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Effects of Olive Mill Wastewater on Seed Germination and Seedling Growth

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ABSTRACT

Olive mill wastewater (OMW) is considered as phytotoxic and thus an environmentally hazardous material. Therefore, alternative areas of application have been discussed; one of these might be its use as an additive in growing media for agricultural purposes. However, phytotoxic responses might occur at higher concentrations. The objective of the present study was to determine threshold levels for application of OMW in agriculture. The hypotheses were that (i) high concentrations of OMW are detrimental to seed germination and seedling growth, (ii) toxicity caused by OMW may be removed through sedimentation in a settling basin prior to addition. Data obtained showed that low concentrations of OMW did not reduce seed germination and seedling growth. However, higher concentrations showed significant differences in almost all the evaluated parameters. The addition of raw OMW at high concentrations (over 50%) reduced strongly *in vitro* seed germination, α- and β-amylase activities. The great toxicity of the OMW observed in an *in vitro* experiment decreased drastically when the same OMW was utilized onto soil in microcosm, showing a decrease at about 35% in germination for fava (*Vicia faba* L.), sulla (*Hedysarum coronarium* L.), chicory (*Cichorium intybus* L.) and wheat (*Triticum durum* Desf.) seeds. These mean that the phytotoxic effects of OMW might be modified by chemical and biological processes in soil.

Keywords: amylase activities, germination, olive mill wastewater, seedling growth

INTRODUCTION

Olive mill wastewater (OMW), a by-product of the olive mill industry, is produced by traditional and industrial olive mills in large amounts in Mediterranean countries over a limited time period (usually from October-December). Olive oil production worldwide continues to increase and is estimated to be about 2.8×10^6 ton/year (IOOC 2006). A major portion of the oil production (76%) comes from European Union Countries. Consequently, disposal of olive mill wastes (waste-water and olive husk) produced at a rate in excess of 10×10^6 ton/year becomes a significant environmental problem in all Mediterranean Countries (Sabbah *et al*. 2004; Khoufi *et al.* 2006). The production of OMW is estimated to be more than 30 million m^3 annually in the Mediterranean regions, where the culture of the olive-tree has an important socio-economical place (Casa *et al*. 2003). The removal of this waste is a problem for the whole community in general and for the producers and millers in particular. This is due to the great toxicity that OMW exhibited against microorganisms and plants during seed germination and plant growth (Paixão *et al*. 1999; Fiorentino *et al*. 2003; Isidori *et al*. 2005; Mekki *et al*. 2008). Olive mill wastewater contains a high organic load, substantial amounts of plant nutrients but also several compounds with recognized toxicity towards living organisms. The toxicity of olive mill wastewaters is commonly attributed to monomeric phenols, high percentage of salt, acidic pH, as well as short and long-chain fatty acids (Casa *et al*. 2003; Kistner *et al*. 2004; Isidori *et al*. 2005; Celano *et al*. 2008) and antimicrobial (Gonzalez *et al*. 1990; Paixão *et al*. 1999; Fiorentino *et al*. 2003; Isidori *et al*. 2005; McNamara *et al.* 2008). Several phenolic compounds present in OMW have considerable phytotoxic effects (Wang *et al*. 1967; Pérez *et al*. 1992; Quarantino *et al*. 2007). Piotrowska *et al.* (2006) indicated that the impact of OMW on soil properties was the result of opposite effects, depending on the relative amounts of beneficial and toxic organic and inorganic compounds. The toxic compounds contained in OMW most likely counteracted the beneficial effect of organic substrates provided, which promoted the growth and activity of indigenous microorganisms. Saadi *et al*. (2007) found direct short-term effect of OMW application on soil phytotoxicity. However, the soil was partly or completely recovered between successive applications. No further phytotoxicity was observed in treated soils as compared with control soil, 3 months after OMW application. Such short-term phytotoxicity was not in correlation with measured EC and total polyphenols in the soil extracts. Komilis *et al.* (2005) showed that OMW may have beneficial effects when applied to plants at certain loading rates. Flouri *et al*. (1988) mentioned that after application of OMW onto soil, the latter was enriched with nitrogen-fixing bacteria and acquires fighting properties against phytopathogenic fungi. Generally, according to Flouri *et al*. (1988), OMW irrigation increases soil fertility. These findings were confirmed by Di Serio *et al*. (2008) that evidenced the economic and environmental validity of the OMW spreading on cultivated soils since it does not induce toxic effects on the soil, helps to reduce, or avoid, the chemical fertilizer with macronutrients. Balis *et al*. (1995) mention that an OMW biologically derived compost was successfully used as a fertilizer during the cultivation of olive trees, grapes and potatoes. Rousan (2007) utilized OMW without preliminary treatments to barley crops as a soil amendment, showing that OMW spreading caused no significant differences in barley yield compared to untreated plantation. Bonari *et al*. (1993) performed irrigation experiments using OMW and concluded that if OMW is applied to the soil 60 days after seeding, no detrimental effects are observed on the newly grown seeds as long as the OMW annual dosage is kept between 4 and 8 ton per 1000 m^2. Fiestas Ros de Ursinos (1986) obtained the same results as Bonari *et al.* (1993).

Moreover, OMW may represent a low-cost source of water in region with scarce presence of rain or irrigation water. These liquid effluents, in the majority of cases are

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collected in pits located near the mills, or discharged in non adapted wastewaters canalization, which can constitute an environmental pollution for ground water and stations of wastewaters treatment. To this very significant production of OMW and in absence of all adapted treatment processes, controlled spreading can constitute a less expensive alternative for the evacuation of a great quantity of these liquid effluents. Some characteristics of this material are favourable for agriculture, since this effluent is rich in organic matter, N, P, K and Mg (Casa *et al*. 2003; Rinaldi *et al*. 2003).

OMW management has been a major issue of environmental concern for olive oil-producing countries. OMW can be a serious nuisance, when disposed of untreated, due to its significantly high organic load, its phytotoxic properties and its relatively low biodegradability (Komilis *et al* 2005). Field and plant irrigation with raw or pre-treated OMW may represent an easy and relatively inexpensive method to treat and dispose of OMW. Typical pre-treatment techniques could be comprised of phase separation through a settling basin, dilution with water, aeration to promote biological degradation and pH neutralization.

The aim of this study is the evaluation of the effects of OMW without preliminary treatments, on seed germination of *Triticum durum*, *Cichorium intybus*, *Vicia faba* and *Hedysarum coronarium*. Germination trials were carried out *in vitro* and in microcosm. α- and β-amylase enzymes hydrolysing starch responsible for the germination process and the amount of chlorophyll in leaves of seedling grown in microcosm were tested.

MATERIALS AND METHODS

The original OMW used in the present study was obtained from an olive oil production plant located in the city of Lametia Terme, Italy, which uses a continuous process for extraction of olive oil. The OMW utilised in the trials came from an olive mill equipped with a 3-phases centrifugal decanter. It was collected and stored in 200 m^3 cement tanks in October 2006 and then taken in February 2007 for the experiments. The meteoric contribution was 0.32 m^3. The OMW, obtained as above described, was refrigerated at 4°C until analysis was performed.

OMW characterization

The pH was measured using a glass electrode; electrical conductivity (dS m^{-1}) was detected using a conductivity meter (Hanna Instruments, Hi 8733). The phenolic content of the OMW was determined by extraction with distilled water (200 g l^{-1}) after agitation for 18 h. Total phenolic content was determined by a colorimetric method using Folin–Ciocalteu's reagent (Box 1983). Organic C was estimated by the Walkley-Black procedure (Nelson and Sommers 1982). Total extractable polyphenols (TEP) were determined according to Julkunen-Tiito (1985), using the original extract. The parameters COD and BOD of the collected OMW samples were determined according to the "Standard Methods for the Examination of Water and Wastewater, 20th Edition 1998". All chemicals of analytical grade were purchased from Sigma. Chemical characteristics of OMW are reported in **Table 1**.

Table 1 Chemical characteristics of olive mill wastewater.

Parameters	
Organic carbon (mg ml^{-1})	5.52 ± 0.05
Water-soluble phenol (mg TAE l^{-1})	5.47 ± 0.5
pH	5.50 ± 0.1
BOD_5 (g l^{-1})	4.1 ± 0.8
COD (g l^{-1})	10.5 ± 1.3
EC (dS m^{-1})	4.59 ± 0.03
TEP (mg l^{-1})	11.3 ± 0.7

Each value represents the mean of 5 independent observations ± S.E.

Table 2 Chemical properties of soil into microcosm.

pH (H_20)	8.10 ± 0.2
O.C. %	0.96 ± 0.1
O.M. %	1.65 ± 0.1
Sandy %	82 ± 2.0
Silt %	2 ± 1.0
Clay %	16 ± 1.0
Textural class	Sandy-loam
$CaCO_3$ %	7.0 ± 0.7
Humic carbon %	0.23 ± 0.03
Humification ratio	24% ± 0.8
Phenols (mg TAE l^{-1})	30 ± 1.2

Each value represents the mean of 5 independent observations ± S.E.

Germination conditions

1. In vitro experiments

Seeds of *T. durum*, *C. intybus*, *H. coronarium* and *V. faba* (purchased by Istituto del Germoplasma Bari Italy) used in the experiment were surface-sterilized for 20 min in 30% (v/v) H_2O_2, rinsed and soaked in distilled water for 1 h.

A randomized complete block experimental design with 5 replicates and 50 representative seeds per *T. durum*, *C. intybus*, *H. coronarium* and 20 for *V. faba* per Petri dish were used. Seeds were placed on a filter paper in 9 cm Petri dishes containing 3 cm^3 of distilled water (control), or 10, 25, 50, 75 and 100% of OMW. The Petri dishes were hermetically sealed with Parafilm® "M" I (Pechiney Plastic Packaging, Chicago) to prevent evaporation and then care kept in a humidity chamber at 25 ± 1°C in the dark. The seeds were considered germinated when there was radicle protrusion through the seed coat. Data are reported as mean ± standard error.

2. Soil microcosm test

The experiment was carried out in microcosm. A microcosm consisted of a soil filled in plastic pots with a mean of 23 cm diameter and a height of 21 cm. The inner cup was filled with soil that had been passed through a 2-mm sieve. Soil microcosm characteristics are reported in **Table 2**. The total number of seeds per pot was calculated on the basis of total surface area of each pot which came to about 20 seeds of wheat, fava, chicory and sulla per pot. The soil in the control pots were sprayed with deionised water. The OMW were utilized at the concentration of 10, 25, 50, 75 and 100%. Dilutions were obtained using distilled water. Five replicate microcosms were used for each species and each soil treatment. The microcosms were kept in a growth chamber under controlled temperatures (25°C) and 16 h lighting cycles a photon fluence rate of 300 μmol m^{-2} s^{-1} at plant height and 70% relative humidity.

The superior side of each microcosm was covered with parafilm to limit evaporation and maintain constant soil moisture. Growth index, determined as rate between fresh weight of treated seedlings and fresh weight of untreated seedlings X 100 after 30 days of growth in microcosm. Chlorophyll content was measured in leaves of 30 day old seedlings by using a Chlorophyll detector (SPAD 502, Minolta).

Enzyme assay

α- and β-amylase activities in the crude extracts of each species were determined. The seeds of each species, in deionised water (control) and treated with different concentrations of OMW (10, 25, 50, 75, 100%) were homogenised in a chilled mortar with distilled water 1: 4 (w/v) and centrifuged at 14,000 × *g* for 30 min. The supernatants were filtered through a single layer of muslin cloth and were used for α-amylase (EC 3.2.1.1) (Coombe *et al.* 1967) and β-amylase (EC 3.2.1.2) (Bergmeyer *et al.* 1983), estimation.

Statistical analysis

Data were analyzed separately for each species by one way procedure of ANOVA ($P \leq 0.05$) according to a completely randomized design with five replicates. Treatment means were compared using the Student-Newman-Keul test ($P \leq 0.05$) (Sokal and Rohlf 1981).

RESULTS

The OMW is characterized by an intensive violet-dark brown up to black colour, and a strong specific olive oil smell. The chemical properties of OMW (**Table 1**) are similar and generally fall within the ranges commonly reported in the literature for OMW (Paredes *et al*. 1999; 2002). However, with respect to others it has lower COD and BOD values. Seed germination percentage for each species examined decreased with increasing OMW concentrations. Fava and wheat showed a slight decrease in germination (about 5 and 15%, respectively) up to 25% OMW (**Table 3, Figs. 1-2**). At higher OMW concentrations the germination percentage drastically decreased and when 100% OMW

Fig. 1 Effect of different concentrations of olive mill wastewater *in vitro* on seed germination of *Triticum durum*.

Fig. 2 Effect of different concentrations of olive mill wastewater *in vitro* on seed germination of *Vicia faba*.

Table 3 Effects of different concentrations (0, 10, 25, 50, 75 and 100%) of raw OMW on seed germination, of *Triticum durum*, *Cichorium intybus*, *Hedysarum coronarium* and *Vicia faba.* The "*in vitro*" experiment was carried on in a humidity chamber at a temperature of 25 ± 1°C in the dark. The seeds were considered germinated when there was radicle protrusion through the seed coat.

Treatments	*Triticum durum*	*Cichorium intybus*	*Hedysarum coronarium*	*Vicia faba*
0%	100 a*	100 a	100 a	100 a
10%	90 ± 2 b	80 ± 4 b	95 ± 3 b	100 a
25%	85 ± 5 b	70 ± 3 c	75 ± 4 c	95 ± 2 a
50%	50 ± 2 c	40 ± 2 d	45 ± 2 d	55 ± 3 b
75%	15 ± 1 d	10 ± 1 e	10 ± 0.8 e	18 ± 2 c
100%	10 ± 1 e	10 ± 1 e	0 f	10 ± 1 d

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

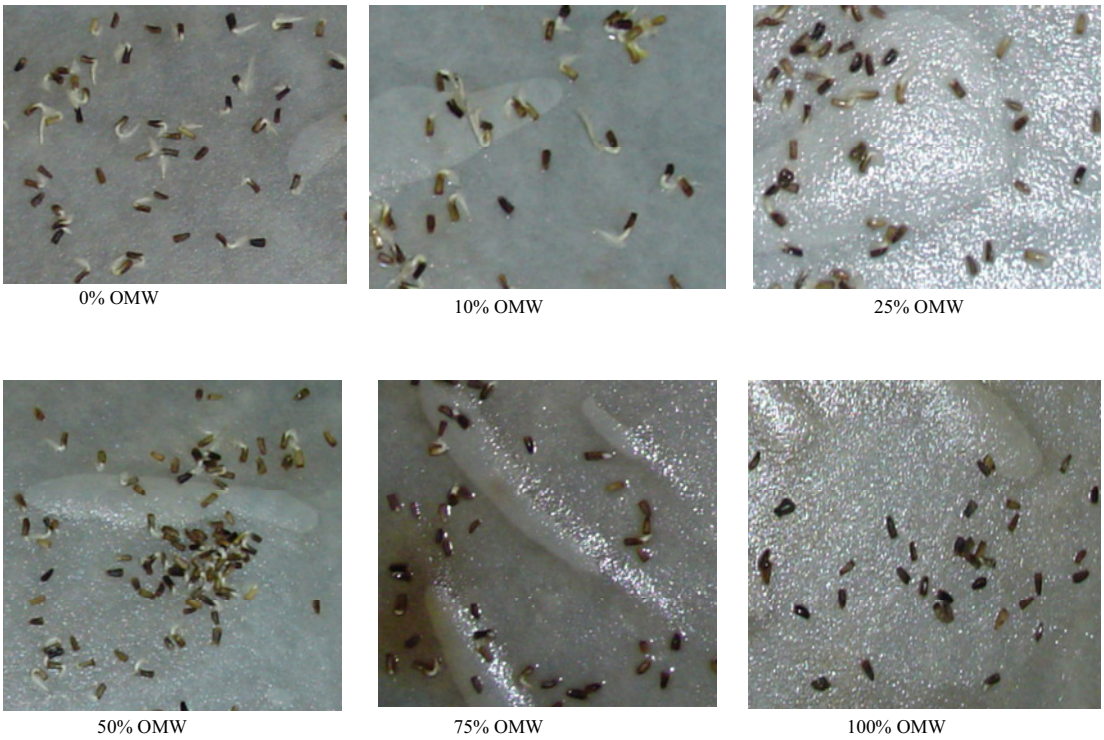

Fig. 3 Effect of different concentrations of olive mill wastewater *in vitro* on seed germination of *Cichorium intybus*.

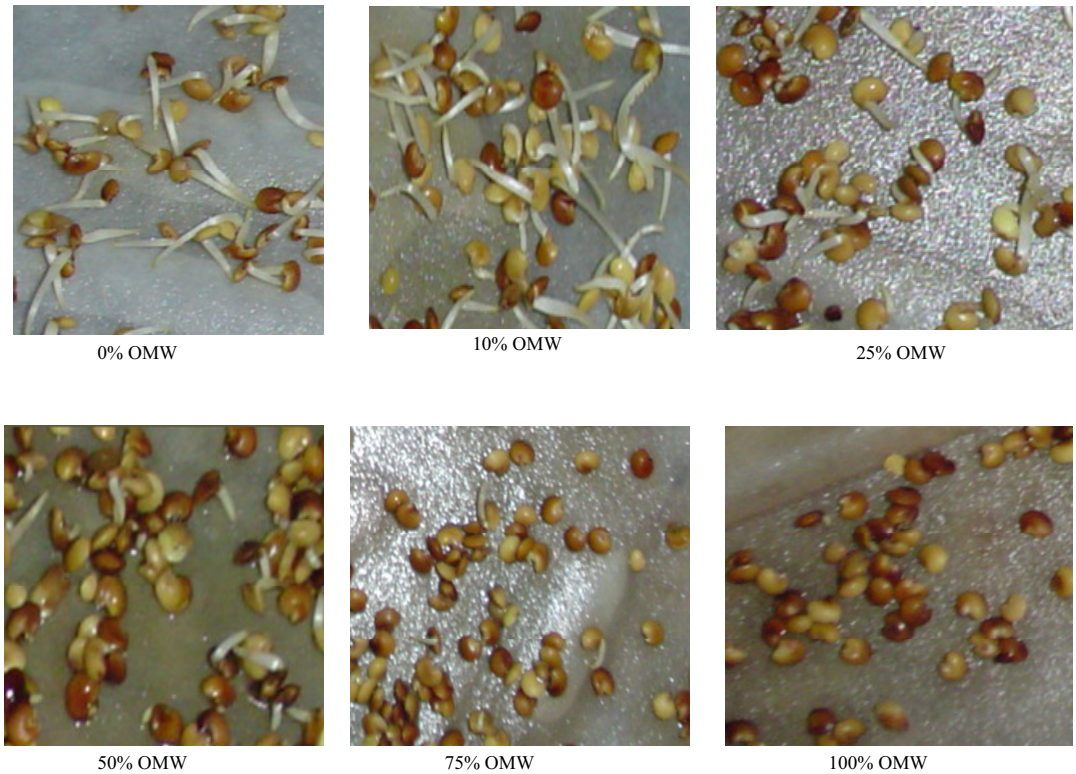

Fig. 4 Effect of different concentrations of olive mill wastewater *in vitro* on seed germination of *Hedysarum coronarium*.

was used only 10% of wheat and fava seeds germinated (**Table 1, Figs. 1-2**). *C. intybus* and *H. coronarium* showed a similar trend (**Table 3**, **Figs. 3-4**), but they resulted more sensitive to OMW toxicity than fava and wheat. The results were confirmed by data on α- and β-amylase activities (**Tables 4-5**). α-Amylase activity was more pronounced than β-amylase in each species. Both enzymes decreased with increasing OMW concentrations, in each species examined. α- and β-amylase activities in wheat and *V. faba* decreased less rapidly compared to the other two species. In addition, and in contrast in *T. durum* the α-amylase activity decreased up to 70% in the presence of undiluted OMW; the activity of β-amylase decreased less than α-amylase. A similar behaviour in *V. faba* was observed. In the other two crop species, in presence of 100% OMW both enzyme activities were undetectable. In microcosm germination experiment all the species examined were less affected by irrigation with higher concentrations of OMW than *in vitro* germination experiment. In fact comparing the results obtained for *T. durum*, increasing the OMW concentration seed germination decreased slowly and gradually. When the highest concentration of OMW was used, 65% of seeds were able to germinate. A similar trend was observed for the other species. Plant growth expressed as mean plant height over 30 days, decreased with increasing OMW concentration for both species, and varied among the four species studied (**Tables 6-9**). The detrimental effects of the highest concentration of OMW were more evident in the first stage of

Table 4 Effects of different concentrations of raw OMW on α-amylase activity (μg maltose/g fresh weight) in *Triticum durum*, *Cichorium intybus*, *Hedysarum coronarium* and *Vicia faba* seeds.

Treatments	*Triticum durum*	*Cichorium intybus*	*Hedysarum coronarium*	*Vicia faba*
0%	91.4 ± 3.5 a*	98.5 ± 3.2 a	126.4 ± 4.5 a	110 ± 4.9 a
10%	88.1 ± 2.8 a	95.9 ± 3.0 a	118.1 ± 5.6 b	99.1 ± 4.8 b
25%	74.3 ± 4.3 b	70.4 ± 4.1 b	84.3 ± 4.7 c	85.2 ± 3.5 c
50%	48.7 ± 2.5 c	55.6 ± 3.9 c	68.7 ± 3.3 d	73.8 ± 2.3 d
75%	33.2 ± 1.8 d	25.1 ± 10 d	13.2 ± 1.0 e	23.2 ± 1.0 e
100%	28.2 ± 0.9 e	Nd	Nd	21 ± 2.0 e

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Table 5 Effects of different concentrations of raw OMW on β-amylase activity (μg maltose/g fresh weight) in *Triticum durum*, *Cichorium intybus*, *Hedysarum coronarium* and *Vicia faba* seeds.

Treatments	*Triticum durum*	*Cichorium intybus*	*Hedysarum coronarium*	*Vicia faba*
0%	45.8 ± 1.2 a*	38.5 ± 3.2 a	51.8 ± 2.8 a	61.2 ± 2.9 a
10%	44.5 ± 3.5 a	35.5 ± 2.5 b	50.5 ± 1.5 a	55.1 ± 1.8 b
25%	39.8 ± 3.7 b	22.4 ± 2.1 c	44.8 ± 3.5 b	40.2 ± 2.6 c
50%	31.5 ± 1.2 c	14.6 ± 1.9 d	22.5 ± 0.9 c	23.8 ± 2.1 d
75%	26.1 ± 0.8 d	5.1 ± 1.5 e	6.3 ± 0.6 d	23.1 ± 1.2 d
100%	20.1 ± 1.0 e	Nd	Nd	21.4 ± 2.0 e

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Table 6 Effects of different concentrations of raw OMW sprayed on soil into microcosm on *Vicia faba* seed germination percentage and seedling growth (height cm) monitored over 30 days in a growth chamber under controlled temperatures (25°C) and 16 h lighting cycles.

Treatments	Germination (%)	Height (cm) 5 days	Height (cm) 8 days	Height (cm) 15 days	Height (cm) 30 days
0%	100 a*	4.0 ± 0.2 a	5.7 ± 0.2 a	11.0 ± 0.3 a	34 ± 1.2 a
10%	100 a	3.8 ± 0.3 ab	5.6 ± 0.1 a	9.9 ± 0.2 b	33 ± 1.0 a
25%	94 ± 1.2 b	4.0 ± 0.2 a	5.5 ± 0.1 a	9.8 ± 0.4 b	33 ± 1.2 a
50%	89 ± 1.2 c	3.7 ± 0.2 b	5.5 ± 0.1 a	9.9 ± 0.3 b	30 ± 1.0 b
75%	85 ± 0.6 c	3.5 ± 0.1 c	5.4 ± 0.4 a	9.3 ± 0.1 bc	29 ± 1.5 bc
100%	71 ± 0.6 d	3.0 ± 0.1 d	4.7 ± 0.2 b	9.0 ±0.7 c	28 ± 1.0 c

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Table 7 Effects of different concentrations of raw OMW sprayed on soil into microcosm on *Triticum durum* seed germination percentage and seedling growth monitored over 30 days in a growth chamber under controlled temperatures (25°C) and 16 h lighting cycles.

Treatments	Germination (%)	Height (cm) 5 days	Height (cm) 8 days	Height (cm) 15 days	Height (cm) 30 days
0%	100 a*	2.0 ± 0.5 a	6.0 ± 0.7 a	16.8 ± 1.3 a	28 ± 1.0 a
10%	90 ± 3.1 b	2.0 ± 0.4 a	5.6 ± 0.5 b	15.8 ± 1.1 ab	28 ± 1.1 a
25%	87 ± 3.6 bc	2.0 ± 0.6 a	5.5 ± 0.8 b	15.6 ± 1.5 b	28 ± 1.0 a
50%	83 ± 2.1 cd	2.0 ± 0.3 a	5.0 ± 0.4 c	13.7 ± 1.1 c	27 ± 1.2 ab
75%	79 ± 3.0 d	2.0 ± 0.2 a	4.5 ± 0.5 d	13.7 ± 1.0 c	28 ± 1.1 a
100%	65 ± 2.9 e	1.0 ± 0.3 b	4.1 ± 0.4 e	12.6 ± 1.0 d	26 ± 0.9 b

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Table 8 Effects of different concentrations of raw OMW sprayed on soil into microcosm on *Cichorium intybus* seed germination percentage and seedling growth (height cm) monitored over 30 days in a growth chamber under controlled temperatures (25°C) and 16 h lighting cycles.

Treatments	Germination (%)	Height (cm) 5 days	Height (cm) 8 days	Height (cm) 15 days	Height (cm) 30 days
0%	100 a*	1.0 ± 0.2 a	3.3 ± 0.2 a	9.8 ± 0.2 a	22 ± 1.2 a
10%	100 a	1.0 ± 0.3 a	3.1 ± 0.1 ab	9.9 ± 0.2 a	21 ± 1.0 a
25%	99 ± 1.2 a	1.0 ± 0.2 a	3.1 ± 0.1 ab	9.2 ± 0.3 b	21 ± 1.2 a
50%	90 ± 1.1 b	1.0 ± 0.2 a	3.0 ± 0.1 b	9.3 ± 0.3 b	19 ± 1.0 b
75%	84 ± 0.8 b	0.8 ± 0.1 b	3.1 ± 0.4 ab	9.0 ± 0.1 b	18 ± 1.5 bc
100%	69 ± 0.5 c	0.8 ± 0.1 b	2.7 ± 0.2 c	8.9 ± 0.7 b	17 ± 1.0 c

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Table 9 Effects of different concentrations of raw OMW sprayed on soil into microcosm on *Hedysarum coronarium* seed germination percentage and seedling growth (height cm) monitored over 30 days in a growth chamber under controlled temperatures (25°C) and 16 h lighting cycles.

Treatments	Germination (%)	Height (cm) 5 days	Height (cm) 8 days	Height (cm) 15 days	Height (cm) 30 days
0%	100 a*	1.0 ± 0.2 a	3.3 ± 0.2 a	10.8 ± 0.2 a	24 ± 1.2 a
10%	100 a	1.0 ± 0.3 a	3.4 ± 0.1 a	10.9 ± 0.2 a	22 ± 1.0 b
25%	99 ± 1.2 a	1.0 ± 0.2 a	3.2 ± 0.1 ab	10.2 ± 0.3 b	20 ± 1.2 c
50%	88 ± 1.1 b	1.0 ± 0.2 a	3.0 ± 0.1 bc	9.8 ± 0.3 b	19 ± 1.0 c
75%	80 ± 0.8 b	0.9 ± 0.1 b	3.0 ± 0.4 bc	9.0 ± 0.1 c	19 ± 1.5 c
100%	68 ± 0.5 d	0.8 ± 0.1 c	2.9 ± 0.2 c	9.0 ± 0.7 c	19 ± 1.0 c

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Table 10 Growth index, determined as rate between fresh weight of treated *Vicia faba*, *Triticum durum*, *Cichorium intybus* and *Hedysarum coronarium* seedlings and fresh weight of untreated seedlings X 100 after 30 days of growth in microcosm in a growth chamber under controlled temperatures (25 °C) and 16 h lighting cycles.

Treatments	*Vicia faba*	*Triticum durum*	*Cichorium intybus*	*Hedysarum coronarium*
0%	100 a*	100 a	100 a	100 a
10%	103 a	100 a	100 a	103 a
25%	101 a	99 a	99 a	101 a
50%	99 a	100 a	100 a	99 a
75%	101 a	100 a	100 a	101 a
100%	99 a	100 a	100 a	99 a

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Table 11 Chlorophyll content (SPAD units; SPAD 502 Minolta λ=650, 940) in leaves of the different seedlings grown in microcosm with different concentrations of OMW over 30 days in a growth chamber under controlled temperatures (25 °C) and 16 h lighting cycles.

Treatments	*Vicia faba*	*Triticum durum*	*Cichorium intybus*	*Hedysarum coronarium*
0%	48 ± 1.1 a*	41 ± 1.2 a	45 ± 1.2 a	49 ± 1.1 a
10%	49 ± 1.0 a	40 ± 1.1 a	44 ± 1.1 a	48 ± 1.1 a
25%	45 ± 1.4 c	39 ± 1.0 ab	43 ± 1.0 a	47 ± 1.0 a
50%	47 ± 0.9 b	36 ± 0.9 c	38 ± 0.9 b	44 ± 0.9 b
75%	41 ± 0.6 d	33 ± 1.1 d	35 ± 1.1 c	39 ± 1.1 c
100%	41 ± 1.1 d	31 ± 1.5 e	32 ± 1.5 d	39 ± 1.5 c

Each value represents mean of five independent observations ± S.E.
[#] Values in the same column followed by the same letter are not statistically different at $P \leq 0.05$.

Fig. 5 Seedling of *Triticum durum* grown for 5 days in soil into microcosm sprayed with different concentrations of OMW [100%, 75%, 50%, 25% 10% and water (control)].

seedling growth (5 days). Increasing the concentration, root and leave length progressively decreased. After five days of treatments, by increasing the OMW concentrations, root length was more affected than leaf length, in wheat and fava (**Figs. 5-6**). Increasing the time of growth the negative effects of OMW decreased. 30 day old *T. durum* seedlings did not show differences in height compared to control seedlings even if in presence of the high OMW concentrations. A slight decrease was instead observed in the other three species when high OMW concentrations were used. The Growth Index confirmed these data (**Table 10**) showing no significant differences among treated seedlings and control. The green values detected in 30 day old seedlings, grown in microcosm, showed that the OMW did not modify up to 50% concentration the chlorophyll content in leaves of treated seedlings compared to control (**Table 11**).

DISCUSSION

The results obtained in the present study show that "*in vitro*" seed germinability of all the species examined was markedly reduced by the high concentrations of OMW (50 and 75%) and that the undiluted effluent leads to a complete suppression of the germinability. OMW phytotoxicity has been mainly attributed to the phenolic and organic acid content (Capasso *et al.* 1991; Isidori *et al.* 2005). The phytotoxic effect on higher plants is especially severe during germination and seedling development (Krogmeier and Bremner 1989). For this reason, it has been suggested that land spreading should be performed at least three weeks before sowing (Proietti *et al.* 1995). OMW phytotoxicity is a complex property, since more than one compound can be responsible for it. Polyphenols are not necessarily the sole compounds responsible for the phytotoxic properties of OMW; however, they have been claimed as the major ones responsible for phytotoxicity (Pèrez *et al.* 1986). Della-Greca *et al.* (2001) mention that it is generally accepted, though not clearly proven, that OMW phytotoxic properties are attributed to their phenolic constituents. Flouri *et al.* (1988) mention that except for the low molecular fatty acids, which are phytotoxic, OMW phytotoxicity is also attributed to phenols (Aliotta *et al.* 2000). El Hadrami *et al.* (2004) tested OMW phytotoxicity on seed germination of maize, wheat, chickpea and tomato. They observed significant reduction in seed germination specially for tomato and wheat with either OMW solution or their related soluble phenolic extracts, suggesting a predominantly inhibition effect of

Fig. 6 Seedling of *Vicia faba* grown for 5 days in soil into microcosm sprayed with different concentrations of OMW [100%, 75%, 50%, 25% 10% and water (control)].

seed germination by OMW phenolics. These findings may explain the seed germination reduction of wheat, chicory, sulla and fava observed in our study. Numerous works (Muscolo *et al*. 2001, 2002), have in fact suggested that phenolic compounds are implicated in the OMW germinability suppression or reduction and that they are able to affect the germinability of different species. Isidori *et al*. (2005) studied the effects of 15 phenolic compounds with low molecular weight (<350 Da), isolated from the reverse osmosis in the fractionation of OMW, on seeds of *Cucumis sativus*, *Lepidium sativum*, and *Sorghum bicolor*. Results of phytotoxicity showed the acute toxicity of these compounds confirming that phenols in OMW may represent an elevate risk for germination. Kim *et al.* (2005) tested allelochemical effects of phenols from leaves of three *Phytolacca* species, on seed germination of *Sonchus oleraceus* and *Lactuca indica*. Analysis of aqueous extracts by HPLC showed seven phenolic compounds (gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *m*-hydroxybenzoic acid, *p*-coumaric acid, and cinnamic acid). The results showed a marked inhibition of seed germination for both species, confirming the high toxicity of phenols. Muscolo and Sidari (2006), after having identified and quantified different soil phenolics, tested the effects of phenolic extracts, single phenolic acids and synthetic phenol mixtures on germination and glyoxylate of *Pinus laricio* Poiret, *Pinus pinaster* Aiton and *Pinus halepensis* Mill seeds. Addition of phenol extracts to germination medium reduced seed germination showing phytotoxic effects which differed, depending on the species and the fractions tested.

The results obtained in the present study show that OMW can markedly reduce seed germinability *in vitro* over 25% dilution and that the undiluted effluent leads to a complete suppression of the *in vitro* germinability in each species. In addition α- and β-amylases enzymes hydrolysing starch the major constituent of mature seeds substrate were negatively affected by high concentration of OMW. Germination activates respiration, protein synthesis and other metabolic activities. The first step in the process is related to water penetration, which occurs equally well in dead and living tissues. The seed undergoes continued swelling as the water penetrates the seed. At this point non-dormant seeds have an active metabolism in preparation for germination. The increase in water content in the wetted areas leads to germination and subsequent growth (Sidari *et al*. 2008). Starch is the major constituent of mature seeds and as the seeds are soaked in water, enzymes present in dry seeds become active. The major categories of starch degrading enzymes in germinated grains are α- and β-amylases. α-Amylase is the predominant enzyme synthesised during germination and serves to mobilise the starch reserves in the endosperm, it acts by randomly hydrolysing α-1,4-glucan linkages in the starch polymers: amylose and amylopectin (Helland *et al.* 2002). β-Amylase is an exoenzyme that attacks starch from the nonreducing ends of the dextrin products yielded by alpha-amylase and limit dextrinase. It attacks α-1,4 glucosidic bonds and breaks every second bond to release maltose. The combination of α- and β-amylase activity degrades starch faster and more completely to glucose the main respiratory substrate. Numerous works correlated germination performance with α-amylase, but in a study of the comparative importance of α- and β-amylase in determining germination ability, Das and Sen-Mandi (1992) demonstrated greater importance of β-amylase compared to α-amylase, during the early hours of germination in wheat scutella. In addition, Nandi *et al.* (1995) showed that β-amylase activity becomes detectable immediately before visible germination becomes evident, whereas α-amylase activity is initiated at later stage of germination, suggesting that α-amylase affects rate of seedling growth while β-amylase activity is associated with initiation of germination. Therefore, β-amylase is a crucial and essential enzyme for germination. The variation in stress sensitivity of different species may be linked to a strong decrease in the hydrolytic enzyme activities.

The great toxicity of the OMW *in vitro* decreased drastically when the same OMW were sprayed onto soil in microcosm, showing a 35% decrease in seed germination

for the species examined. These mean that OMW phytotoxic effects might be modified by chemical properties and biological processes in the soil.

The soil capability to reduce the phenolic load of OMW is ascribable both to its biotic components (microbial cells, free enzymes or associated to the organic or organ-mineral component in soil) and to abiotic constituents. This detoxifying potential of soil explains why OMW in microcosm decrease its phytotoxic effects. Reduction of the above constituents by any soil means will eventually reduce phytotoxicity. OMW spreading procedures, carried out with a reasonable anticipation with respect to the sowing period, do not result in a phytotoxic effect. In fact the OMW utilized in this study after four months of stockage have a low organic load due to the low amount of carbohydrate, fatty acid and phenols in particular, which represent the main source of toxicity of OMW. The data suggest that four months of stockage in the open air, are sufficient to decrease OMW phytotoxicity. Therefore, reduction in phenols and organic acids is achieved along with reduction in nutrient salts, particulate matter and organic content, minimizing the negative effects on seed germination and seedling growth. Aeration was found to be the second most important technique affecting phytotoxicity. Aeration apparently reduced BOD concentration through biological decomposition – induced by the inherent microbial population present in OMW – by transforming several phytotoxic compounds to less phytotoxic metabolic organic byproducts and CO_2. Aeration also resulted in slight pH increase but not in an electric conductivity increase. This is probably attributed to the loss of volatile acids and CO_2 to the atmosphere during stockage as well as to the biological decomposition, and therefore loss, of organic acids. Based on our results both dilution or stored OMW in an open air may represent a potential OMW treatment technique that can be adopted in some situations in the Mediterranean according to the irrigational needs, types of soil and groundwater characteristics of the land adjacent to an olive mill as well as according to local water availability. Application rates of both water and OMW should be adjusted so that desired dilution rates are maintained. Since irrigation is mainly required during the dry season, which does not coincide with the OMW production season, storage of OMW could be implemented prior to irrigation. Irrigation, however, can still be practiced during the winter months, if merely viewed as a disposal technique. Settling aids in the removal of solids that could potentially clog piping during irrigation, and in the removal of an oily fraction (supernatant) that could be recovered.

CONCLUSIONS

- All types of seeds used had comparable germination behaviours, though chicory seeds had a lower germination compared to the other seeds.
- Dilution and aeration were the primary pretreatment techniques affecting OMW phytotoxicity. Dilution with water at a high ratio reduced phytotoxicity compared to when dilution was kept at a low ratio. In particular, dilution at the 50% reduced phytotoxicity the most compared to the other factors investigated in this study. Low seed germination was observed *in vitro* when raw OMW, without any dilution, was applied to all types of seeds.
- Aeration of OMW obtained with stockage in open air resulted in reduced phytotoxicity and should be the second most important main effect after dilution. In addition, aeration resulted in slight pH increase without a corresponding conductivity increase. Our results recommend safe soil application rates in terms of 20 l OMW m^{-2} per month, considering that the OMW utilized contains about 1% COD, 1.2% TEP and 0.6% WSP.
- The recommendations should consider that OMW can be used for irrigation some months after their production, taking in account the kind of cultivation and the volumes of OMW to be used. It seems not safe to indicate limits of OMW volumes for irrigation independently of the pedologic characteristic of each soil to be irrigated. Detailed information on each agricultural site is required to delineate the correct strategies to mitigate the OMW impact on agricultural field.

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