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Phytotoxic Effects of retentates Extracted from Olive Mill Wastewater Suggest a Path for Bioherbicide Development

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Abstract: The aim of this study was to screen the phytotoxicity of different retentates concentrated in polyphenols and extracted from olive mill wastewater (OMW), namely, nano filtration retentate (RNF) and inverse osmosis retentate (ROI). The activity of both retentates was evaluated using bioassays on dry seeds (with concentrations of 0.0, 0.1, 0.5, 1.0, 5.0, and 10.0% and compared with $CaCl_2$ solutions to evaluate the salinity effects), on germinated seeds (with concentrations of 0.0, 5.0, and 10.0%), and on the emergence of seedlings from the soil (with concentrations of 0.0, 5.0, and 10.0%). Three indicator plant species were used: *Lepidium sativum* L. (cress), *Solanum lycopersicum* L. (tomato), and *Triticum turgidum* subsp. *durum* Desf. (durum wheat). The results were expressed as the germination rate or emergence rate (GR or ER, respectively) and as the average germination time or average emergence time (AGT or AET, respectively) depending on the bioassays. Salinity showed a certain effect on the GR. Total or near-total inhibition of germination was obtained with the highest concentrations (5.0–10.0%). The dose of 1.0% of RNF and that of 0.5% of ROI caused delays in the germination of cress. The germination of tomato was delayed by RNF and ROI at concentrations of 0.5% and 1.0%. The AGT of durum wheat was not affected by RNF, but was slightly affected by ROI. The development of the seedlings was inhibited by both retentates. The results in the Petri dishes were also confirmed in pots. Retentates could be evaluated as a basis for the development of bioherbicides.

Keywords: polyphenols; allelopathy; weed control; nano filtration; inverse osmosis

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1. Introduction

Chemical weed control with herbicides is still widely used, and several reasons make farmers reluctant to use alternative strategies to weed control [1]. However, chemical herbicides must be considered an "exhaustible resource that can be depleted over time" [2], and, in recent years, farmers have been experiencing a visible decrease in the number of active ingredients for chemical weed control.

The increasing attention of institutions towards possible environmental and health problems has led to more stringent rules on the use of pesticides and therefore also of herbicides [3]. Moreover, decades of chemical weed control have led to many herbicide-resistant weed populations, and few new herbicide modes of action are available to counter this trend [4,5]. Several strategies must be introduced to overcome these problems.

Integrated weed management is a holistic approach consisting of all techniques aiming at preventing infestation, improving crop competitiveness, gaining a better understanding of the biological and ecological characteristics of the weed species, making decision based on the critical period, and direct control [6]. Additionally, in this modern scenario, chemical weed control still retains its importance despite it being associated with other

strategies [7]. Therefore, the possibility of substituting synthetic active ingredients with natural compounds can lead to the discovery of new herbicides and biopesticides with new modes of action and that are more environmentally friendly [4,8].

In the last few years, many studies have been carried out on natural products with biological action. The reuse of products results from olive cultivation, such as biomasses derived from tree pruning or those obtained in olive mills by separating the leaves and thin branches from the drupes, and byproducts from olive oil extraction such as pomace and mill wastewater, has attracted growing interest [9–15]. De-oiled olive pomace, for example, proved to be effective as a mulching material for grapevines or young super-high-density olive orchards [10,11]. The reuse and significance of these byproducts is an important topic, mainly in the Mediterranean region where the most important olive oil production area in the world resides [9,14], and huge quantities of waste in short periods of time are generated yearly.

Olive mill wastewater (OMW) is a key problem because it causes concern due to its organic compounds, high salinity, acidity, and polyphenol content [16]. OMW can be used to enrich animal feed, to extract compounds useful in the pharmaceutical and cosmetic industries, to absorb heavy metals in aqueous solutions, to recover energy, and for application during or after composting on soil as fertilizers or herbicide [13,17–24]. In addition, several studies showed the phytotoxic activity of OMW [25,26]. Particularly, El Herradi et al. [27] observed a complete inhibition of radish (*Raphanus sativus* L.) and turnip (*Brassica rapa* L.), as well as also tomato (*Solanum lycopersicum* L.) and alfalfa (*Medicago sativa* L.), when seeds were treated with OMW in Petri dishes. Ghidaoui et al. [28] and Tubeileh et al. [29] observed the inhibition of germination in *Vicia faba* L. and *Malva parviflora*, respectively. L. Enaime et al. [30], in addition to recording a low germinability of tomato and maize (*Zea mays* L.) seeds treated with raw OMW, also concluded that phytotoxicity was determined by biophenolic content and other factors such as high salinity, acidity, and short- or long-chain fatty acids [31]. It was hypothesized that the OMW phytotoxicity was mainly due to the concentration of heavy metals and total phenols, but other organic and inorganic parameters such as pH, conductivity, and residual lipid fractions can contribute to phytotoxicity [32]. However, if used directly in fields, OMW could have a polluting effect on the soil, the aquifers, or the air [13,33–35]. As shown by Paraskeva et al. [36], the filtration process of OMW results a reduction in the chemical oxygen demand (COD) values and obtaining retentates containing mainly the bioactive fraction. Furthermore, retentates are also more easily stocked and therefore usable for a longer period than OMW, which needs to be distributed as soon as it is produced.

The chemical composition of OMW depends on many factors including the olive mill extraction technology. In a study, OMW from a two-phase centrifugation olive oil production process was found to be more phytotoxic than a three-phase process for cress, although both OMWs had similar total phenolic contents [26]. Pretreatment is necessary to manage OMW through the use of technologies that minimize environmental impact, improve efficiency, and allow the sustainable use of these resources [37]. Several methods of treatment have been studied [21]; among these, Bellumori et al. [38] proposed an integrated centrifugation–ultrafiltration system that reduces pollution and allows the separation of some useful compounds such as polyphenols. Olive mill byproducts generally contain 98% of the total drupe polyphenols [25] depending on the oil mill technologies [12]. During crushing and mixing, oleuropein, one of the major phenolic compounds found in olives, is enzymatically converted by β-glucosidase into other polyphenols and secoiridoid derivatives, which are phenols with a complex structure derived from the secondary metabolism of terpenes. Hydroxytyrosol, tyrosol, oleuropein, and caffeic acid are the main phenolic compounds that remain in an aqueous phase of OMW [26]. These biophenols can be extracted and concentrated from OMW through industrial membrane filtration systems, allowing one to obtain retentates [38].

Polyphenols have been studied for the control of fungi, nematodes, and insects because of their bioactive properties [39–41]. Moreover, they are also a category of compounds

implicated in plant allelopathy [42,43], and some researchers have proposed enhancing these properties to increase the availability of the active ingredients and as tools for weed control [44,45]. Once the phytotoxic activity of the polyphenols is confirmed, they could be useful to the development of herbicides to increase the effectiveness and sustainability of weed control practices.

The aim of this study was to provide a screening for the phytotoxicity of retentates concentrated in polyphenols derived from OMW using germination and emergence bioassays carried out on three indicator plant species genetically distant one from each other.

2. Materials and Methods

2.1. Retentates Production

Two retentates were provided by a commercial plant. They were obtained with technologies used for the treatment and recycling of OMW. Before being processed, the OMW was pretreated with a double set of 100, 60, and 25 μm mechanical filters with automatic regeneration to eliminate solid residues. The technologies were based on the extraction and concentration process of polyphenols from OMW through four steps corresponding to four sequential membrane operations: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO), respectively. In each step, a permeate and a retentate were obtained. The retentate is the part that does not pass through the membrane, while the permeate is that part that does pass through the membrane. Permeates were used in the next step. The MF and UF operated in the particle size ranges of 0.1–1.0 μm and 0.01–0.1 μm, respectively. In this study, we used only retentates derived from NF (particle size range of 1.0–10.0 nm) and RO (particle size range of 0.1–1.0 nm), henceforth indicated as RNF and ROI, respectively [38]. The retentates were stored in a controlled nitrogen atmosphere for 24 h to allow the breakdown of oleuropein into the most valuable compounds: hydroxytyrosol, tyrosol, and verbascoside [46].

2.2. Characterization and Quantification of Phenolic Compounds in the Retentates

The characterization and quantification of the phenolic compounds in the retentates were carried out using a reverse-phase high-performance liquid chromatography analysis (HPLC) on an LC-10AD Shimadzu (Milan, Italy) liquid chromatograph equipped with an SPD M10A VP diode array detector (Shimadzu). A binary gradient elution was used. A maximum absorbance of 279 nm was used for wavelength detection. The retentates were injected after appropriate dilution with a solvent system composed of solvent A (water: trifluoroacetic acid, 97:3, v/v), and solvent B (acetonitrile: methanol, 80:20, v/v). A step gradient, from 5% to 98% B (45 min), was applied at a flow rate of 1 mL min^{-1}. More details on the applied methodology can be found in the work of De Marco et al. [47]. The total phenolic content, obtained through ultraviolet/visible spectroscopy, was obtained as the sum of all the phenolic compounds, and it was expressed as percentage by weight of the hydroxytyrosol equivalent. The quantitative composition of the RNF and ROI was provided by Azienda Agricola Fangiano, and more details about the analysis protocols and their detailed characterization can be found in the work of Bellumori et al. [38].

The retentates were diluted into six concentrations (0.0%, 0.1%, 0.5%, 1.0%, 5.0%, and 10.0%) using distilled water. A portable conductivity meter (Mettler Toledo-Seven2Go Pro) was used to measure the electrical conductivity (EC) and pH.

2.3. Evaluation of the Phytotoxic Activity of Retentates

The seeds of three different indicator plant species were used to detect the activity of retentates: cress (*Lepidium sativum* L., manufacturer code: IT080589), tomato (*Solanum lycopersicum* L. cv 'San Marzano', manufacturer code: IT080589), and durum wheat (*Triticum turgidum* subsp. *durum* Desf., provided by a local farm). The species listed here were used to allow a quick and easy response because their seeds germinate quickly and uniformly, grow fast, and are highly susceptible [45,48]. They had not undergone any pre-treatment before being used for the experiments. Moreover, they belong to botanical families genetically

distant one from each other; thus, they can provide much information about the activity of the compounds.

A preliminary test was carried out in order to evaluate if salinity stress, induced by the retentates, was the only cause of inhibition. The seeds (n = 25) of each indicator species were placed in Petri dishes (Ø = 9 cm), with a double layer of filter paper (Whatman No. 1) wetted with 2.0 mL of increasing concentrations of each retentate (0.0%, 0.1%, 0.5%, 1.0%, 5.0%, and 10.0%) or with solutions of $CaCl_2$ having the same EC of the concentrations of the retentates (see Table 1).

Table 1. Electrical conductivity of RNF and ROI concentrations.

Concentration (%)	Electrical Conductivity (mS cm^{-1})	
	RNF	ROI
0.0	0.0	0.0
0.1	0.8	1.1
0.5	3.8	4.6
1.0	6.0	7.3
5.0	24.6	33.2
10.0	41.4	56.3

The germinated seeds in each dish were counted daily. Seeds were considered completely germinated when they showed both the root and the cotyledons (or the first leaf for wheat). Seeds that only showed roots were not counted because the germination growth stage starts from dry seed (or caryopsis) and ends with the emergence of the coleoptile. The trial was stopped when no newly germinated seeds were recorded in the control for 3 continuous days. The results were expressed in terms of the germination rate (*GR*). This is summarized in Equation (1):

$$GR(\%) = \frac{n}{N} \cdot 100 \quad (1)$$

where n is the number of germinated seeds, and N is the total seeds in the dish.

2.3.1. Bioassay on Dry Seeds

The same experimental protocol, previously described (see Section 2.3), was repeated to evaluate only the effects of RNF and ROI. Six concentrations of each retentate (as in the preliminary experiment) were used, and, in this case also, the results were expressed in terms of the germination rate (*GR*) When the *GR* was other than 0.0, the average germination time (*AGT*) was calculated to provide information about a possible delay in germination. This is summarized in Equation (2):

$$AGT(d) = \frac{\sum n \cdot t}{N} \quad (2)$$

where n is the daily number of germinated seeds; t is the days of incubation; and N is the number of germinated seeds.

2.3.2. Bioassay on Germinated Seeds

The bioassay was conducted on germinated seeds, namely, those showing the radicle (dicotyledons) or coleoptile (*Triticum turgidum* subsp. *durum* Desf.). This was performed to evaluate whether retentates act only as germination inhibitors or also have an effect on the development of the seedling once the first germination step is completed.

The concentrations with the highest phytotoxic inhibition effects from the previous bioassay (5.0–10.0%) were tested. The seeds of the three different indicator plant species were allowed to germinate on a double layer of filter paper (Whatman No. 1) in Petri dishes (Ø = 9 cm) wetted with distilled water and incubated in a growth chamber under the same conditions as those used in the bioassay on dry seeds. As soon as the seeds reached the desired stage (i.e., elongation of the radicle for dicotyledons, or the emergence

of the coleoptile from the caryopsis with a maximum length of 2 mm), the germination process was continued by placing twenty-five seeds on filter paper wetted (just enough of a uniform amount to moisten the filter paper) with 2 mL of different concentrations of retentates. The seeds whose roots continued to lengthen and with cotyledons (or the first leaf for wheat) appearing and not showing any necrotic areas were counted. Additionally, in this case, the results were expressed as the GR, namely, the % of seedlings (with respect to the control) continuing to grow. The trial was stopped when no new seedings continuing to grow were recorded in the control for 3 continuous days.

2.3.3. Bioassay on the Seedling Emergence from the Soil

This bioassay allowed us to observe the activity of the compounds in a more complex physicochemical system and also one that is closer to the real field conditions. The seeds (n = 25) of each indicator plant species used in the previous bioassays were sown in plastic pots of 15 cm (length) × 10 cm (height) containing a commercial substrate (Brill Type 3 Special—Gebr. Brill Substrate GmbH & Co., Georgsdorf, Germany) composed of a mixture (50:50 v/v) of very fine black and white peat. Peat-based mixes represent the most common plant growing media due to their optimal physicochemical properties. Immediately after sowing, three increasing concentrations of retentates (0.0, 5.0, and 10.0%) were spread on the soil surface of each pot. A volume of 100 mL per pot was used. The pots were irrigated from the bottom, soaking up the water placed in the tray. The number of emerged seedlings in each pot was counted daily. The results were expressed both in terms of the emergence rate (*ER*) and as the average emergence time (*AET*) to verify a possible delay in emergence. They are summarized in Equation (3) and Equation (4), respectively. Seeds were considered emerged when the cotyledons (or the first leaf for wheat) were visible. The trial was stopped when no newly emerged seeds were recorded in the control for 3 continuous days.

$$ER(\%) = \frac{n_e}{N} \cdot 100 \quad (3)$$

where n_e is the number of emerged plants, and N is the total seeds sown in the pot.

$$AET(d) = \frac{\sum n_e \cdot t_e}{N_e} \quad (4)$$

where n_e is the daily number of emerged plants; t_e is the days from sowing; and N_e is the number of emerged seeds.

2.4. Environmental Conditions

All the experiments were conducted using putting pots or dishes in a growth chamber (Fitotron® SGC 120 LED Production Chamber Weiss Umwelttechnik GmbH, Reiskirchen, Germany) at a temperature of 27 °C, with 85–90% relative moisture, and constantly illuminated (150 µmol m^{-2} s^{-1}).

2.5. Statistical Analysis

A preliminary experiment to evaluate the EC effects was conducted according to a two-way completely randomized design, assigning the type of solution (retentates or $CaCl_2$) and concentrations as factors. The other experiments were conducted according to a one-way completely randomized design. Four replications (Petri dishes or pots) were performed for each treatment. The results were subjected to analysis of variance (ANOVA), and the significance between treatments was determined using Duncan's multiple range test at $p \leq 0.05$. The CoStat statistics software (www.cohort.com, accessed on 5 March 2020) was used to perform the analysis [49].

3. Results

The main chemical parameters are presented in Table 2. Retentates showed the highest total polyphenol concentrations in RNF compared to ROI. In both retentates,

hydroxytyrosol was the main phenol. Verbascoside and tyrosol were more concentrated in RNF than ROI, while hydroxytyrosol was prevalent in ROI. According to their chemical structure, hydroxytyrosol, tyrosol, and verbascoside were classified as benzoic acids with only hydroxyl groups (-OH) [26]. Note that the number of -OH substituents in the chemical structure of these polyphenols is high: nine for verbascoside, three for hydroxytyrosol, and two for tyrosol.

Table 2. Dry weight of the chemical compounds of retentates (1).

Parameters	RNF	ROI
Water content (g 100 g^{-1})	31.3	47.4
Total polyphenols (% by weight of equivalent hydroxytyrosol)	14.0	12.0
Verbascoside (g L^{-1})	5.5	3.9
Tyrosol (g L^{-1})	11.8	8.1
Hydroxytyrosol (g L^{-1})	33.1	48.8
Carbohydrates (g 100 g^{-1})	54.0	38.5
Ashes (g 100 g^{-1})	11.4	12.5
Lipids (g 100 g^{-1})	0.1	0.2
Protein (N ×6.25 g 100 g^{-1})	3.0	1.3
Insoluble fiber (g 100 g^{-1})	0.31	0.29
Na (mg 100 g^{-1})	66.8	72.7
K (mg 100 g^{-1})	1955.7	2236.5

(1) Data are the mean of three determinations.

3.1. Physicochemical Effects

The salinity of the retentate solutions, measured using the EC (mS cm^{-1}), increased with the concentration. It was more elevated in ROI (0.8–41.4 mS cm^{-1}) than RNF (1.1–56.3 mS cm^{-1}) (Table 1). The pH values of the 0.1, 0.5, 1.0, 5.0, and 10.0% concentrations were in the range of 5.0–5.5 and 5.0–5.6 for RNF and ROI, respectively.

Salinity stress showed a certain effect on the germinability of the seeds of all three indicator species. However, the statistical analysis showed highly significant effects of both the treatments and their interaction with EC. In particular, for all the species, the seeds treated with the retentates had a lower germinability than those treated with $CaCl_2$ at each level of EC (data are shown in the Supplementary Materials, Tables S1–S3). This allowed us to exclude that the observed effects were only due to the salinity level of the solutions.

3.2. Effects of Retentetates on Dry Seeds

Cress germination was completely inhibited with concentrations of 5.0 and 10.0% of RNF and ROI (Table 3). With RNF at a concentration of 1.0%, the GR was 82.0%, which was statistically lower than that recorded with 0.1–0.5 and 0.0%. The dose of 1.0% ROI was able to reduce the GR (88.0%) with respect to the other concentrations. Additionally, for tomato, the highest concentrations (5.0 and 10.0%) of both RNF and ROI inhibited germination. With RNF at concentrations of 0.5 and 1.0%, a higher GR was recorded, although this was still statistically lower with respect to the 0.1% concentration and the control. The GR of durum wheat was the lowest at the doses of 5.0 and 10.0% of both retentates. With ROI at concentrations of 0.5 and 1.0%, the GR was still significantly lower than at concentrations of 0.1% or 0.0%.

For the treatments in which no germinated seeds were recorded, the AGT value was not calculable. The AGT of cress was 4.0 d with a 1.0% concentration of RNF, and there were no significant differences between the control or those with doses of 0.1 and 0.5% (Table 4). When the seeds were treated with ROI, the highest AGT (3.9 d) was observed with the concentration of 1.0%, although also the dose of 0.5% gave an AGT (3.4 d) higher than those recorded for the 0.1 and 0.0% doses. The AGT of tomato was 8.0 d with the 0.5% dose and 8.9 d with a 1.0% dose of RNF, whereas, with the dose of 0.1%, it was not significantly different from that of the control. The doses of 0.5 and 1.0% of ROI gave data significantly

higher than those of the control and with the 0.1% dose. No significant differences between treatments were observed for the durum wheat treated with RNF, whereas the AGT of the seeds treated with ROI was not statistically different from the control with all doses except for the 1.0% dose.

Table 3. Effects of different concentrations of retentates on germination rate of cress, durum wheat, and tomato (1).

Concentration (%)	Germination Rate (%)					
	Lepidium sativum L.		*Solanum lycopersicum* L.		*Triticum turgidum durum* Desf.	
	RNF	ROI	RNF	ROI	RNF	ROI
0.0	100.0 ± 00.0 a	100.0 ± 00.0 a	84.0 ± 4.6 a	67.0 ± 10.0 a	74.0 ± 15.5 a	84.0 ± 07.3 a
0.1	98.0 ± 02.3 a	100.0 ± 00.0 a	87.0 ± 2.0 a	78.0 ± 06.9 a	67.0 ± 13.6 a	84.0 ± 05.7 a
0.5	93.0 ± 06.0 a	98.0 ± 04.0 a	72.0 ± 5.7 b	58.0 ± 27.4 a	64.0 ± 15.0 a	71.0 ± 05.0 b
1.0	82.0 ± 13.7 b	88.0 ± 09.8 b	52.0 ± 7.3 c	57.0 ± 14.4 a	69.0 ± 10.5 a	61.0 ± 10.0 c
5.0	0.0 ± 00.0 c	10.0 ± 10.1 c	0.0 ± 0.0 d	0.0 ± 00.0 b	2.0 ± 04.0 b	0.0 ± 00.0 d
10.0	0.0 ± 00.0 c	0.0 ± 00.0 d	0.0 ± 0.0 d	0.0 ± 00.0 b	0.0 ± 00.0 b	0.0 ± 00.0 d

(1) Within each column, data followed by different letters are significantly different at a p value of 0.05 (Duncan's test). ± shows the standard deviation.

Table 4. Effects of different concentrations of retentates on average germination time of cress, tomato, and durum wheat (1).

Concentration (%)	Average Germination Time (d)					
	Lepidium sativum L.		*Solanum lycopersicum* L.		*Triticum turgidum durum* Desf.	
	RNF	ROI	RNF	ROI	RNF	ROI
0.0	3.1 ± 0.06 b	3.0 ± 0.00 c	7.6 ± 00.30 c	7.6 ± 0.17 b	4.2 ± 0.32	4.1 ± 00.06 b
0.1	3.1 ± 0.10 b	3.0 ± 0.05 c	7.7 ± 0.24 bc	7.5 ± 0.34 b	4.3 ± 0.49	4.2 ± 0.10 ab
0.5	3.2 ± 0.10 b	3.4 ± 0.29 b	8.0 ± 00.22 b	8.2 ± 0.53 a	4.2 ± 0.15	4.1 ± 00.08 b
1.0	4.0 ± 0.37 a	3.9 ± 0.22 a	8.9 ± 00.22 a	8.5 ± 0.37 a	4.3 ± 0.17	4.3 ± 00.13 a

(1) Within each column, data followed by different letters are significantly different at a p value of 0.05 (Duncan's test). ± shows the standard deviation.

3.3. Effects on Germinated Seeds

For both retentates and concentrations, the value of the GR was 0.0% for germinated seeds and was statistically lower with respect to the control (Table 5).

Table 5. Effects of different concentrations of retentates on pre-germinated seeds of cress, tomato, and durum wheat (1).

Concentration (%)	Germination Rate (%)					
	Lepidium sativum L.		*Solanum lycopersicum* L.		*Triticum turgidum durum* Desf.	
	RNF	ROI	RNF	ROI	RNF	ROI
0.0	98.8 ± 2.0 a	76.8 ± 38.1 a	72.8 ± 10.0 a	78.0 ± 4.0 a	66.0 ± 8.3 a	66.0 ± 5.2 a
5.0	0.0 ± 0.0 b	0.0 ± 00.0 b	0.0 ± 00.0 b	0.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b
10.0	0.0 ± 0.0 b	0.0 ± 00.0 b	0.0 ± 00.0 b	0.0 ± 0.0 a	0.0 ± 0.0 b	0.0 ± 0.0 b

(1) Within each column, data followed by different letters are significantly different at a p value of 0.05 (Duncan's test). ± shows the standard deviation.

3.4. Effects on the Seedling Emergence in the Soil

The ER of cress and durum wheat (Table 6) in the pots treated with both retentates was statistically lower than in the control. Concentrations 5.0 and 10.0% of both RNF and ROI completely inhibited the emergence of tomato.

Table 7 shows that, for both retentates and concentrations, the AET of cress was higher in the treated pots than in the control. No significant differences were observed for durum wheat treated with RNF, whereas ROI gave higher values with respect to the control.

Table 6. Emergence rate of cress, tomato and durum wheat in response to different concentration of retentates (1).

Concentration (%)	Emergence Rate (%)					
	Lepidium sativum L.		*Solanum lycopersicum* L.		*Triticum turgidum durum* Desf.	
	RNF	ROI	RNF	ROI	RNF	ROI
0.0	96.8 ± 3.8 a	88.0 ± 19.0 a	56.0 ± 14.2 a	58.0 ± 9.5 a	68.8 ± 11.5 a	74.8 ± 03.8 a
5.0	10.8 ± 6.8 b	30.8 ± 14.0 b	0.0 ± 00.0 b	0.0 ± 0.0 b	26.8 ± 21.0 b	34.0 ± 21.8 b
10.0	12.0 ± 5.6 b	20.8 ± 10.0 b	0.0 ± 00.0 b	0.0 ± 0.0 b	14.0 ± 10.6 b	26.0 ± 10.1 b

(1) Within each column, data followed by different letter are significantly different at a p value of 0.05 (Duncan's test). ± shows the standard deviation.

Table 7. Average emergence time of cress, tomato, and durum wheat in response to different concentration of retentates (1).

Concentration (%)	Average Emergence Time (d)					
	Lepidium sativum L.		*Solanum lycopersicum* L.		*Triticum turgidum durum* Desf.	
	RNF	ROI	RNF	ROI	RNF	ROI
0.0	4.1 ± 0.08 a	4.2 ± 0.23 a	13.9 ± 0.70	12.8 ± 0.67	6.0 ± 0.42	6.6 ± 0.75a
5.0	10.8 ± 1.03 b	9.2 ± 1.76 b	-	-	8.9 ± 1.33	9.9 ± 0.52 b
10.0	9.7 ± 1.60 b	10.6 ± 0.60 b	-	-	6.6 ± 4.57	10.0 ± 1.39 b

(1) Within each column, data followed by different letters are significantly different at a p value of 0.05 (Duncan's test). ± shows the standard deviation.

4. Discussion

Despite the extensive literature on OMW phytotoxicity [16,25–31], there is still a strong need for studies on biophenols' herbicide effects for the development of bioherbicides and to reduce environmental impacts. Filtration processes such as the integrated centrifugation–ultrafiltration system allow one to simultaneously extract phenols and remove polluting compounds from OMW, reducing the risk of potential damage to the environment and giving biophenols significant value [37] (e.g., carbohydrates decrease after NF and RO membranes since polysaccharides are large molecules [50]). Tundis et al. [51] observed the complete detention of phenolic compounds by the RO membrane, while for NF membranes, the rejection values measured for the compounds were similar to those observed for the RO membrane. Our analysis revealed the highest polyphenols content, with high tyrosol and verbascoside concentrations in RNF and a higher concentration of hydroxytyrosol in ROI. In the literature, the higher levels of phenols and flavonoids have been attributed to compounds reported as phytotoxic (e.g., gallic, coumaric, salicylic protocatechuic, benzoic, caffeic, p-coumaric, and ferulic acids, etc.) to *Phaseolus aureus* L. [52], *Cassia sophera* L., *Allium sepa* L. [53], and *Lactuca sativa* L. [54].

According to the physicochemical properties of RNF and ROI, the ranges of pH were not inhibitory for the indicator plant species, although pH = 6 is generally optimal for plant growth [55]. Therefore, a slight change in pH (5.0–5.6), according to stability of the tested retentates, could be tolerated without creating significant differences in germination [55]. The EC values of the RNF and ROI concentrations were much higher than the inhibitory range of 2 dS m^{-1} (= 2 mS cm^{-1}) [56,57]. However, the bioassay on dry seeds carried out to compare the GR of the $CaCl_2$ groups with the RNF and ROI groups, respectively, highlighted significant differences for most concentrations with the same EC. Thus, there was an inhibition action of the retentates on the seeds' germination and plant growth, excluding phytotoxicity determined by high salinity and pH as reported by some authors [30,31]. These effects are due to natural substances that act as allelopathic compounds. Allelopathy is defined as "the direct or indirect (stimulating or inhibitory) effect of a plant on another through the release of natural-chemical substances into environment" [58], and phenols originating from the secondary metabolism of olive trees and recovered from OMW can perform this action.

The relevant results of this study demonstrated the total or near-total inhibition of germination at the stage of dry seed and the total inhibition of seedlings' development and

emergence when the seeds were treated with the highest concentrations of the retentates (5.0 and 10.0%) for all the species. Even Ghidaoui et al. [28] and Tubeileh et al. [29] reported the same results in Petri dishes and soil, respectively, using, however, raw OMW. Results comparable to those obtained in the Petri dishes were observed when the olive mill wastewater was directly sprayed on the soil, reducing weed numbers and the biomass of purslane (*Portulaca oleracea* L.) and malva (*Malva parviflora* L.) [29]. The phytotoxic effects and delays in the germination of plants belonging to the *Brassicaceae* family were observed in soils treated with OMW [16]. In our study, the delays in germination and emergence were detected with different doses according to the retentate and species. In cress, a significant increase in AGT was observed for lower ROI and higher RNF concentrations (0.5 and 1.0%, respectively). On the other hand, in durum wheat, a significant increase in AGT was recorded only for ROI with a lower concentration (0.1%), whereas no differences were observed between different concentrations of RNF. In tomato, the results showed the opposite behavior of the two retentates with respect to cress, even if a significant increase in AGT resulted from an even lower RNF concentration (0.1%). At the highest concentration (1.0%), the AGT was the same for RNF and ROI and for all the species. AET was increased by ROI but not by RNF in durum wheat, and by both RNF and ROI in cress. From these results, cress and durum wheat seem to be more susceptible to ROI and tomato more susceptible to RNF. In addition, focusing on the GR in the dry seed stage, durum wheat seemed to be the species that tolerated the most RNF and tomato the one that tolerated the most ROI, although in tomato this was not confirmed by an AGT. The different phytotoxic behavior of RNF and ROI is probably related to their chemical characteristics and, particularly, to their hydroxytyrosol, tyrosol, and verbascoside content. These phenols belong to benzoic acids with only hydroxyl groups (-OH) [26]. Even Capasso et al. [59] observed the phytotoxicity of tyrosol and hydroxytyrosol (isolated and characterized using chemical tools) on *Cucurbita pepo* L. and *Solanum lycopersicum* L., respectively. Our investigations support the correlation between the allelopathic activity of the phenol groups and their potential phytotoxicity. In addition to the well-known bioactivity of phenols belonging to the group of cinnamic acids (cinnamic acid, p-coumaric acid, and caffeic acid) observed by Pinho et al. [26], and supporting the study of Capasso et al. [59], our results extend the effectiveness of biophenols' phytotoxicity to other benzoic acids with only -OH groups. The phytotoxic effects depend on their lipophilic character determined by quantity of -OH substituents and thus on their capacity to cross cell membranes [60]. It is likely that the higher overall number of -OH in ROI than in RNF reduced its phytotoxic activity. However, this behavior was clearly not evident for durum wheat.

These results could raise questions about selectivity; this property relates to several factors such as the absorption, translocation, and metabolism of crops [61]. Boz et al. [62] observed the herbicidal effect of OMW on some important weed species with no adverse effects on maize, sunflower, and wheat crops. On the other hand, a study by Tubeileh and Souikane [63] showed a toxic or stimulating effect according to plant species. Therefore, specific experiments can be conducted to properly manage the herbicidal effect of these compounds and allow their effective field use. For example, Ursinos, 1986 [64], determined that the toxic effects caused by OMW could be overcome after one month; thus, retentates could be used, simulating a pre-sowing herbicide treatment, especially in transplanted vegetable crops that are highly susceptible to weed competition [65] and that are increasingly in need of new tools for weed control [66].

5. Conclusions

In our study, we found that both OMW and retentates from nano filtration and reverse osmosis proved their effectiveness on species belonging to different families, allowing one to assume rather broad allelopathic activity on their bioactive compounds. The results open a window to the possibility of using retentates extracted through sequential membranes to integrate weed control techniques and tools, particularly to formulate "bioherbicides" [36], namely, compounds of natural origin acting as pre-emergence herbicides that are able

to prevent germinated weed seedlings from becoming established [67]. The use of this type of agrochemical has proved to be very useful in numerous crops such as wheat [68], maize [69], and vegetable crops [70,71]. Although our data show that the lower doses may have only a delaying effect, this property could be exploited in many crops to keep the field free of weeds only during the critical period of weed interference [72]. In addition to providing an opportunity for the alternative use of byproducts, the use of products obtained with filtration processes are less potentially harmful to the environment. Indeed, integrated centrifugation–ultrafiltration systems allow one to concentrate polyphenols and to remove some potentially polluting OMW compounds from the environment [37].

Our study (i) shows an opportunity to use OMW, thus reducing the environmental impact of the olive oil sector; (ii) it offers a screening of retentates' phytotoxicity in inhibiting the seed germination of indicator plant species, and (iii) it improves the knowledge on the phytotoxic effects of retentates extracted from olive byproducts, extending the general biophenols' phytotoxicity to hydroxytyrosol, tyrosol, and verbascoside, which are benzoic acids with only -OH groups. Further studies and trials should be carried out to learn more about the efficacy, selectivity, inhibitory activity, synergistic action, and mechanism of action of the single polyphenolic components and, finally, to evaluate their persistence and chemical stability in the environment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12061378/s1. Table S1. Comparison between effects of different levels of EC corresponding to concentrations of retentates and $CaCl_2$ on germination rate of cress. Table S2. Comparison between effects of different levels of EC corresponding to concentrations of retentates and $CaCl_2$ on germination rate of tomato. Table S3. Comparison between effects of different levels of EC corresponding to concentrations of retentates and $CaCl_2$ on germination rate of durum wheat.

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