Ecotoxicology and Environmental Safety 80 (2012) 69–75

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

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

Toxicity effects of olive-mill wastewater on growth, photosynthesis and pollen morphology of spinach plants

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ARTICLE INFO

Article history:
Received 19 October 2011
Received in revised form
10 February 2012
Accepted 12 February 2012
Available online 26 March 2012

Keywords:
Olive mill wastewater
Pollen
Phenolic compounds
Phytotoxicity
Proline
Sugars

ABSTRACT

Olive mill-wastewater (OMW), a by-product of the olive oil extraction process, represents a significant environmental problem in Mediterranean areas. We studied the impact of OMW dilutions (1:10 and 1:20) on growth, photosynthesis, proline and sugar accumulation as well as on pollen morphology of spinach (*Spinacia oleracea* L.) plants, to evaluate the application of OMW dilutions as pretreatment technique, prior to land disposal. Biomass, height, total chlorophyll and leaf area of spinach declined progressively with decreasing OMW dilution. Since fatty acids and phenolic compounds (present in the OMW) are considered precursors in the polymerization of sporopollenin, we suggest that under OMW treatment spinach plants seem to 'direct' the excess of these substances in the production and formation of increased pollen grains. Proline did not accumulate under OMW stress, but decreased possible due to transport to pollens in response to increased demand to over-production of pollens. Both OMW dilutions resulted in a decreased efficiency of PSII functioning and an increased excitation pressure ($1-q_P$). It is concluded that, higher than 1:20 OMW dilutions should be used, and/or additional treatment should be applied before use of the OMW in the environment.

1. Introduction

The olive oil processing industry represents an important socio-economical activity in the Mediterranean areas (Mechri et al., 2011). There are approximately 750 million productive olive trees worldwide, 98% of them located in the Mediterranean region, where more than 97% of olive oil is produced. The three major olive oil producers worldwide are Spain, Italy, and Greece, followed by Turkey, Tunisia, and to a lesser extent Portugal, Morocco and Algeria (Tsagaraki et al., 2007). Olive-mill wastewater (OMW) is a liquid waste produced from the olive oil extraction process (Bodini et al., 2011). The annual OMW generation in the Mediterranean olive oil producing countries varies between 7×10^6 and 30×10^6 m^3 (Kavvadias et al., 2010). In Greece, over the last years, the OMW production has been approximately 1.660.000 t $year^{-1}$ and this huge amount is generated between November and February (Kapellakis et al., 2006). Olive-mill wastewaters (OMW) generated during production of olive-oil are significant sources of environmental/industrial problems in olive-oil-producing countries, which is a consequence of high content in polyphenols, organic matter, salt and low molecular weight organic acids, seasonal operation and high territorial scattering (Aggelis et al., 2002; Hafidi et al., 2005). A long contact of plant roots with OMW would allow phytotoxicity substances existing in OMW such as phenols, organic acids and fats (Capasso et al., 1992; Paredes et al., 1999) to affect root membrane structure and to modify its functions including metabolic efficiency and stability (El Hadrami et al., 2004). Therefore, increasing concern has been expressed about the effective treatment and safe disposal of OMW in the environment (Mekki et al., 2008).

Research efforts have been directed towards the development of efficient treatment technologies including several physical, chemical and biological processes as well as various combinations of them (Sayadi et al., 2000; Dhouib et al., 2006a, b; Ginos et al., 2006). However, the disposal of OMW is still predominantly carried out via spreading or by means of evaporation ponds (Karpouzas et al., 2009; Ouzounidou et al., 2010). The direct application of OMW to the soil (Chartzoulakis et al., 2010) or dilutions of OMW can be considered as a treatment allowing the

Abbreviations: F_v/F_m, maximal photochemical efficiency of PSII; OMW, olive mill wastewater; PSII, photosystem II; Q_A, the first stable quinone electron acceptor of PSII; q_N, non-photochemical quenching; q_P, photochemical quenching; $1-q_P$, the relative reduction state of Q_A referred as PSII excitation pressure; Φ_{PSII}, actual PSII efficiency

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0147-6513/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ecoenv.2012.02.030

reduction of OMW toxicity and irrigation of fields with either raw or pretreated OMW is a relatively inexpensive disposal technique (Cegarra et al., 1996) that could be implemented by small sized three-phase centrifugal olive mills. Many authors warmed that OMW disposal in the nature causes serious environmental problems due to its antibacterial effects and its phytotoxicity (Martins et al., 2008; Mekki et al., 2008; Ouzounidou et al., 2008; Peixoto et al., 2008). Thus, this practice represents now a controversy discussion and a debate of actuality between those that are for and those that are against this strategy (Mekki et al., 2008). However, despite the research interest that OMW has attracted, relevant studies on effects on photosynthesis of crop plants are rare (Mechri et al., 2008, 2009, 2011; Ouzounidou et al., 2008, 2010) and on pollen grains practically non-existent.

In recent years, chlorophyll fluorescence measurements have become a widely used method to study the functioning of the photosynthetic apparatus and are a powerful tool to study the plant's response to environmental stress (Krause and Weis, 1991; Maxwell and Johnson, 2000). Photosynthetic apparatus acts as not only a transducer of photon energy but also as a primary sensor of environmental changes (irradiance, radiation quality, temperature, water availability, nutrient status etc) through modulation of the chloroplast redox signal as a consequence of imbalances between energy supply and energy consumption (Ouzounidou et al., 1997).

Accumulation in plants of proline and sugars in response to environmental stresses has been reported by various workers (Hare et al., 1998; Verbruggen and Hermans, 2008; Szabados and Savouré, 2010), but there is no investigation in response to OMW application. Little is known also on the effect of various stress conditions on pollen morphology and production (Prasad et al., 1999, 2002; Cross et al., 2003; Koti et al., 2005). In pollen grains have been identified different fatty acids and phenolic compounds that are considered precursors in the polymerization of sporopollenin (Scott, 1994; Boavida et al., 2005) and all of these compounds can be found in OMW (Ouzounidou et al., 2008, 2010). To our knowledge, this is the first report on effects on pollen grains following application of OMW. Therefore, we studied the impact of olive mill-wastewater (OMW) dilutions (1:20 and 1:10) on growth, photosynthesis, proline and sugar accumulation, and pollen morphology of spinach (*Spinacia oleracea* L.) to evaluate the application of OMW dilutions as pretreatment technique, prior to OMW land disposal.

2. Materials and methods

2.1. Materials and growth conditions

The OMW used in the treatments obtained from a three-phase olive mill plant located near the city of Kalamata (Southern Greece), and exhibited the characteristics summarized in Table 1. The samples were stored in big PVC vessels tightly closed at 4 °C before use. The cultivated soil exhibited the following characteristics: pH 7.2, water holding capacity (%) 85, electrical conductivity (mS cm^{-1}) 4.23, organic matter (%) 12.0, C.E.C. (meq 100 g^{-1}) 30.4, total salt content (%) 0.28, exchangeable Na (meq 100 g^{-1}) 0.56.

The seeds of *Spinacea oleracea* L. cv Virofly were sown in sterilized sand and left for 10 day germination. Then the seedlings were transferred into cultivated soil filled pots (three plants per pot). The pots (5 l each) were daily irrigated with 500 ml of full strength Hoagland nutrient solution (Ouzounidou et al., 1997). A total of 15 pots were placed in a randomized block designed in a growth chamber programmed for 16 h photoperiod with photosynthetic photon flux density of 300 μmol m^{-2} s^{-1} at plant level, temperature $21 \pm 1/19 \pm 1$ °C day/night and relative humidity $65 \pm 5/75 \pm 5$%. One-month-old plants cultivated in soil were subjected to OMW treatments for another month and irrigated with either 500 ml of Hoagland (control) or 500 ml 1:20 OMW dilution (OMW: Hoagland) or 500 ml 1:10 OMW dilution (OMW: Hoagland) every day, adjusted to pH 5.2 ± 0.1. The total volume of raw OMW used in our study, was approximately 25 l for both OMW treatments (1:20 and 1:10) in the two replications. The experiment lasted up to pollen production of spinach (*Spinacia oleracea* L.) plants and after this, pollen grains were collected, and then spinach plants were separated to roots and leaves, and used for chemical analysis.

Table 1
Physicochemical properties of raw OMW used as nutrient solution (modified from Ouzounidou et al. (2008)).

Parameters	3-Phase OMW
pH	4.8 ± 0.2
Total proteins (g l^{-1})	2.6 ± 0.75
COD (mg l^{-1})	$36,800 \pm 170$
TOC (mg l^{-1})	$26,600 \pm 670$
Total phosphorus as P_2O_5 (mg l^{-1})	9.51 ± 1.2
Total phenols (mg l^{-1})	27.5 ± 3.5
Potassium as K_2O (mg l^{-1})	3840 ± 310
Total nitrogen (mg l^{-1})	370 ± 58
Tannins (g l^{-1})	2.2 ± 0.8
Total Suspended Solids (TSS, %)	1.1 ± 0.5
Fatty acids (%)	
Myristic acid (C 14:0)	2.82 ± 0.9
Palmitic acid (C 16:0)	10.75 ± 1.3
Palmitoleic acid (C 16:1)	0.84 ± 0.02
Stearic acid (C 18:0)	3.10 ± 0.5
Oleic acid (C 18:1)	69.70 ± 2.1
Linoleic acid (C 18:2)	9.40 ± 0.9
Arachidic acid (C 20:0)	0.49 ± 0.01
Linolenic acid (18:3)	1.6 ± 0.1

Values are the mean of three measurements ±SE.

2.2. Chlorophyll content

After one month OMW treatment, chlorophylls (a+b) of the youngest fully expanded leaf of control and OMW treated plants were quantitatively measured in 100% acetone extract by spectrophotometry using the re-determined extinction coefficients (Lichtenthaler and Buschmann, 2001; Ouzounidou et al., 2006).

2.3. Proline and sugar content

Proline and sugar content of control and one month OMW treated plants were determined as described previously (Giannakoula et al., 2010). Fresh roots and shoots-leaves of spinach were cut into small pieces, weighed, placed separately in glass vials containing 10 ml of 80% (v/v) ethanol, and heated at 60°C for 30 min. The extract was then filtered and diluted with 80% (v/v) ethanol up to 20 ml (Khan et al., 2000). The concentrations of free proline and sugars were determined in this extract following the acid-ninhydrin reagent method and the anthrone method, respectively (Khan et al., 2000; Plummer, 1978).

Two milliliters of the aqueous alcohol extract was transferred into test tubes and 2 ml of acid-ninhydrin was added. With glass marbles on top, to minimize evaporation, test tubes were maintained at 95 °C for 60 min in a water bath and then allowed to cool at room temperature (Giannakoula et al., 2010). Four milliliters toluene was added to each replicate and mixed thoroughly. After separation of solution layers, the toluene layer was carefully removed and placed in glass cuvetes, and absorption was determined at 518 nm with a spectrophotometer (UV-1700 Shimadzu, Tokyo, Japan). Ethanol extracts, as used for proline assay were diluted 10 times with 80% (v/v) ethanol for the assay of sugars. The diluted extract was added drop-by drop in 2 ml anthrone reagent in test tubes in ice bath and left to mix the content (Giannakoula et al., 2008). Fully mixed samples were incubated in a water bath at 90 °C for 15 min, cooled and absorbance was read at 625 nm with a spectrophotometer (UV-1700 Shimadzu, Tokyo, Japan).

2.4. Total polyphenol content

The total polyphenols were extracted according to the method described by Ayaz et al. (2007) and determined by the Folin–Ciocalteu method with calibration curves for gallic acid. Absorption was determined at a wavelength of 725 nm with a spectrophotometer (UV-1700 Shimadzu, Tokyo, Japan). Data were expressed as mg gallic acid equivalents per kg FW spinach.

2.5. Chlorophyll fluorescence measurements

In vivo chlorophyll fluorescence was measured as described previously (Ouzounidou et al., 2008) on the upper surface of the fully expanded younger leaves after they were left for 30 min to dark adaptation, at room temperature. We measured the maximal photochemical efficiency of PSII photochemistry in the