam-pretreated GP were determined.

2.4. Preparation of methanol extracts of SF

To prepare GP methanol extracts, 30 mg of steam-pretreated GP SF was extracted with methanol (150 µL) in an ultrasonic bath for 20 min. After sonication, the samples were filtered through Whatman filter No. 4, and supernatants were evaporated in a stream of nitrogen to dryness. The extracts were stored at room temperature for further analyses (Devrnja et al., 2017).

2.5. Structural instrumental analysis

2.5.1. Chromatographic analysis

The methanol extracts were derivatised with BSTFA for 45 min at 60 °C and analysed using a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) comprehensive two dimensional gas chromatograph-quadrupole mass spectrometer (GC×GC-MS) with ZX2 thermal modulation system (Zoex Corp.). A RtxR-1 (RESTEK, CrossbondR 100% dimethyl polysiloxane, 30 m, 0.25

mm ID, df=0.25 μm) and a BPX50 (SGE Analytical Science, 1 m, 0.1 mm ID, df=0.1 μm) columns were connected through the GC×GC modulator as the first and second capillary columns, respectively. The oven was programmed at an initial temperature of 40 °C for 5 min, than ramped at 5.2 °C min^{-1} to 300 °C. The modulation period was 9 s. The GC×GC-MS data were analysed using Chrome Square 2.1 software, and MS spectra were compared with the MS libraries NIST 11, NIST 11s, and Wiley 8.

2.5.2. FT-IR measurement

Fourier Transform Infrared (FTIR) spectra were recorded using a Thermo Scientific Nicolet 6700 FT-IR Spectrophotometer, using attenuated total reflectance (ATR) technique from Smart accessory with diamond crystal (Smart Orbit, Thermo Scientific, Madison, WI, USA). Spectral data were collected in the mid-IR range (1800–600 cm^{-1}) with 64 scans and resolution of 4 cm^{-1} for all methanol extracts of: uninoculated GP (UGP) before incubation (0 days); UGP after 90 days' incubation, and; GP inoculated with fungi after 90 days from inoculation. Methanol solutions were applied on the ATR crystal using a capillary. Solvent was evaporated by a nitrogen stream in order to obtain thin ATR films. The spectra were fitted using OMNIC software (Version 7.0, Thermo Scientific, USA). A curve-fitting procedure was used to decompose each original spectrum to its Voigt curve constituents that could be assigned to certain structural features. The contribution of each curve was assessed by integrating the area under the curve, and then normalising it to the total area of all spectra. Vibrational bands were assigned based on literature data (Pretsch et al., 2000).

2.5.3. Elemental analysis

To assess the content of carbon, hydrogen, nitrogen, oxygen and sulphur, the steam-pretreated GP SF powders were analysed using a Vario EL III CHNS/O Elemental Analyzer.

2.6. Germinability assays

The effect of a WSF_d obtained from each steam-pretreated GP SF powder from both uninoculated and fungal-inoculated GP after 90 days' incubation was determined on germination of lettuce (*Lactuca sativa*) and tomato (*Lycopersicon esculentum*) seeds according to the method described by Zucconi et al. (1981). Each WSF_d at 10% (p/v), obtained according to Gopinathan and Thirumurthy (2012), was filtered through a 0.2 μm pore membrane before use. The seeds were surface-sterilised using 1% sodium hypochlorite (NaClO) solution at for 1 minute and washed 6-7 times with sterile distilled water to remove traces of NaClO. In sterile Petri plates, sterile filter paper was kept soaked in WSF_d, and sterile distilled water soaked filter paper was used as a control. Twenty (lettuce) or fifteen (tomato) seeds were kept in each Petri plate and the experiment was conducted in triplicate. These experiments were performed on undiluted and variably diluted (1:2, 1:4, 1:8 in water) WSF_d. Seed germination was observed for seven days. The experiment was conducted in the dark at 25 °C. The number of germinated seeds was counted and radial growth measured. The germination index (GI) was calculated according to the formula $GI = (G/Go) \times (L/Lo) \times 100$, where G and Go are the number of grown seeds in sample and control and L and Lo are the average sum of root lengths in sample and control, respectively.

2.7. Statistical analysis

Mean and standard deviation were calculated from data obtained for each treatment. Results were analysed by a one-way ANOVA and means of all variables were contrasted by Tukey's test (Statistix 8.0).

177 **3. Results**

178 *3.1. Physical and chemical properties of steam-treated grape pomace*

179 We monitored several physical and chemical properties of steam-treated GP inoculated
180 with fungi after 90 days' incubation and its corresponding WSF_w. Only some slight differences
181 were found in ammonium and phenolic levels of WSF_w obtained from UGP after 90 days'
182 incubation compared to those in WSF_w from UGP at the beginning of the incubation. The six
183 fungal strains examined were able to grow and colonise steam-pretreated GP, using it as the only
184 substrate source in SSF conditions and reducing its weight and modifying several physico-
185 chemical features (**Table 1)**. The greatest weight loss was caused by *U. botrytis* (up to 32% after
186 90 days), which degraded the GP much faster than the other fungal species. Substrate weight loss
187 caused by *C. rigida*, *G. sepiarium*, *Pe. albobadia*, *Py. sanguineus* and *T. harzianum* was between
188 18 and 26% at the end of the incubation period, although differences were not always statistically
189 significant. The chemical composition of the WSF_w extracted from UGP and GP treated with
190 each fungus differed widely. The pH of all fungi-inoculated steam-pretreated GP increased
191 slightly compared to the UGP control after 90 days. The most prominent pH increase was
192 measured in the steam-pretreated GP inoculated with *U. botrytis*. All the fungi examined
193 increased the NH_4^+-N content in WSF_w, except *U. botrytis*. While lower TRSC was found in the
194 WSF_w from material inoculated with *Pe. albobadia*, followed by those inoculated with *C. rigida*
195 and *T. harzianum*, *G. sepiarium* and *Py. sanguineus* slightly increased the TRSC. Regarding the
196 phenolics in WSF_w, apart from in the steam-pretreated GP inoculated with *G. sepiarium*, there
197 was a drastic decrease in all other inoculated, steam-pretreated GP. The highest phenol reduction
198 was caused by *Pe. albobadia*. The level of chromophores in WSF_w was only increased by *U.*
199 *botrytis* compared to the UGP.

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).

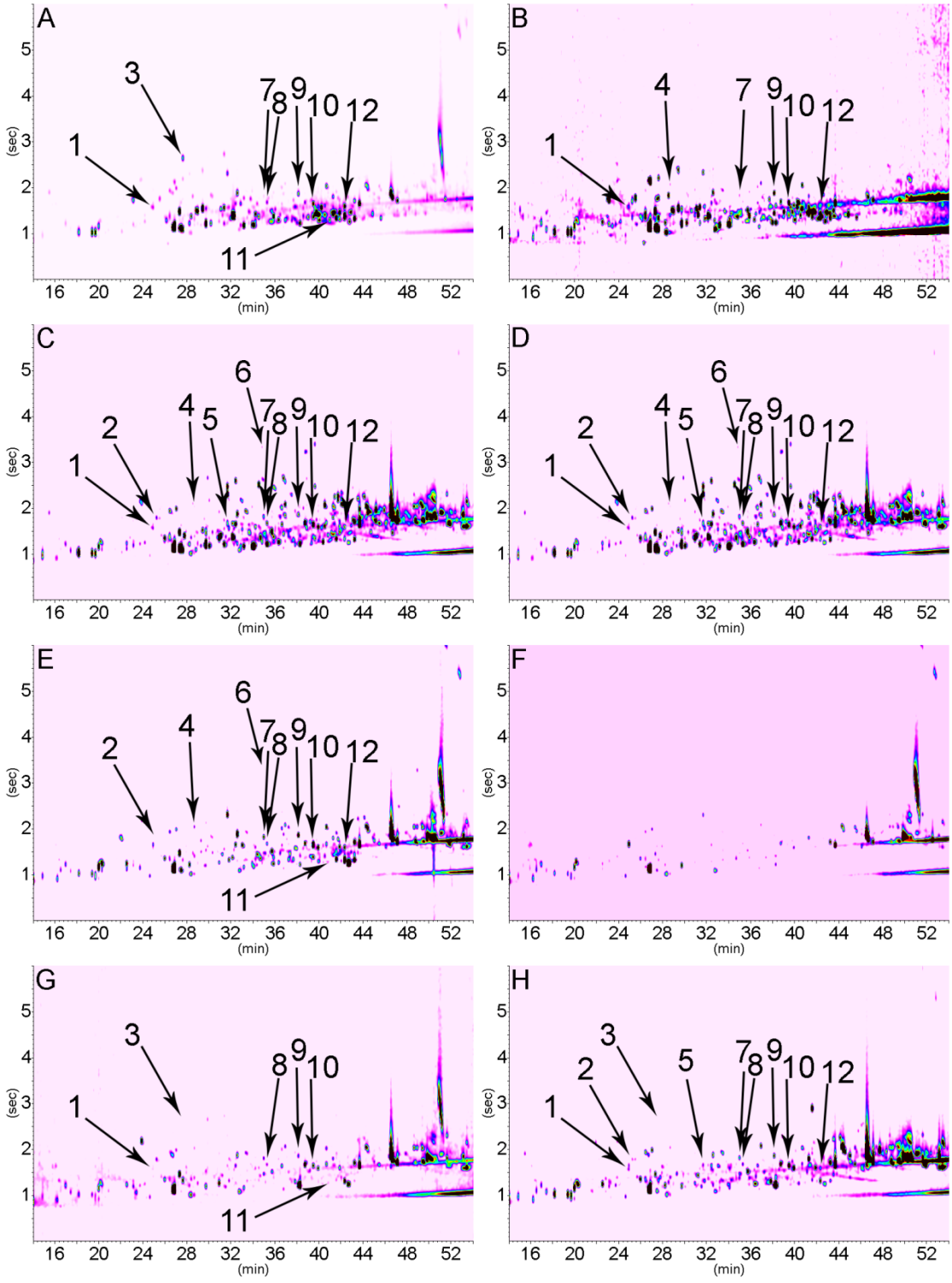
204

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.

However, in uninoculated steam-pretreated GP after 90 days' incubation, additional monoaromatics appeared (isovanillic, protocatechoic, cinnamic). All steam-pretreated GPs inoculated with fungi had several other monoaromatics present in addition to those found in the UGP after 90 days' incubation, and the list of compounds present in each is given below (**Table 2**; **Fig. 1C-H**). The exception was steam-pretreated GP inoculated with *U. botrytis* (**Fig. 1F**), which did not contain any monoaromatics.



222

223 **Figure 1.** GCxGC-MS of methanol extracts of: **(A)** grape pomace immediately after steam treatment; **(B)**

224 uninoculated steam-treated grape pomace after 90 days' incubation; **(C)** steam-treated grape pomace inoculated with

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].

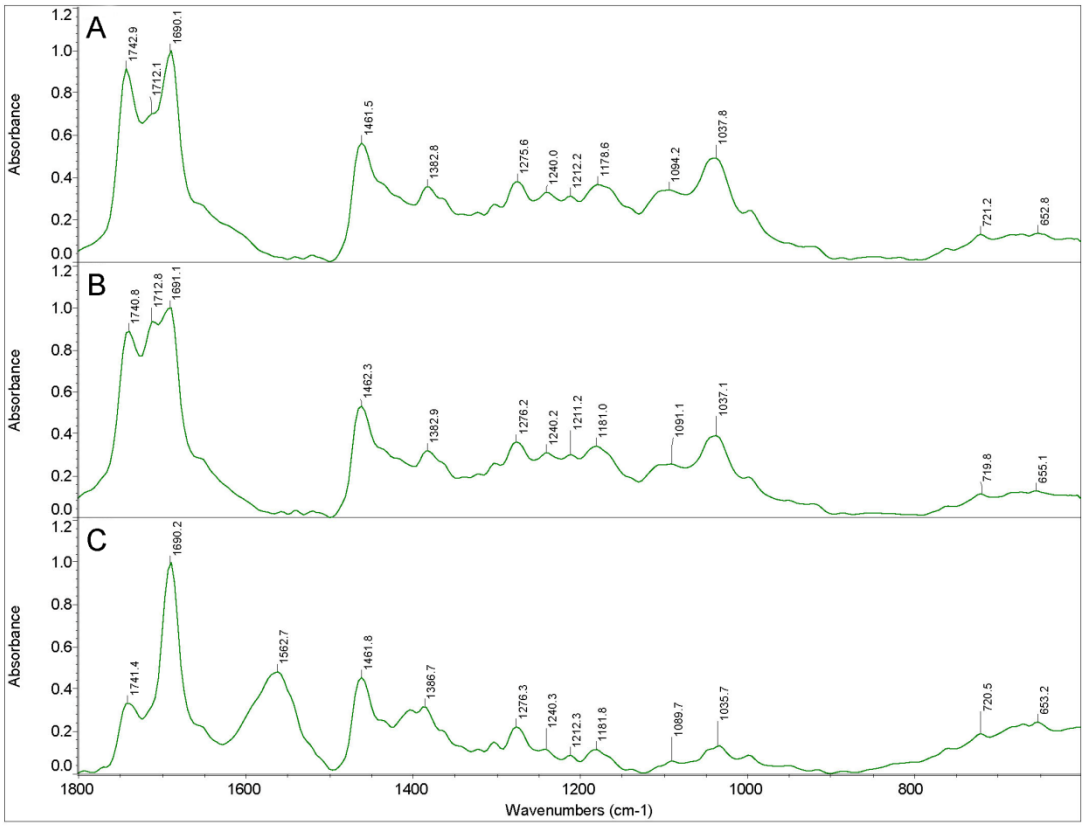
No.	Compound	tR	2tR	0 days	90 days						
				UGP	UGP	LPSC 232	LPSC 285	LPSC 163	LPSC 735	FALH 18	LPSC 813
1	2-Phenylethanol, TMS	24.944	0.81	+	+	+	+	+	+	-	-
2	Benzoic acid, TMS	25.248	1.89	-	-	-	+	-	+	+	-
3	Benzeneacetic acid, TMS	26.749	2.64	+	-	+	-	+	+	-	-
4	4-Hydroxybenzaldehyde, TMS	28.702	1.5	-	+	-	+	+	-	+	-
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

(UGP at the beginning of the experiment and after 90 days). The bands at 1740 cm^{-1} and 1712 cm^{-1} were attributed to the stretching vibrations of the acid carbonyl groups, and bands from 1690 cm^{-1} belong to vibrations of the fatty acid carbonyl groups. Numerous bands attributed to aromatic C-H and O-H in plane deformation vibrations of phenols were present from 1460 to 1020 cm^{-1}. An additional, wide peak at 1563 cm^{-1} was present in steam-pretreated GP inoculated with *U. botrytis* after 90 days; that peak corresponded to amide I and amide II of methanol soluble proteins from fungus (Pretsch et al., 2000; Naumann, 2009; Lecellier et al., 2015). The content of soluble phenols and absorbance at 395 nm, which depend on the presence of aromatic groups (such as tyrosine, tryptophan, phenylalanine), were higher in steam-pretreated GP inoculated with *U. botrytis* than in the controls. This, together with the appearance of the band at 1563 cm^{-1}, could be due to increased soluble protein content resulting from fungal growth in this treatment. The significant reductions of a carbonyl band at 1741 cm^{-1} and a C-O anti-symmetrically stretched band at 1036 cm^{-1} in steam-pretreated GP with *U. botrytis* after 90 days' 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 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

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

Among organisms involved in revalorising organic wastes, fungi play a great role due to their ability to degrade severely recalcitrant plant polymers and various toxic compounds, such as phenolic compounds, as well as recycle nitrogen more efficiently than bacteria (Karpe et al., 2017; Hodge et al., 2000). Six fungi, including wood-rotting Basidiomycota and two Ascomycota, were inoculated in steam-treated GP from *V. labrusca* under SSF axenic conditions. When compared, these fungi differently degraded GP and its components such as free phenolic compounds. This could be due, at least partly, to their ability to detoxify inhibitory compounds present in GP and/or to synthesise different enzymes to degrade lignocellulose. However, after 90 days' incubation, *U. botrytis* caused the highest mass loss of GP and the highest reduction of GP phytotoxicity compared with other fungi used. Also, it was the only strain that degraded the monoaromatics found in steam-treated GP. Previous reports showed the outstanding ability of *U. botrytis* LPSC 813 to attack *Scutia buxifolia* leaf-litter using its cellulolytic enzyme complex and extracellular enzymes with peroxidase (EC 1.11.1.7) activity, which might be considered ecologically advantageous in the colonisation of a broad spectrum of aromatic-rich substrates, which might also possibly extend to the ones from GP (Saparrat et al., 2008, 2010b). Extracellular oxidative enzymes in another non ligninolytic fungus (*Ciliochorella buxifolia*) growing on *Scutia buxifolia* leaf-litter have been reported to be involved in mechanisms of detoxification of phenolics (Troncozo et al. 2015). So, this latter information hypothesizes that the peroxidase activity of *U. botrytis* LPSC 813 might play a key role in detoxification reactions of GP phenolics. However, we did not evaluate any enzyme activity in this study.

WSF_w indirectly reflects alteration of the insoluble lignocellulose macromolecular fractions (Dorado et al., 1999). Variations in sugar levels of GP transformed by our six fungi,

including those that generated reducing substances in WSF_w after 90 days' incubation, could be due to the relationship between the activity of their carbohydrate depolymerising enzymes and their efficiency to absorb the resultant soluble sugars. However, biomass and chemical composition were not monitored over the incubation period, and the different fungi likely have different ecological strategies to obtain nutrients. Although fungi belonging to different ecophysiological groups have different substrate decomposition strategies, lignocellulose decomposition can vary among fungi belonging to the same ecophysiological group (Presley and Schilling, 2017). *U. botrytis* LPSC 813 is a litter fungus with little ligninolytic activity (Saparrat et al., 2008), however we speculate it might have, a yet unknown, strategy to colonise GP, giving it greater ability to degrade GP than the other fungi examined and enabling it to degrade monoaromatics.

Levels of colour and free phenols can be used as indicators of organic matter quality in WSF_w (Dorado et al., 1999, Saparrat et al., 2008). We found *U. botrytis*-treated GP produced a strongly coloured WSF_w, likely due to chromophoric pigments derived from fungal transformation of phenols. While our white rot fungi and ascomycetes severely reduced the free phenol levels in GP, *G. sepiarium* was unable to do so. This might have some relation with fungal ability to detoxify GP compounds, since *G. sepiarium* did not reduce the phytotoxicity of the steam-pretreated GP when it was evaluated on lettuce and tomato seed. However, free phenols cannot be uniquely responsible for the phytotoxicity of GP, as *Py. sanguineus* decreased phenol levels, but this did not result in increased seed germinability. The presence of heavy metals in GP and its wastewater could be another plausible cause of phytotoxicity. Although we didn't analyse the content of metals in GP, Speltini et al (2011) have reported that inferior levels of Cu, Pb and Cd than the maximum limit allowed by the Instituto Nacional de Vitivinicultura

(Argentina) are present in water samples taken from a creek that passes through vineyards in a region neighbouring Rio de La Plata, Argentina (the Arroyo Sarandí basin).

In our study, monoaromatic compounds were the main biotransformation products of lignin and related compounds. Monoaromatics were found in the uninoculated GP at the beginning of the experiment (day 0), but also in the uninoculated GP after 90 days. This was probably due to heat and spontaneous oxidation by molecular oxygen and its reactive species (Flores et al., 2016). Removal of monoaromatics from GP by *U. botrytis* could involve two possible mechanisms. Firstly, the fungus could use these compounds as sources of carbon and energy (Prenafeta-Boldú et al., 2006). Secondly, *U. botrytis* might detoxify monoaromatics by using its extracellular oxidative enzymes (Saparrat et al., 2008) to polymerise them. Since *U. botrytis* is a melanin-producing fungus (Bell and Wheeler, 1986), it could consequently immobilize these polymerised products in its own cell walls. The removal of monoaromatics from GP by *U. botrytis* LPSC 813 could be the basis for the reduced phytotoxicity of this waste towards tomato and lettuce seeds.

Alkalinisation of GP by *U. botrytis* can facilitate the availability of nutrients to plants, so this fungal transformation could improve the ecological management and utilisation of the lignocellulosic byproduct as an effective fertilizer or soil amendment. Although ammonia has been considered as a key player in environmental alkalinisation by several fungi, it is not a well-understood phenomenon (Vylkova, 2017). In the current study, *U. botrytis*-treated GP had a lower concentration of ammonia compared to UGP or GP treated with the other five fungi. In the GP treated with these five remaining fungi ammonium contents in WSF_w increased compared to the UGP, although they were only related to the low pH increments measured. The higher ammonium levels in WSF_w from these fungal cultures compared to the control could indicate

these fungi mineralise organic N in the GP. Nitrogen in GP is mainly incorporated in proteins (6-15% dry matter in GP) (García-Lomillo and Gonzalez-San José, 2017). Therefore, the chemical mechanism behind the rise in pH by *U. botrytis*-treated GP might be, at least in part, independent of fungal nitrogen metabolism and/or it could be due to a combination of several processes. Potential alkalinisation generating processes involved are the secretion of carbon compounds, the increase in availability of ions such as Ca^{2+}, or the lack of nutrient availability forcing the fungus to consume organic acids as nutrient sources (Danhof et al., 2016). Therefore, our results suggest that *U. botrytis* is an alkalinisation bioagent for the treatment of GP. However, none of these mechanisms were tested during the transformation of GP by *U. botrytis*, so further study is needed.

5. Conclusions

Steam pre-treatment of GP followed by inoculation with six fungi results in considerable alteration of the GP and its WSF_w after 90 days' incubation. The combination of a thermal treatment followed by incubation with *U. botrytis* reduces the phytotoxic monoaromatic compounds in GP and opens the way for guidelines on management of these wastes and their potential use as organic amendments in agricultural soils. The mechanisms of removal of monoaromatics by *U. botrytis* LPSC 813 likely involve detoxification and/or degradation reactions that should be further studied.

Acknowledgements

We wish to thank Dr Marina C. Stocco from Centro de Fitopatología-CIDEFI (UNLP-CICBA, La Plata, Argentina) for providing the isolate *T. harzianum* FALH 18. M.I.T. is a recipient of a scholarship from the UNLP. This research was supported by Agencia Nacional de

Promoción Científica y Tecnológica (PICT 2015-1620 to MCNS), CONICET (PUE INFIVE), CICPBA, UNLP, Argentina; Ministry of Education, Science and Technological Development of the Republic of Serbia (Project III 43004) and by the Japan International Cooperation Agency (JICA) (grassroot project "Capacity building for analysis and reduction measures of persistent organic pollutants in Serbia").

Author Contributions

M.I.T. and M.C.N.S. were responsible for conception and design of research. M.I.T., 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. 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. wrote the first draft of the manuscript. V.P.B. and M.C.N.S. wrote the final version of the 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 ± 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

[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	90 days						
				UGP	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