Chemosphere 185 (2017) 258–267

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Phytotoxicity assessment of olive mill solid wastes and the influence of phenolic compounds

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HIGHLIGHTS

- Phytotoxicity of olive mill solid wastes (OMSW) was evaluated with *Lepidium sativum*.
- Phytotoxicity of OMSW was related with specific phenolic compounds.
- Among the phenolic compounds, cinnamic acid revealed to be the most phytotoxic.
- Lipophilic nature of phenolic compounds is associated with the phytotoxic activity.

ARTICLE INFO

Article history:
Received 21 December 2016
Received in revised form
6 June 2017
Accepted 1 July 2017
Available online 3 July 2017

Handling Editor: Frederic Leusch

Keywords:
Phytotoxicity
Phenolic compounds
Germination
Germination index
Lepidium sativum L.
EC50

ABSTRACT

The main objective of this work is to evaluate the phytotoxicity of olive mill solid wastes (OMW) produced in two different centrifugation technologies and also the toxicity associated with specific phenolic compounds. Two samples of waste were collected in two-phase (2P-OMW) and three-phase (3P-OMW) centrifugation olive oil production processes, and cress bioassays with *Lepidium sativum* L. were employed to evaluate phytotoxicity. Although both OMW have similar total phenolic content (TPh), results confirmed that 2P-OMW is more phytotoxic than 3P-OMW. When extracts from 2P-OMW at liquid to solid ratio of 10 L kg^{-1} were applied none of the seeds germinated, i.e. germination index (*GI*) was 0%, while for 3P-OMW GI was 94.3%. Growth tests in soil and mixtures with OMW also led to more favorable results for 3P-OMW, whereas worse results than those obtained in the control experiments were observed.

In order to discriminate the individual influence of eleven phenolic compounds, gallic acid, protocatechuic acid, cinnamic acid, syringic acid, 3,4,5-trimethoxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, caffeic acid, veratric acid and phenol were tested in the concentration range of 5 −500 mg L^{-1}. Results showed that cinnamic acid is the most phytotoxic, with EC_{50} of 60 mg L^{-1}, which is related with its hydrophobicity. Moreover, increasing -OH and -OCH_3 groups in these molecules seem to reduce phytotoxicity. Tests with a mixture of six phenolic compounds demonstrated there are neither synergistic nor additive effects. The phytotoxicity appears to be determined by the presence of the most lipophilic phenolic molecule.

1. Introduction

Olive oil production is an important industry in the Mediterranean region. Although Spain, Italy and Greece are the principal world producers of olive oil (Roig et al., 2006), Portugal is the fourth producer in the European Union (Niaounakis and Halvadakis, 2006). At the industrial scale, olive fruit is transformed into olive oil through pressing or centrifugation processes, but wastes are generated requiring further management. The traditional discontinuous pressing process has been gradually substituted by the three-phase (3P) centrifugation technology, and then by two-phase (2P) centrifugation systems. While 3P-centrifugation processes generate large amounts of wastewaters, 2P-centrifugation systems create a semi-solid waste, commonly called *alperujo*.

Both wastewaters and solid wastes are often considered phytotoxic due to phenolic, lipidic and others organic compounds (Roig et al., 2006; Niaounakis and Halvadakis, 2006). Indeed, olive fruit contains phenolic compounds, but the type and quantity are

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http://dx.doi.org/10.1016/j.chemosphere.2017.07.002
0045-6535/

influenced by the olive cultivar, degree of maturation, climate conditions and agriculture practices. The oil extraction technology is responsible for the phenolic profile not only in olive oil but also in solid residues (Roig et al., 2006; Niaounakis and Halvadakis, 2006; Klen and Vodopivec, 2012; Morillo-Pérez et al., 2009). Some phenols are originally present in the olive fruit, but others may be formed during the processing phase (Klen and Vodopivec, 2012). Depending on the partition coefficients, temperature and catalyzed oxidative reactions phenols of olive paste are more or less soluble in water and oil (Niaounakis and Halvadakis, 2006; Baddi et al., 2004).

According to the literature, the olive mill solid waste from two-phase centrifugation process (2P-OMW) may contain a higher concentration of phenolic compounds than those from three-phase process (3P-OMW) (Niaounakis and Halvadakis, 2006; Fernández-Bolaños et al., 2006; Leouifoudi et al., 2015).

Previous studies indicated that hydroxytyrosol and tyrosol are the main phenolic compounds in OMW, together with oleuropein, *p*-coumaric acid, vanillic acid, verbascoside, elenolic acid, catechol, rutin, ferulic acid, cinnamic acid, protocatechuic acid, 4-hydroxybenzoic acid and others (Niaounakis and Halvadakis, 2006; Alu'datt et al., 2010; Dermeche et al., 2013; Lesage-Meessen et al., 2001). However, it is still unclear the role of individual phenolic molecules in phytotoxicity. This is an important issue since OMW is often disposed of or used as soil conditioner (Azbar et al., 2004).

Phytotoxicity is a measure of the delay or inhibition of seed germination, inhibition of plant growth or any adverse effect on plants caused by specific substances (Baumgarten and Spiegel, 2004). Thus, phytotoxicity bioassays can detect any substance capable of generating temporary or long-term stress on the germination capacity of seeds, roots growth and dry matter evolution (Pavel et al., 2013). Bioassays must respond not only to known compound but also to complex mixtures of phytotoxins (Trautmann and Krasny, 1997), must be simple, reproducible and fast (to avoid plants adapting to the toxic compounds) (Barral and Paradelo, 2011). Although various plant species have been tested, *Lepidium sativum* L. (commonly called cress or garden cress) is often chosen because of its rapid and sensitive response and cost-effective (Trautmann and Krasny, 1997; Zucconi et al., 1981). The bioassays results may be expressed as germination index (GI) evaluated in an extract solution, which is indicative whether if a medium contains detrimental substances for seed germination or for the growth of the radicle (Trautmann and Krasny, 1997). Direct growth tests take into account not only the fraction dissolved in the aqueous extracts, but also the fraction associated with the solid matrix. Although provide reliable data, direct growth tests are slow and may require complex installations with controlled temperature, humidity and illumination (Barral and Paradelo, 2011).

The main objectives of this study are to evaluate the phytotoxicity of 2P-OMW and 3P-OMW based on germination tests in aqueous extracts and on direct growth tests, along with the effect of eleven individual phenolic molecules. Literature indicates that phenolic compounds are major contributors for phytotoxicity, but to the best of our knowledge their individual effects were never before assessed, thus this is the main novelty of this study.

2. Experimental methodology

2.1. Materials

Two types of olive mill solid wastes were characterized (2P-OMW and 3P-OMW). 2P-OMW was collected from a 2-phase olive mill in the Spanish region of Extremadura and subsamples were frozen until use. 3P-OMW was collected from a 3-phase olive mill in the center of Portugal and the sample was stored at room temperature in an airtight and dark container. Seeds of *Lepidium sativum* L. employed in the germination tests were kept in dark and dry conditions.

Soil used in the growth tests was collected from a superficial horizon in a cultivated field. In the laboratory, the soil was dried at 38 °C, aggregates separated by gentle pressing and then sieved with a 2 mm mesh. About 80% of the soil is fine earth (<2 mm). The sample contains 2.0% of organic matter, pH 6.4 and electrical conductivity 0.18 mS cm^{-1}. The total content of specific elements are: 0.56 mg P_2O_5 100 g^{-1}, 2.10 mg K_2O 100 g^{-1}, 6.02 mg CaO 100 g^{-1} and 0.66 mg MgO 100 g^{-1}.

2.2. Analytical techniques

2P- and 3P-OMW samples were characterized by measuring several properties at least in triplicate and using blank experiments as control. Moisture content (MC) and total solids (TS) were obtained by drying samples at 105 °C until constant weight. Volatile solids (VS) was obtained by weighing the sample before and after calcination in a muffle at 550 °C for 2 h. Total organic carbon (TOC) was estimated dividing SV by a factor of 1.8 (Jiménez and García, 1992).

Water holding capacity (WHC) of 2P- and 3P-OMW and soil were assessed using small plastic containers perforated at the bottom and over the holes three nets with different mesh sizes were placed. These nets retain solid but allow water diffusion. Containers with solids were soaked in water for 24 h. After this period, water was drained off by gravity for 24 h. WHC corresponds to the ratio between the absorbed water amount to the soaked and drained sample weight (in percentage). pH and electrical conductivity (EC) were analyzed in aqueous extracts at a liquid to solid ratio (L/S) of 10 L kg^{-1} ds (dry substance), under agitation for 1 h, using a CRISON micro pH 2002 and Multiparameter analyzer Consort C863, respectively.

Chemical oxygen demand (COD) was determined according to Noguerol-Arias et al. (2012), in which samples were digested with potassium dichromate in acid conditions in a ECO25 thermoreactor (VELP Scientifica) and after cooling down the absorbance was read in a photometer PhotoLab S6 (WTW) at 605 nm.

Total phenolic content (TPh) was determined by Folin-Ciocalteau Method (Golsmith et al., 2014), and the calibration curve was built using standard solutions of gallic acid dissolved in methanol/water (80/20 v/v%), in a linear range of 10–100 μg/mL. TPh was measured by reading absorbance at 760 nm in the UV/Vis Spectrophotometer T60.

Total Kjeldahl nitrogen (TKN) and total nitrogen (TN) determination consisted of three steps: digestion (DKL Fully Automatic Digestion Unit from VELP Scientifica), distillation (UDK Distillation Unit from VELP Scientifica) and titration. For TKN analysis 0.5 g of dry sample were mixed with a Kjeldahl catalyst tablet and 10 mL of H_2SO_4 (96%). Digestion was carried out at 420 °C during 2 h. After cooling, 10 mL of distilled water and 50 mL of NaOH solution (40 g/L) were added before distillation. In TN determination, 0.5 g of dried waste were digested at 420 °C during 1 h with 0.5 g of chromium metal powder, 20 mL of HCl (7%), 7 g of potassium sulfate anhydrous, 100 mg of HgO and 100 mL of H_2SO_4 (96%). Before distillation, 50 mL of water, 25 mL H_3BO_3 and 70 mL NaOH were added. In both cases, 100 mL of distillate were collected, mixed with 10 mL of a pH indicator solution (boric acid with bromocresol green and methyl red) and titrated with HCl 0.1 M.

Total phosphorus (TP) quantification involved digestion of 37.5 mg of dry sample, 50 mL of water, 1 mL H_2SO_4 (11 N) and 0.4 g of ammonium persulfate in the DKL Fully Automatic Digestion Unit