Inês Almeida Oliveira Pinho

Phytotoxicity Assessments of Olive Mill Solid Wastes: Influence of Phenolic Compounds

Master's Dissertation in Chemical Engineering, supervised by Prof. Doctor Margarida Maria João de Quina and Doctor Rui Carlos Martins submitted to the Department of Chemical Engineering, Faculty of Science and Technology, University of Coimbra

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ABSTRACT

Olive oil production plays an important role in economy and customs in Mediterranean countries. Although, it originates solid wastes and wastewater (OMSW and OMWW, respectively) that cause negative environmental impacts when disposed into the environment, mainly due to high organic loads and phenolic content. In this work, solid wastes from 2- and 3-phase centrifugation processes (2P- and 3P-OMSW, respectively) were analyzed in terms of phytotoxicity through cress bioassays with *Lepidium sativum* L. (garden cress). The results confirmed that 2P-OMSW is more phytotoxic than 3P-OMSW. For a L/S ratio of 10 L/kg, germination index (GI) of 2P-OMSW was 0% (none of the seeds had germinated), while in 3P-OMSW extracts was 94.3% by comparison with the distilled water control. Moreover, the influence of phenolic compounds in phytotoxicity was assessed, revealing that this parameter is highly influenced by compounds chemical structure and hydrophobicity. Ten phenolic compounds were tested and cinnamic acid revealed to be the most phytotoxic. Their toxic character is also influenced by synergistic effects between them. Using these wastes as soil amendments was also evaluated in growth tests. Cress germination and growth led to more promising results in 3P-OMSW trials than in 2P-OMSW, although results show worse results than those obtained in the control experiments. To find and environmentally friendly and economic viable solution for their treatment and dispose has been a widely discussed issue. Therefore, Fenton's oxidation process was tested in both 2P- and 3P-OMSW and in a synthetic effluent composed of six phenolic acids. Results showed that the treatment intensified phytotoxicity of both solid wastes, but led to the reduction of phytotoxicity in the liquid effluent. However, even after treatment, synthetic effluent remained phytotoxic, since GI increased from 54.5 to 64.1%.

Keywords: phytotoxicity, olive mill solid waste, phenolic compounds, germination, germination index, *Lepidium sativum* L., hydrophobicity, Fenton's.

RESUMO

A indústria da produção de azeite é muito importante na economia e costumes dos países mediterrânicos. Contudo, o processo de produção origina resíduos sólidos e líquidos (OMSW e OMWW, respetivamente) que têm um impacto ambiental negativo quando lançados para o ambiente, sobretudo, devido à sua carga orgânica e conteúdo fenólico elevados. Neste trabalho, foi analisada a fitotoxicidade de resíduos sólidos provenientes dos processos de extração de duas e de três fases (2P- e 3P-OMSW, respetivamente), através de ensaios de germinação com *Lepidium sativum* L. (agrião de jardim), cujos resultados confirmaram que 2P-OMSW é mais fitotóxico que 3P-OMSW. Para o primeiro resíduo, uma razão L/S de 10 L/kg conduziu a um índice de germinação (GI) de 0% (nenhuma das sementes germinou), enquanto que para 3P-OMSW o GI foi de 94.3% por comparação com o branco (água destilada). Foi também analisada a influência que os compostos fenólicos têm na fitotoxicidade, tendo-se concluído que este parâmetro é afetado pela estrutura química desses compostos, bem como pelo seu carácter hidrofóbico. Dos 10 compostos testados, o ácido cinâmico foi o que revelou maior fitotoxicidade. Também se verificou que existem interações sinergéticas entre os compostos que aumentam a fitotoxicidade. Não obstante, g*rowth tests* para avaliar a possibilidade de usar estes dois resíduos como corretores de solos mostraram que nenhum dos dois produziu efeitos positivos no desenvolvimento dos agriões quando comparados com o branco, mas que os resultados dos ensaios em 3P-OMSW foram melhores que em 2P-OMSW. Por fim, testou-se o processo de oxidação de Fenton na redução da toxicidade dos dois resíduos e de um efluente sintético composto por 6 dos compostos fenólicos anteriormente testados. Para os dois resíduos sólidos, o tratamento não cumpriu o objetivo e até aumentou o grau de fitotoxicidade dos resíduos. Para o efluente líquido, o processo permitiu o aumento do GI, embora ainda num nível fitotóxico.

Palavras-chave: fitotoxicidade, compostos fenólicos, bagaço de azeitona, germinação, índice de germinação, *Lepidium sativum* L., hidrofobicidade, Fenton.

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ACRONYMS

2P-OMSW	Two-phase Olive Mill Solid Waste
3P-OMSW	Three-phase Olive Mill Solid Waste
ANOVA	Analysis of Variance
C	Carbon
COD	Chemical Oxygen Demand
EC	Electrical Conductivity
EU	European Union
GI	Germination Index
H	Moisture
H_0	Null Hypothesis
H_1	Alternative Hypothesis
K	Potassium
L	Box plot length
L/S ratio	Liquid/solid ratio
N	Nitrogen
OMSW	Olive Mill Solid Waste
OMW	Olive Mill Waste (liquid and/or solid)
OMWW	Olive Mill Wastewater
P	Phosphorus
Q_1	1st Quartile
Q_2	2nd Quartile
Q_3	3rd Quartile
RRG	Relative Root Growth
RSG	Relative Seed Germination
TKN	Total Kjeldahal Nitrogen
TOC	Total Organic Carbon
TS	Total Solids
VS	Volatile Solids
WFD	Waste Framework Directive

1 INTRODUCTION

1.1 WORK MOTIVATION AND SCOPE

Among all environmental problems being faced in Mediterranean Sea area, the generation of large amounts of wastes from olive oil production requires special attention.

Olive oil is produced in mills, where olive fruit is transformed in olive oil through pressing or centrifugation processes, with generation of liquid and solid wastes. The most ancient process of olive oil production is the traditional press, where olives are processed by pressure, generating olive oil, solid waste and wastewater. This sector was modernized by the three-phase centrifugation process. However, this methodology has huge water requirements and generates large amounts of wastewaters, so this technology evolved to a centrifugation process named the two-phase process. In this system, only two phases are generated at the end: olive oil and semi-solid waste that is a combination of wastewater and solid residues, commonly called *alperujo*.

Both liquid and solid olive mill wastes (OMW) are characterized by high phytotoxicity, so their release in the environment has negative effects. This property is mostly related with their high phenol, lipid and organic concentrations [1]. Indeed, olive fruit has a high concentration of phenolic compounds by itself. However, the olive oil extraction process will influence the phenolic composition of both the wastes and the oil. It is usually noticed a lower concentration of phenols in the oil phase and a loss with the wastewater, justified by factors such as the relative polarities and partition coefficients of this compounds. For this reason, it is known that olive mill wastewaters (OMWW) have higher phenolic content than olive mill solid wastes (OMSW) and, even among OMSW, those resulting from a two-phase centrifugation process show also higher phenolic concentration than those from a three-phase process. This is due the fact that the first ones are a combination of two wastes: wastewater and solid olive cake.

There are a huge number of polyphenols already identified in OMW. The most common are hydroxytyrosol and tyrosol, together with oleuropein, *p*-coumaric acid, vanillic acid, verbascoside, elenolic acid, catechol, rutin, ferulic acid, cinnamic acid, protocatechuic acid and 4-hydroxybenzoic acid. In the literature, several studies have been done for assessing the major phenolic compounds in OMW, for evaluating their antioxidant properties or even about methodologies for extracting them from these wastes. However, it is still not clear their role in phytotoxicity. This is an important issue since these wastes

are generally disposed in the soils and thus may represent an environmental risk for plants [2].

Lepidium sativum L. (commonly known as garden cress) is the species most frequently used to assess phytotoxicity of any substrate, because it is sensible to toxins and has a rapid response. Bioassays using cress may be performed in different ways: germination into liquid extracts and growth tests.

Cress bioassays with liquid extracts consist of determining if a certain substrate has substances capable of inhibiting seed germination and growth. In an aqueous extract of the medium to be tested, a number of seeds are placed during few days in dark and controlled conditions of temperature.

On the other hand, growth tests allow simulating the soil application of a certain substance. There are many different ways of performing this type of experiments: seeds can be sown in the substrate to be tested or can be watered by an aqueous extract of it.

In both cases, at the end of the experiments, it is possible to quantify the development of the seedlings, by measuring the number of germinated seeds, their root length, steam length or even chlorophyll content, among some other parameters.

However, there is other ways to express phytotoxicity. It can be quantified by other parameters as EC_{50}, which is the concentration of a pollutant or toxic substance that leads to the reduction of 50% percent of a certain response, that may be number of germinated seeds, root length, steam length, etc., when compared with a blank experiment (without contaminants).

After concluding that a waste is phytotoxic, it is necessary to evaluate the best treatment option to detoxification. The case study in this work are olive mill by-products. Thus, after investigate phytotoxicity issues it will be analyzed the management. Three-phase solid wastes are usually burned as solid fuel after a second oil extraction, but it is impossible to implement the same procedure to the two-phase solid waste because of its high moisture. Thus, further research is required to find a suitable procedure. Fenton's process, based on the oxidant power of hydrogen peroxide enhanced by iron salts, arises as an alternative since it operates at room conditions of pressure and temperature which reduces costs. Moreover, it requires environmentally friendly reactants.

1.2 OBJECTIVE

The main objective of this study is to evaluate phytotoxicity of olive mill solid wastes from 2- and 3-phase centrifugation processes, using germination assays with garden cress. Therefore, the study will involve the following phases: phytotoxicity assessments of 2P- and 3P-OMSW; phytotoxicity assessments of individual phenolic compounds and a synthetic liquid effluent; and growth tests with mixtures of waste and soil.

Globally, it is expected to predict how some of the major phenolic compounds present in these wastes individually influence phytotoxicity and the potential synergistic effect of mixtures of these contaminants. Moreover, the application of a Fenton's process in the reduction of phytotoxicity of OMSW and a typical agro-industrial effluent will be also assessed.

1.3 THESIS STRUCTURE

The present work is divided in six chapters. The first chapter includes the work motivation and scope, the objectives that are expected to achieve and the structure of the work. In the second chapter it is presented the existing technology for olive oil production and the resulting products and wastes. The methods to assess phytotoxicity and detoxification methodologies are also discussed. The third chapter resumes the state of art of some critical aspects for this study, such as the main methodologies to assess phytotoxicity and detoxification/valorization strategies. In the fourth chapter the experimental procedures and methodologies adopted are described, whose results are discussed in the fifth chapter. At last, in the sixth chapter, the main conclusions of this work are listed and suggestions for future works on this subject are indicated.

2 THEORETICAL BACKGROUND

2.1 OLIVE OIL INDUSTRY AND BY-PRODUCTS

The olive oil production is an important industrial sector worldwide, especially in Mediterranean areas, where this product plays an essential role, not only in economy, but also in terms of tradition. Nowadays, the main world producers are: Spain, Italy, Greece and Portugal. Others such as Turkey, France, Cyprus, Syria and Tunisia are also notable. Although, some other countries such as Argentina, Australia and South Africa are becoming emergent producers due to increasing promotion of olive trees cultivation [1, 2]. Therefore, for economic competitiveness of this sector, it is necessary improvements in environmental and quality profiles of the whole production chain [2].

The olive is a fruit known for not requiring high quantities of chemicals for growing and there is no need of large amounts of energy for its processing. Consequently, olive oil is also recognized as environmentally friendly, because its manufacturing process is low-energy and chemicals-free [2].

However, the increasing demand of olive oil worldwide is becoming a growing environmental problem. The generation of large amounts of olive mill liquid and solid wastes (OMW) is inevitable and to find an ecofriendly and economically viable solution in handling and dispose of OMW is one challenging concern olive oil producers have been facing. Nonetheless, it is necessary regulation that safeguard the environmental values without harming producers and olive oil markets [2].

Olive oil production is carried out in mills and there are two ways of extracting the oil: a batch process called the traditional press and two continuous processes of centrifugation [1, 3]. In Fig. 2.1, the three different processes are summarized. Depending on the type of olive oil production, products and by-products have different characteristics.

Mill residues consist in solid wastes (OMSW), as olive pulp and pits left over pressing the olives, and liquid effluents - wastewaters (OMWW) - composed of vegetable water of the fruit and additional water generated during decantation. Solid and liquid wastes are dark-colored containing high amounts of organic materials and complex substances not easily degradable, that disturb natural water bodies and plant life when disposed into the environment. Olive oil production facilities and OMW management are associated with unpleasant odors caused by emissions of volatile compounds [2]. But some studies have indicated OMW as an economic resource, because it can be used as soil conditioner,

biomass fuel, for producing compost, irrigation water or as a source of valuable products such as antioxidants, enzymes or biogas fuel [2].

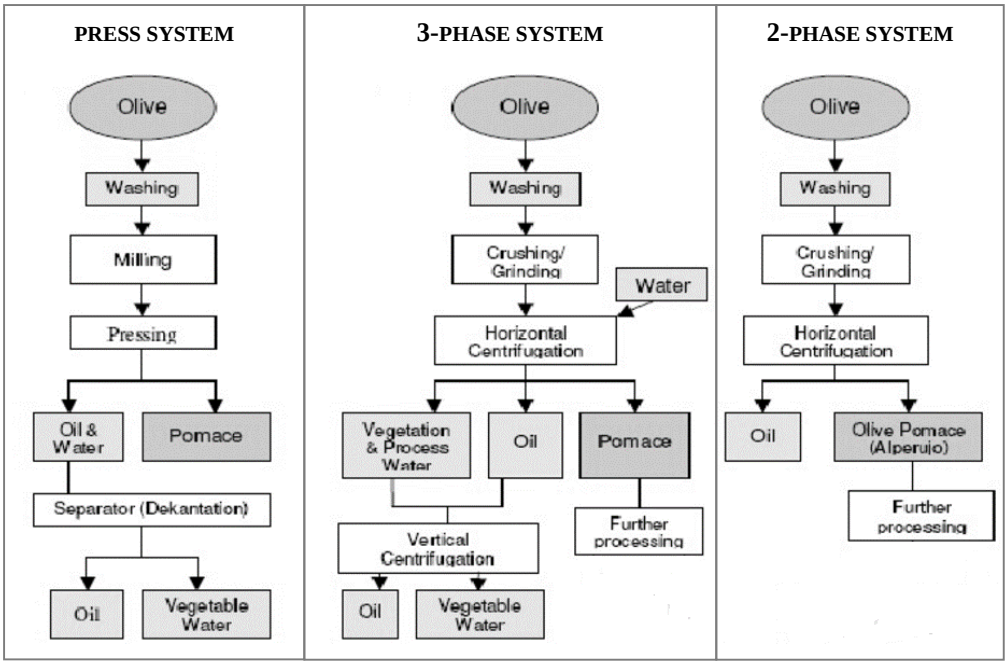


Fig. 2.1 Olive oil extraction technologies.

Traditional press is used by centuries with only a few modifications and, despites being a relatively archaic technology, there are still many press mills in full operation (Fig. 2.2). In order to circumvent this ancient process, three-phase system was developed that brought automation to this sector. Even though, it has some inconveniences which led to the development of the two-phase extracting system.

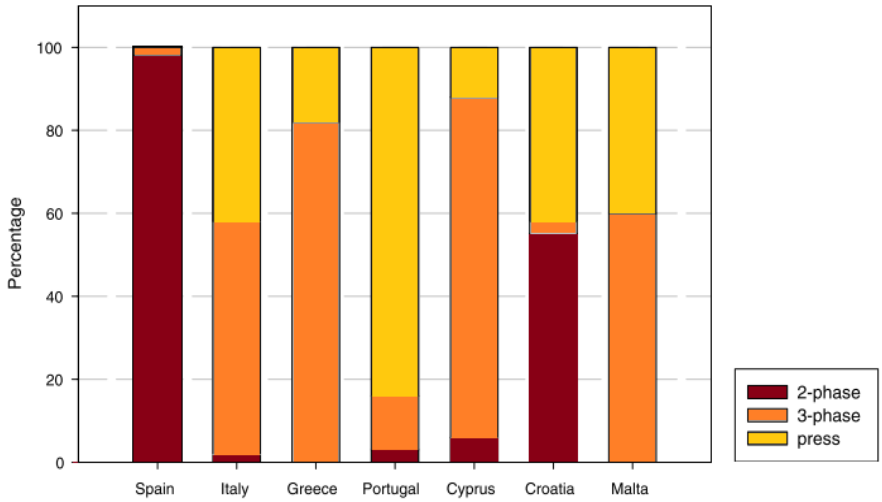


Fig. 2.2 Technologies used by European olive oil mills (2003) (adapted from [1]).

After extraction by pressing, it is obtained a solid fraction, named olive husk, and an emulsion containing the olive oil that is separated from the remaining olive mill wastewater (OMWW) by decantation [1]. The cake composed of pressed solids (the olive husk) can be further de-oiled elsewhere. De-oiled solids are usually burned for energy and the resulting oil is used in the soap manufacture or as edible oil when quality is assured after refining [2].

In a three-phase system, three fractions are produced at the end of the process: a solid (olive husk, olive pomace or olive cake (3P-OMSW)) and two liquids (olive oil and wastewater). When compared to press technology, a continuous process increases production, produces better oil quality due to the elimination of mat flavor, needs smaller areas, minimizes labor costs due to automation. However, the three-phase system requires greater water and energy consumptions, more expensive installations and leads to more wastewater production [1, 3].

Water requirements and wastewater production have been the main environmental concern. OMWW from press and three-phase systems have been illegally dumped to soil and aquatic ecosystems, affecting soil microbial populations, aquatic life and even air medium, due to its high phytotoxicity and organic matter concentration. One valid option to reduce these impacts was to create a processes with lower water consumption during the extraction stage. So, in consequence, the two-phase system appeared. This "new" process uses less water and only produces two fractions at the end: a solid waste also called *alperujo*, olive wet husk, wet pomace, two-phase olive mill waste (2P-OMSW) or water-solid mixture; and a liquid phase – the olive oil [1, 3]. This solid fraction is in fact a semi-solid by-product, because it is a combination of OMWW and olive husk.

The cake resulting from a two-phase process has peculiar physical-chemical properties that complicate its handling. It has high moisture and carbohydrate concentration, therefore it cannot be treated like the three-phase olive husk, that suffers a second oil extraction (de-oiling) with organic solvents after its drying. 2P-OMSW is not used as fuel, because of its high moisture content [1, 3].

In the last years, a significant number of works has been done for characterizing OMSW and determining their impact on soils and plants. Residual olive cakes after oil extraction can be used in furfural production, as animal food additive, fertilizer, compost or even as a raw material in biotechnology. Although 2P-OMSW has high nutrients concentrations, especially potassium, that makes it possible to be used as soil amendment, it cannot be transported and dried easily, as the transport, storage and drying machinery and

equipment in residual oil recovery plants are not quite suitable. Its pollutant load is more concentrated which brings some harmful effects: besides being less phytotoxic than OMWW, it causes great nutritional imbalances, since it modifies the nitrogen cycle in soil due to its high C/N ratio. Thus, for 2P-OMSW, composting of this wet material together with other agricultural wastes may be the most reasonable technology [1, 3]. The 3P-OMSW, in turn, is mostly used as solid fuel after de-oiling [4, 5]. When these wastes are disposed on soils, they inhibit seed germination and early plant growth, alter soil characteristics and create reducing conditions, affecting microbial diversity in soil [6]. Olive phenols are main contributors to this impact due to their toxicity and antimicrobial activity [5]. In fact, many studies have been done on the antioxidant and antimicrobial potential of olive oil and OMW, due to its high phenolic content [5–8]. Thus, suitable management methodologies must be developed to minimize the potential impact of these materials if disposed into soils. An approach comprises the recovery of such phenolic compounds with interesting antioxidant characteristics.

In what regards OMWW, in press system, since it requires the addition of a low quantity of water, it produces a small volume of OMWW (40 to 60 L/ 100 kg of olives) [5]. The three-phase centrifugation adds 1.25-1.75 times more water compared to the press extraction, producing 80-120 L of OMWW/ 100 kg of olives [5] (about 30 million m^3 per year), and 500-600 kg of OMSW/ 1 ton of olives [2]. The 2P-OMSW leads to only about 10 L of wastewater/ 100 kg of olives [5].

It is estimated that in the olive growing countries worldwide, the uncontrolled spreading of olive husk on farm lands is around 100-200 m^3/h/year [3].

Due to the complexity of OMW, the classification according to European Union (EU) and National Legislation on Waste, Water and Soil cannot be an easy task. Within its waste life, regardless containing dangerous substances or not, it is included in the Waste Framework Directive (WFD)[1] and not the REACH regulation[2]. As it contains phenols, which are (potential) dangerous substances, the waste is rendered as (potentially) hazardous. However, if it is considered a by-product, it is excluded from WFD as well as REACH regulation, if is consumed by the same legal entity while the by-product placed on the market is regarded as a substance that does not need to be registered. Substances

[1] Core legislative act of EU laws that regulate waste management (2008/98/EC), which also includes rules on hazardous waste and waste oils, which were previously covered by separate legislation.

[2] Regulation1907/2006 of the European Parliament and of the Council of 18 December 2006. Concerns the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH).

recovered from wastes usually fall within the scope of REACH regulation; and when a material ceases being considered a waste in the basis of WFD provisions, REACH regulation requirements apply in principle in the same way as to any other material. In addition, olive waste is a natural material as defined in REACH regulation but as contains potentially dangerous substances is not absolutely excepted from the scope regulation [3].

2.1.1 Polyphenols in olive mill solid wastes

According to [7], the main organic compounds present in OMW are sugars, nitrogenous compounds, volatile acids, polyalcohols, pectins, fats and polyphenols.

Phenolic compounds generally include a huge number of organic substances which common characteristic is possessing an aromatic ring with one or more substitute hydroxyl group and a functional chain [4]. Polyphenols are considered the main antioxidant compounds in OMW with high bioactive properties. In recent years, there is a rising interest in the recovery of these compounds rather than their synthetic production. Phenolic compounds properties arise from their ability to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation [7, 8]. Polyphenols are characteristic of the olive fruit. This fruit contains high concentrations of hydrophilic and lipophilic phenolic compounds in the range 1-3% of the fresh pulp weight. The main lipophilic phenols are cresols and the main hydrophilic phenols are phenolic acids, phenolic alcohols, flavonoids and secoiridoids. Oleuropein, demethyloleuropein, ligntroside and nuzhenide are the most abundant secoiridoids glucoside in olive fruit [4]. In Table 2.1 is indicated a typical chemical characterization of olive fruit (seed) and in terms of pulp and stones.

Table 2.1 A representative chemical composition of olive fruit (%) [4].

Components	**Olive pulp**	**Stones**	**Seed**
Water	50 – 60	9.3	30
Oil	15 – 30	0.7	27.3
N-contg. compounds	2 – 3	3.4	10.2
Sugar	3 – 7.5	41	26.6
Cellulose	3 – 6	38	1.9
Minerals	1 – 2	4.1	1.5
Polyphenols	2.25 – 3	0.1	0.5 – 1
Other compounds	–	3.4	2.4

The majority of the polyphenols is in the olive pulp, while stones have mainly sugars and cellulose [4].

In this context, olive oil is now recognized as one of the healthiest lipid sources worldwide, especially because it is proven that its high phenol content possess antioxidant, anti-inflammatory, antibacterial and anticancer properties [9]. Therefore, there is an increasing attention for using OMW extracts as potential agents for preventing and treating oxidative stress-related diseases [8].

The type and quantity of phenols in olive fruit are influenced by cultivar and genetics, degree of maturation, climate conditions and agriculture practices. The oil extraction technology is responsible for the phenol profile of the olive oil and in the residues as well [9].

Table 2.2 shows the influence of extraction processes in the chemical composition of the solid wastes produced.

Table 2.2 Characteristics of olive cakes from the three different processes (adapted from [4]).

Parameter	**Pressure system**	**3P-OMSW**	**2P-OMSW**
Moisture (%)	27.21 ± 1.05	50.23 ± 1.94	56.80 ± 2.19
Phenolic Compounds (%)	1.15 ± 0.06	0.33 ± 0.04	2.43 ± 0.15
Fats and oils (%)	8.72 ± 3.25	3.89 ± 1.45	4.65 ± 1.74
Proteins (%)	4.77 ± 0.02	3.43 ± 0.02	2.87 ± 0.01
Total sugars (%)	1.38 ± 0.02	0.99 ± 0.01	0.83 ± 0.01
Cellulose (%)	24.14 ± 0.28	17.37 ± 0.20	14.54 ± 0.17
Hemicellulose (%)	11.00 ± 0.61	7.92 ± 0.44	6.63 ± 0.37
Lignin (%)	14.18 ± 0.29	0.21 ± 0.21	8.54 ± 0.16
Total Carbon (%)	42.90 ± 3.42	29.03 ± 2.32	25.37 ± 2.02

Table 2.2 indicates that 2P-OMSW is richer in phenols, followed by the traditional press system and 3P-OMSW.

The extraction process has effect not only on the wastes composition, but also on the quality of the virgin oil. Some phenols are originally present in the olive fruit, but others are formed during the processing of olive into oil [4]. Indeed, during olive oil processing, phenolic compounds present in olive fruit are transferred from paste (crushed fruits) to oil depending on the relative polarities of phenols, the presence of surfactants, temperature and the composition and relative amounts of the resulting phases. Others are newly formed through various enzymatic biotransformation-reaction pathways [9].

Depending on their partition coefficients and temperature, phenols of olive paste are more or less soluble in water and oil. Thus, the addition of water to the paste alters the partition equilibrium between aqueous and oil phases and causes a reduction of phenol concentration through dilution of the aqueous phase [4]. However, solubility is not the only mechanism responsible for the distribution of hydrophilic phenols between the oil

and the water phase. Also some catalyzed oxidative reactions can promote the phenolic oxidation during the process. The interaction between polysaccharides and phenolic compounds present in the olive pulp may also contribute to the loss of phenols during processing [4]. Olive oil producers aim to obtain an oil rich in phenols. Moreover, these substances are not welcome in OMW due to their environmental impact related with their potential microbiota inhibition [10].

Several authors identified the following main families of phenolic compounds present in olive mill wastes, both wastewaters and olive pomaces: phenolic acids, secoiridoids and flavonoids [5]. Others divided phenol in: simple phenols, phenolic acids, derivatives secoiridoids, flavonoids and lignans [8]; other possibility is: phenolic acids, phenolic alcohols, phenyl alcohols, secoiridoids, flavonoids and lignans [4]. To date, a wide range of phenolic compounds were identified in olive mill wastes, as indicated in Table 2.3. Between all of these polyphenols, literature indicates hydroxytyrosol and tyrosol as 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 [4, 5, 12, 13].

Table 2.3 Phenolic compounds assayed by different authors.

Compound	**IUPAC name** [14]	**Typical Concentration**	**Ref.**
3,4,5-Trimethoxybenzoic acid	3,4,5-Trimethoxybenzoic acid		[9, 15]
4-Hydroxybenzoic acid	4-Hydroxybenzoic acid		[4]
Apigenin	5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one		[8]
Caffeic acid	3-(3,4-Dihydroxyphenyl)prop-2-enoic acid	2P and 3P-OMSW: ~14 mg/100 g dry residue	[8, 9, 12, 13, 16]
Catechol	Benzene-1,2-diol		[4]
Cinnamic acid	3-Phenylprop-2-enoic acid		[4, 12]
o-Coumaric acid	(2E)-3-(2-Hydroxyphenyl)prop-2-enoic acid		[4]
p-Coumaric acid	3-(4-Hydroxyphenyl)prop-2-enoic acid	2P-OMSW: ~28 mg/100 g dry residue; 3P-OMSW: ~18 mg/100 g dry residue	[9, 12, 13, 15]
Elenolic acid	2-[(2S,3S,4S)-3-formyl-5-methoxycarbonyl-2-methyl-3,4-dihydro-2H-pyran-4-yl]acetic acid		[8, 15, 18]
Ferulic acid	(2E)-3-(4-Hydroxy-3-methoxyphenyl)prop-2-enoic acid	2P-OMSW: ~8 mg/100 g dry residue; 3P-OMSW: ~4 mg/100 g dry residue	[9, 12, 13, 16]
Gallic acid	3,4,5-Trihydroxybenzoic acid		[9, 12, 16]
Hesperidin	(2S)-5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4-oxo-3,4-dihydro-2H-chromen-7-yl 6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside		[12]

Table 2.3 Phenolic compounds assayed by different authors (continued).

Compound	**IUPAC name** [14]	**Typical Concentration**	**Ref.**
Hydroxybenzoic acid	Hydroxybenzoic acid		[12]
Hydroxytyrosol	4-(2-Hydroxyethyl)benzene-1,2-diol	2P-OMSW: 1.16% (w/w dry residue); 3P-OMSW: ~0.8% (w/w dry residue)	[8-10, 13, 15-17]
Hydroxytyrosol glucoside	2-(3,4-Dihydroxyphenyl)ethyl β-D-glucopyranoside		[8, 10, 15]
Ligstroside	Methyl (4S,5E,6S)-5-ethylidene-4-[2-[2-(4-hydroxyphenyl)ethoxy]-2-oxoethyl]-6-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4H-pyran-3-carboxylate		[8, 10, 15, 19]
Ligstroside aglycone	methyl (2R,3E,4S)-3-ethylidene-2-hydroxy-4-{2-[2-(4-hydroxyphenyl)ethoxy]-2-oxoethyl}-3,4-dihydro-2H-pyran-5-carboxylate		[8, 16, 20]
Luteolin	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one		[8, 15]
Luteolin-7-glucoside	7-(β-D-Glucopyranosyloxy)-5-hydroxy-2-(3,4-dihydroxyphenyl)-4H-chromen-4-one		[8, 15]
Luteolin-7-rutinoside	2-(3,4-Dihydroxyphenyl)-5-hydroxy-4-oxo-4H-chromen-7-yl 2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranoside		[8, 15]
Oleuropein	methyl (2S,3Z,4S)-4-{2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl}-3-ethylidene-2-{[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}-3,4-dihydro-2H-pyran-5-carboxylate		[8-10, 15-17, 20]
Oleuropein aglycone	methyl (2R,3E,4S)-4-{2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl}-3-ethylidene-2-hydroxy-3,4-dihydro-2H-pyran-5-carboxylate		[8, 10, 15, 16, 20]
Protocatechuic acid	3,4-Dihydroxybenzoic acid		[9, 12, 15]
Quercetin	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one		[8, 12]
Rutin	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl 6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside		[8, 10, 12]
Sinapic acid	3-(4-Hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid		[8, 9, 12]
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid		[8, 9, 12, 16]
Tyrosol	4-(2-Hydroxyethyl)phenol	2P-OMSW: ~0.2% (w/w dry residue); 3P-OMSW: ~0.1% (w/w dry residue)	[8, 9, 13, 15-17]
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	2P-OMSW: ~16 mg/100 g dry residue; 3P-OMSW: ~6 mg/100 g dry residue	[8, 9, 12, 13, 15, 16]
Veratric acid	3,4-Dimethoxybenzoic acid		[4]
Verbascoside	6-[2-(3,4-dihydroxyphenyl)ethoxy]-5-hydroxy-2-(hydroxymethyl)-4-[(3,4,5-trihydroxy-6-methyloxan-2-yl)oxy]oxan-3-yl (2E)-3-(3,4-dihydroxyphenyl)prop-2-enoate		[8, 9, 15, 20]

In olive mill wastes (liquid and solid), the phenolic fraction has been reported to exhibit antimicrobial activity against certain nonindigenous strains, which is even greater than the respective activities induced by the individual phenolic compounds. This indicates a synergistic action of OMW phenols [6].

In [9], it was concluded that fruits, paste and wastes presented similar phenol composition, which diverged significantly from that of oil, indicating that phenols are not only transferred but also transformed during oil processing. No qualitative differences in phenol profiles were observed between the 3 extraction systems, whereas significant ones at the quantitative level. Crushing and malaxation resulted in the highest phenols lost: only 50 – 60% of total phenols from fruits remain in paste. Only 0.3 – 1.5% of available phenols were found in olive oil, while the rest ended up in wastes (> 40%) depending on the system used. 2-phase centrifugation provided the highest transfer rate of phenols to oil (1.5%) and the highest antioxidant potential, followed by traditional press (1.2%). In 3-phase centrifugation, the transfer rate of phenols to oil was 0.5%, while most of the phenols were flushed away with the wastewater produced (> 30%). These results indicate that the phenolic content is higher in the water phase. So, OMWW will have more concentration of phenols than OMSW [8, 21]. In [8] it was concluded that the amount of total phenols in OMWW is 10 times more than in OMSW. This way, 2P-OMSW has also more phenolic content than 3P-OMSW, because the first is a combination of wastewater and solid waste. It is generated in small quantities, besides being more concentrated and thus richer in fat, dry residue, phenols and *o*-diphenols. Besides its higher moisture, it also has sugars and fine solids that in the 3-phase system are contained in the OMWW [4].

For these reasons, 3P-OMSW are mostly used as a solid fuel so it is a by-product with economic value, while 2P-OMSW cannot be composted or burned without some expensive pretreatment, due to its composition and doughy consistency, that also complicate its transport, storage and handling [4].

The presence of these persistent compounds is one of the main obstacles in the detoxification of OMW [4].

2.2 PHYTOTOXICITY ASSESSMENTS

Phytotoxicity was defined as a delay or inhibition of seed germination, inhibition of plant growth or any adverse effect on plants caused by specific substances or growing conditions [22]. The concept of phytotoxicity covers any substance capable of generate temporary or long-term stress, by damages on growth potential of plants. These substances are called phytotoxic because they affect the germination capacity of seeds, roots growth and dry matter evolution [23].

Impairment of the root system in the presence of toxic substances is a complex phenomenon, intensely influenced by various interacting physical and biological factors. A large number of organic molecules tend to be toxic to the roots and, consequently, to plants. Others become toxic when interact with others or by combining with different phases of organic substrates, which suggest the existence of different toxic groups [24].

Plants can grow in many different substrates varying in physical, chemical and biological properties, because a highly-specific functional differentiation of their roots provides them that flexibility. Each specialized root system is adapted to a distinctive type of absorption and fit for the particular substrate where the roots were formed. Therefore, changes in physical, chemical or biological conditions origin a reduction in root absorption proportional to the time required for adapting to the new environment. Plant responds decreasing the metabolic rate and concentrating on generating a new absorption apparatus (root hair, cortex, root tips, etc.). So, the concept of phytotoxicity combines the effects of environmental changes, specific roots sensitivity and metabolic conditions existing in the plant, that together lead to plant damage [24].

The concept of phytotoxicity is even more complex, since if a substance does not appear to be lethal to a plant, it does not mean that it will not be harmful, i.e. the plant becomes living in unstable conditions. So, it is possible to conclude that plant tolerance to changes is complex. It involves intrinsic resistance to external conditions and adaptation. Adaptability, in turn, may depend on both intrinsic and extrinsic conditions, such as metabolism and amount of change, respectively [24][24][24][24][24][24][24][24][24][24][24][24][24][24][24][24][24].

Analyzing phytotoxicity may provide a wide spectrum of information. The quantification of metabolic toxicity is an important criterion in industrial, agricultural and environmental purposes. It allows not only to assess the influence of specific substances on plants but also to evaluate stabilization processes.

Toxic/phytotoxic effects towards the environment must be avoided when using soil, sludge, bio-waste compost, soil improvers, growing media or any kind of plant substrate. The organisms more likely to suffer immediately and visibly from adverse effects are higher plants. Thus, the phytotoxicity effect of a certain material can be used as an indicator, even without detailed specification of the cause.

There is a variety of methods for examining phytotoxicity, since they can be performed on one species in the laboratory or in a wide range of test substances and test species [23], and the various key parameters of these methods differ to a large extent, mostly depending on the material under investigation [22]. Methods for examining phytotoxicity can be based on germination tests or tests conducted on emerged plants. There are two possible approaches: to grow certain plants directly in the test material or in diluted liquid samples; or to grow them in hydroponic systems supplied with leachate or mixtures of leachate and nutrient solution. When there is the need of studying the effects of volatile phytotoxins closed systems are used [22].

2.2.1 Methods for measuring phytotoxicity

To provide a convenient phytotoxicity quantification, bioassays must respond predictably to a range of concentrations of a known compound and to complex mixtures of contaminants [25]. They should be simple, reproducible and fast, because in long field experiments, plants can adapt to the toxic compounds [26]. Commonly, criteria to evaluate phytotoxicity are the frequency (number of plants at same stage or which have a visual symptom) or based on measurements (height, length or weight of plants). Other criteria are visual estimates (changes in color, plant deformation, etc.). Among the most used parameters to evaluate phytotoxicity symptoms are: changes in root weight, root length, root system development, modifications of germination rate, stem length, color changes, plant necrosis and deformation organs (stem, leaf) [23].

Also a great variety of plant species have been tested. Usually, *Lepidium sativum* L. (commonly, called cress or garden cress) is the chosen species, because it shows rapid response, is sensitive and cost-effective [24, 25]. Thus, it was the species selected for this work. Others include horticultural species (e.g. tomato, carrot, cucumber, cabbages, radish and beans), cereals (like barley, Italian rye grass, rice, wheat, rye, soya or corn) and even sunflowers, petunia, amaranth, among others [26].

- **Seed germination bioassays**

Phytotoxicity bioassays based on germination index assays will be used in this work, promoting the germination of garden cress. This test determines whether if a medium contains substances that can inhibit seed germination or growth of the radicle (the embryo root) [25]. In literature, these tests are mostly used to evaluate maturity of compost [24, 25]. Even mature compost may contain substances that can prevent plant growth, such as heavy metals, salts, pesticides residues or other toxic compounds present in the original substrates [25].

These bioassays with test organisms growing in an extract allow the assessment of combined toxicity of contaminants that might be present. However, they do not allow the identification of which contaminant is responsible for the toxic effects observed [25].

The tests consist of sown seeds in extracts of the tested material at specific conditions and determine the relative seed germination and root growth, as compared with that obtained with distilled water (control).

- **Growth tests bioassays**

Direct growth tests allow to overcome some gaps of the previous ones, because they take into account not only the fraction of contaminants dissolved in the aqueous extracts (i.e. some phytotoxic substances may not be soluble in water), but also the fraction of contaminants associated with the solid matrix. In addition, some authors consider plant growth more sensitive to toxic substances than seed germination, because during seed germination the plant is relatively insensitive to many toxic substances, since the embryo is isolated from the environment and many chemicals are not absorbed by the seed. These experiments can be short tests focused on the assessment of root germination and elongation or longer growth trials focused on the evaluation of the effect of compost at later stages of plant development. Although they provide productivity data, they are slow and may require complex installations with controlled temperature, humidity and illumination [26].

2.2.2 Interpretation of the results

Phytotoxicity is usually expressed in terms of Germination Index, but it can also be quantified in terms to EC_{50} (effective concentration that promotes 50% inhibition of a selected parameter) or to be reported as function of certain criteria (e.g. root elongation, number of germinated seeds, dry weight, etc.), depending on the aim of the study.

- **Germination Index**

Germination Index (GI) is considered the most sensitive parameter for quantifying the phytotoxicity of a matrix and to assess its suitability for use as soil amendment or growing media. It is used to express the results of germination bioassays (e.g. cress bioassays), because it combines the relative seed germination and relative root growth in a single value that is the percentage of phytotoxicity of the tested material in relation to a control trial. GI is calculated by Eq. (2.3), (2.1) and (2.2),

$$RSG(\%) = \frac{N_{GS}}{N_{GS,control}} \times 100 \quad (2.1)$$

$$RRG(\%) = \frac{RL_{GS}}{RL_{GS,control}} \times 100 \quad (2.2)$$

$$GI(\%) = \frac{RSG(\%) \times RRG(\%)}{100} \quad (2.3)$$

where N_{GS} and $N_{GS,control}$ correspond to the mean number of germinated seeds in the treatment and in the control, respectively; RL_{GS} and $RL_{GS,control}$ correspond to the mean root length of the germinated seeds in the treatment and in the control, respectively; RSG is relative seed germination (%) and RRG is the relative root growth (%).

The degree of phytotoxicity of a tested material may be classified as indicated in Table 2.4.

Table 2.4 Phytotoxicity classification according to GI (adapted from [25]).

Germination Index (%)	**Rating**
> 100	The material enhances germination and root growth
100 – 80	No inhibition of plant growth: the material isn't phytotoxic
80 – 60	Mild inhibition: the material is moderately phytotoxic
60 – 40	Strong inhibition: the material is phytotoxic
< 40	Severe inhibition: the material is highly phytotoxic

- **EC50 (median effective concentration)**

EC_{50} is the concentration of a test substance which results in a 50% reduction in a certain response. In this type of experiments, this value may be reported in relation to the number of germinated seeds, root/steam length, GI, etc., by comparison with the control.

2.2.3 Mechanisms of seed germination and plant growth

To understand how a plant is affected by phytotoxic substances, it is necessary to understand the "plant side" of this problem. This analysis will be considered only for *Lepidium sativum* L. because it was the plant species selected for bioassays.

Lepidium sativum L., also known as cress or garden cress, is a species of the rosid clade of the core eudicots [27] and Fig. 2.3 illustrates a mature seed. Cress is a seed plant (spermatophyte) that belongs to the class of angiosperms (flowering plants). In angiosperms, it occurs a double fertilization, which means that it is formed the diploid embryo and the triploid endosperm. The embryo is a young sporophyte that when becomes mature consists of cotyledons (seed leaves), hypocotyl (stem-like embryonic axis below the cotyledons) and radicle (embryonic root). On the other hand, the endosperm is the food storage, nutritive tissue of living cells. A mature seed is also composed by a seed coat named testa, which is an outer protective layer, consisting of maternal tissue (dead cells) [28].

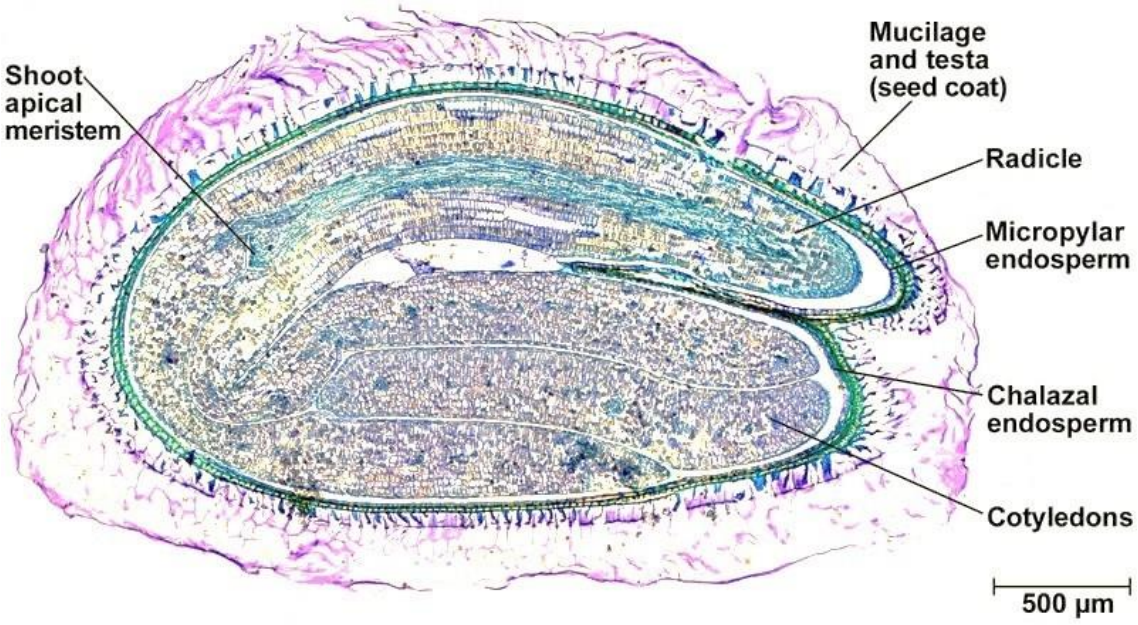


Fig. 2.3 Structure of a mature seed of *Lepidium sativum*. Bright field microscopy of longitudinal sections of 2-3 h imbibed seeds in stained with toluidine blue (adapted from [27]).

In mature cress seeds, besides the embryo is enclosed in a thin endosperm, mostly consisting of a single cell layer, the micropylar endosperm surrounding the radicle tip has up to two cells layers. So, that is why it is often said that mature seeds of cress have 1-2 cell layers of endosperm [28]. In addition, for cress testa and endosperm are the two covering layers of the embryo.

The process of seed germination begins with water uptake by the dry seed through imbibition and ends when the radicle has penetrated all covering layers. So, germination is only completed if the growth potential of the radicle overcomes the tissue resistance of the seed covering layers [27]. Thus, species exhibit a two-step germination: testa rupture and endosperm rupture as indicated in Fig. 2.4.

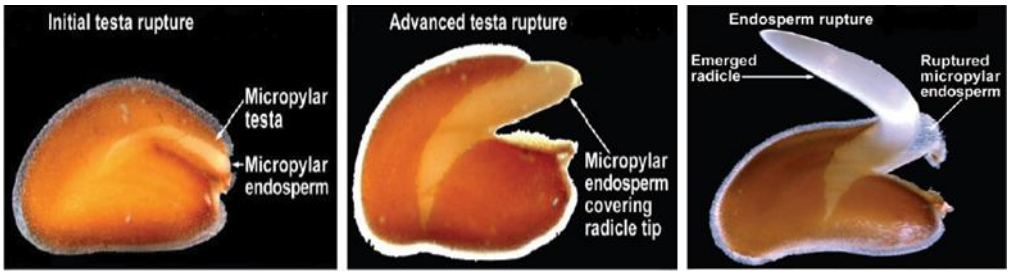


Fig. 2.4 Two-step germination of a mature seed of *Lepidium sativum* (adapted from [27]).

In the first phase (testa rupture), the micropylar endosperm is exposed as a cap-like structure that covers the radicle tip; the endosperm rupture and radicle emergence happen and seed germination is completed [27].

2.3 DETOXIFICATION METHODS

In case of a material (or waste) contain pollutants, it poses risks to human health and environment. If the material is to be used in soil, before its application detoxification should be implemented to remove or stabilize contaminants. Among the different processes available for detoxification, in this study, it was tested the possibility of reducing OMSW phytotoxicity only through Fenton's oxidation process. Although other interesting possibility is the composting route [1–4, 29–31].

2.3.1 Fenton's oxidation process

Fenton's process is an advanced oxidation process (AOP) that has been studied in the depuration of liquid effluents [32]. It is a low expensive treatment, since the reaction takes

place at room temperature and pressure, requires easy-to-use reagents and the reaction occurs in a short time. In this study, Fenton's process was used to treat a synthetic liquid effluent simulating olive mill wastewaters, once this agro-industrial waste has a seasonal and toxic character that makes its treatment by traditional biological systems not appropriate [32]. Moreover, it was also applied in a semi-solid operation for the detoxification of 2P- and 3P-OMSW.

In this case, organic pollutants may be degraded by reacting with strong oxidant hydroxyl radicals that are generated from the decomposition of hydrogen peroxide in the presence of iron ions at acidic conditions (Eq. (2.4)).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \quad (2.4)$$

There is also the catalytic decomposition of hydrogen peroxide which follows a radical mechanism involving hydroperoxyl radicals, traduced by Eqs. (2.5) and (2.6).

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^{+} \quad (2.5)$$

$$HO^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O \quad (2.6)$$

Radical chain oxidations are initiated by hydroxyl radicals reacting non-selectively with the organic molecules. Even though, some radical scavenger effect may be attributed to hydrogen peroxide and iron. Eqs. (2.7) and (2.8) show this effect [32].

$$H_2O_2 + HO^{\bullet} \rightarrow H_2O + HO_2^{\bullet} \quad (2.7)$$

$$Fe^{2+} + HO^{\bullet} \rightarrow Fe^{3+} + HO^{-} \quad (2.8)$$

2.3.2 Composting

Composting is the most used method for stabilization of OMW organic matter. It consists in a biological process that can be carried out in many different scales, from using small indoor bins to huge commercial or industrial operations that process outdoor piles called windrows [25]. Providing the optimal conditions for thermophilic composting is important, since its high temperatures promote rapid decomposition of instable organic matter and kill weed seeds and organisms that can cause diseases [25].

Globally, the process may be represented by the Eq. (2.9):

$$Organic\ matter + H_2O + O_2 + microrganisms \rightarrow resistant\ organic\ matter + H_2O + CO_2 + other\ gases + heat \quad (2.9)$$

This method was applied to OMSW [3, 4, 29–34], with interesting results.

3 STATE OF THE ART

Considering the objective of this study, the state of the art is organized in terms of the following topics: methodologies of germination assays; methodologies of growth tests and live mill waste management option.

3.1 METHODOLOGIES OF GERMINATION ASSAYS

Germination Index (GI) has been widely used as a tool to assess phytotoxicity of different types of wastes. It has been used for testing phytotoxicity of agro-industrial wastes, soil conditioners, irrigation waters or even to evaluate composts maturity. Table 3.1 summarizes some applications of this method.

Table 3.1 Methodologies of germination assays described in the literature.

Ref.	**Tested material**	**Selected species**	**Containers characteristics**	**Test conditions**
[24]	Compost extracts	*Lepidium sativum* L. (cress)	Petri dishes (4-5 cm diameter) lined with filter paper containing 0.5 mL of extract	27 ºC; 24 h; darkness; nº seeds: 6-8; nº of replicates: 10-15.
[25]	Compost extract ("10x dilution" and "full strength)	*Lepidium sativum* L. (cress)	Petri dishes (9 cm diameter) with a 7.5 cm papel filter moisten with 1 mL of the test solution.	27 ºC; 24 h; darkness; nº seeds: 8; nº of replicates: 5. During incubation, the petri dishes are sealed in plastic bags.
[10]	Olive husk, wheat straw and OMWW composted for 1 year	*Lepidium sativum* L. (cress)	Petri dishes	72 h; nº seeds: 10; nº of replicates: 2.
[38]	OMW Undiluted and diluted (1/4, 1/8 and 1/6) in distilled water	*Ordeum vulgare* (barley)	Petri dishes	22ºC; darkness; nº seeds: 50.
[39]	Treated and untreated olive mill effluents	Lettuce	Petri dishes (10 cm diameter) covered with 3 pieces of perforated paper irrigated with 5 mL of sample.	21 ºC; 72 h; nº seeds: 25; nº of replicates: 6.
[15] (adapted from [24])	Dry olive mill residue extracts colonized or not with fungi	*Lepidium sativum* L. (cress)	Petri dishes (9 cm diameter) lined with filter paper with 2 mL	25 ºC; 48 h; darkness; nº seeds: 50; nº of replicates: 4.
[40] (adapted from [24])	Water extracts from mixtures of poultry manure and peat	*Lepidium sativum* L. (cress)	Petri dishes (9 cm diameter) lined with filter paper containing 5 mL of each extract and sealed with parafilm	25 ºC; 48 h; darkness; 40-50% humidity; nº seeds: 10; nº of replicates: 10.

Table 3.1 Methodologies of germination assays described in the literature (continued).

Ref.	Tested material	Selected species	Containers characteristics	Test conditions
[41] (adapted from [24])	Compost from municipal solid waste	*Lepidium sativum* L. (cress)	Petri dishes	Nº seeds: 10; nº of replicates: 4. After 24 h, at 27 ºC, in darkness, it is added ethanol to stop germination.
[23]	Solutions of different concentrations of Cd(II) and Cr(VI) (30, 60, 90, 120, 150 and 300 mg/L)	*Lepidium sativum* L. (cress)	Petri dishes lined with filter paper soaked by 3 mL	Nº seeds: 20; nº of replicates: 3. Petri dishes were covered with lids, at room temperature for 3 days.

These bioassays are easily done and do not need sophisticated equipment or techniques. Besides, the procedure has not suffered great modifications. Most of times it is used the one developed by Zucconi et al, with or without few adjustments. Typically, it is prepared an extract or solution from the compounds/substances/matrixes that are being tested, where the seeds are sown from 24 to 72 h, in darkness, at temperatures that can vary from 20 to 27 °C.

Lepidium sativum L. (garden cress) is the most selected species, because of its good response to toxic materials and also its rapid and easy germination.

In the literature, the calculation formula for GI is also shown in different forms. However, their meaning is the same. It is computed as the product of the percentage of germinated seeds by the percentage of root growth [15, 24, 25, 38, 40, 41]. In this study, these assays were performed to test phytotoxicity of OMSW from 2- and 3-phase systems and phenolic compounds, using cress as the indicator species.

3.2 METHODOLOGIES OF GROWTH TESTS

The response of certain crops to some substances can also be assessed through growth tests. These analyses reproduce the soil application of certain products under conditions more alike to real environments, since germination occurs in real mediums. It is selected a reference substrate, that can be sand, a bedding plant medium or a specific type of soil, where a second substrate will be mixed in different volume proportions. This second substrate is the substance to be tested. These tests can be employed in small pots or even in big areas; some parameters such as the duration of the tests, temperature and light conditions or the watering process may also vary a lot as it is shown in Table 3.2.

Table 3.2 Growth tests methodologies described in the literature.

Ref.	Substrate 1 (reference); Substrate 2 (tested material)	Selected species	Mixture (substrate 1: substrate 2 (%V/V))	Containers characteristics	Conditions and watering
[25]	Natural soil, vermiculite and/ or sand; Compost or vermicompost	Radish; Lettuce; Melon.	100:0 (control) 75:25 50:50 25:75 0:100	Pots or planting trays	Nº of seeds: 6; nº of replicates: 3; well-lit location (sunlight or artificial light). Pots were watered as needed.
[43]	Commercial bedding plant medium; Pig manure vermicompost	*Lycopersicon esculentum* Mill. (tomato)	100:0 (control) 90:10 80:20 70:30 60:40 50:50 40:60 30:70 20:80 10:90 0:100	Polystyrene trays, each one consisting of 50 inverted pyramid cells.	Nº of seeds: 50; nº of replicates: 4; mist house and glasshouse inherent conditions. Twenty days after, 10 plants from each potting mixture were selected randomly and the parameters of interest were measured.
[44]	Not used; There were tested 15 different soils: 5 mineral oil-contaminated soils; 6 soils collected from real environment; soil from an agricultural field; commercial air-dried soil and 2 commercial garden mouls.	*Avena sativa* L. (oat); *Lepidium sativum* L. (cress); *Brassica rapa* Metzg (turnip); *Phaseolus vulgaris* L. (bush bean)	Every 15 soils were tested individually. Note: before use, all of these soils were screened through a 5 mm sieve.	Each pot was 9 cm (top) or 6.5 cm (bottom) diameter and 6.5 cm height, holes on the bottom. A petri dish was placed underneath each pot. Seeds were sown uniformly in each pot to a depth to 0,2 cm for cress.	Nº of seeds: 10 (cress); nº of replicates: 4.; greenhouse conditions of temperature, humidity and light. Test was ended after 14 days of 50% of the seeds in each pot had germinated.
[45]	Commercial bedding plant medium; Pig manure vermicompost	*Lycopersicon esculentum* Mill. (tomato)	100:0 (control) 95:5 90:10 75:25 50:50 0:100	Plastic pots of 10 cm in diameter.	Nº of seeds: 3; nº of replicates: 20. Firstly, pots were placed in a mist house and then moved into a glasshouse. After germination, 5 pots from each potting mixture were selected randomly and the parameters of interest were measured.
[46]	Comercial peat; Composted sewage sludge	*Brassica oleracea* var. *Botryti* cv. Marathon (broccoli)	100:0 (control) 85:15 70:30 50:50	Pots of 10 dm^3.	Nº of seeds: 64; nº of replicates: 6. For the first part of the test, the growth chamber was at 27 ºC for 36h. The growth assay was carried out under the greenhouse conditions. The plants were irrigated in accordance with their water demand with distilled water.

Table 3.2 Growth tests methodologies described in the literature (continued).

Ref.	Substrate 1 (reference); Substrate 2 (tested material)	Selected species	Mixture (substrate 1: substrate 2 (%V/V))	Containers characteristics	Conditions and watering
[47]	Horticultural calcareous soil; Composted sewage sludge	*Capsicum annuum* var. annuum (sweet pepper)	Volume of compost used (kg/m^2): 0 (control) 3 6 9	Two plots of 80 m^2 (one open-air and the other under greenhouse)	Seeds (24) were soaked on a layer of wet cotton using irrigation water for 4 days at 25 °C. Then, they were covered with peat in plastic trays and watered daily. Later, they were transplanted to the experimental plots. Both plots were irrigated regularly twice a week.
[41]	Sand; Commercial compost (municipal solid waste compost)	*Lepidium sativum* L. (cress); *Plantago ovata Forsk* (isabgol); *Cuminum cyminum* L. (cumin)	100:0 (control) 50:50 25:75	No dimensions referred.	Nº of seeds: 10; nº of replicates: 6. The number of emerged seeds were counted after 48, 72 and 96h. Then, seedlings were harvested and GI was measured. The containers were kept saturated by regular watering.

Table 3.2 shows that there are many different ways of performing a growth test, since the authors adapt the test conditions to their needs. Different criteria can be assessed, such as the number of germinated seeds, their radicle length, number of leaves and chlorophyll analysis.

For example, in [25] it is recommended to record on a daily basis the number of seeds that have germinated, plant growth and observations about plant health such as color, vigor or damage due to pests and diseases. Some parameters could be measured, such as plant height, number and size of leaves and dry weight of the entire plant at the end of the experiment (by drying at 105 ºC for 24 h). On other side, the seedling shoots were cut above the soil surface and the fresh biomass was immediately weight [44]. Then, after drying at approximately 80 ºC for 72 h, dry biomass was measured.

In the present work, it was followed the method described in [43]. Plant heights (distance from soil level to the top node) and total leaf numbers (excluding cotyledons) of each of the seedlings were recorded. Then, it was determined the average plant heights and leaf numbers per potting mixture. After removing plants from the pots, they were oven-dried at 60 ºC for 5 days to determine total plant dry weights.

3.3 OLIVE MILL WASTE MANAGEMENT OPTION

Regarding the treatment and safe disposal of OMW, a huge number of studies have been done over the past years, because of the high polluting capacity and the legal restrictions associated with these residues [39].

Table 3.3 aims to identify the best methodologies since it summarizes the main results found in literature on this ambit.

Table 3.3 Overview on olive mill wastes management options described in the literature.

	Reference	Objective	Conclusions
Composting	[29]	Composting 2P- and 3P-OMSW with wheat straw, chicken manure and olive pruning (leaves and stalks), at different proportions. It was added enzymes such as protease, lipase and cellulase.	2P- and 3P-OMSW can be successfully converted into compost. The duration of the composting period ranged between 21-80 days. However, longer periods (i.e. 90-224 days) are needed to achieve detoxification. The decrease in phenol levels achieved around 70% and 50% for 2P- and 3P-OMSW, respectively. The final can be recycled as organic amendment to olive plantation soils.
	[33]	To follow the evolution of the humic acid-like fraction during composting of 3P-OMSW and their co-composting with OMWW by means of chemical and spectroscopy analyses. Humic acids were isolated at different stages of composting and they were characterized aiming to monitor humification processes and the maturity of the compost.	Composting seemed suitable to produce well-humified organic matter to be used as soil amendment. The main transformations were a relative increase of proteinaceous materials and degradation of carbohydrates, due to microbial activity. After 9 months, isolated humic fractions were more similar that those of soil native humic acid. However, at the initial stages, treatment with OMWW seemed to slow down the early mineralization process.
	[36]	Investigating the transformations occurring in organic matter and their relationships with stability during composting of OMSW and a mixture of OMSW and OMWW. The composting was monitored through chemical and biochemical parameters and DSC and FTIR.	For both composts (OMSW and OMSW-OMWW), it was verified an increase of pH and the rate of mineralization of the organic matter. Phenolic removal and thermophilic conditions were concordant with the maturity and stability of the composts, obtained after 9 months. Evolution of the OMSW during composting led to homogenous and stable humic like- materials.
Fenton's Process	[32]	Analyzing the effects of the operating parameters over the efficiency of Fenton's oxidation in the treatment of synthetic OMWW. Toxicity and biodegradability were measured.	Luminescence and respirometric methods showed that the process was efficient on toxicity removal. Fenton's oxidation is promising to be applied before biological treatment.
Extraction of phenols	[7]	Studying the extraction variables for the recovery of phenolic compounds from 2P-OMSW by conventional liquid extraction and supercritical fluid extraction (SFE) using different solvents and CO_2.	Extracts with different phenolic concentrations and activities were obtained from 2P-OMSW. Ethanol was considered the most appropriate, producing extracts with high phenol content and high antioxidant activity. SC-CO_2 was an efficient solvent for recovering phenolic compounds with relatively high antioxidant.
	[48]	Application of hydrothermal treatment to 2P-OMSW to extract phenolic compounds and to test their antioxidant activities. Extraction with ethyl acetate at fixed temperature with different time was evaluated.	Hydrothermal treatment of 2P-OMSW led to a final liquid phase with a high concentration of simple phenolic compounds. HPLC showed variation of the concentration of phenolics with time.

Table 3.3 Overview on olive mill wastes management options described in the literature.

	Reference	Objective	Conclusions
Lime treatment	[49]	Application of lime treatment to OMWW in order to reduce its pollutant effect.	The liquid phase obtained after lime treatment can be discharged into the streams. Lime treatment showed that *o*-diphenols could be removed totally. After lime treatment, the liquid phase is more easily evaporated than raw OMW, because fatty components are removed.
Combined technologies	[39]	To study OMWW treatment by coagulants and poly-electrolytes and assess the effect of operating conditions on TSS, TP and COD removal, sludge produced and phytotoxicity of liquid phase. To enhance organic matter degradation, iron based coagulation was coupled with H_2O_2.	The coagulation-flocculation of OMWW pre-treatment was capable of separating completely the solid fraction with the resulting liquid presenting a reduced concentration of organisms and high phytotoxicity. Phytotoxicity decreased considerably following treatment with lime and cationic poly-electrolytes, due to the removal of phenols and other phytotoxic species from the liquid phase. It was considered a low-budget technology for effective management of OMWW.

DSC – Differential Scanning Calorimetry; FTIR – Fourier Transform Infrared Spectroscopy; HPLC – High Performance Liquid Chromatography; SC-CO_2 – Supercritical Carbon Dioxide; TSS – Total Suspended Solids; TP- Total Phenolic Content

Besides the methodologies for OMW valorization presented on Table 3.3, these wastes can have different applications. Concerning to solid wastes, 3P-OMSW are commonly dried and then a second extraction is performed to recover the remaining oil. However, this cannot be directly applied to 2P-OMSW due to its high moisture content, which causes technical problems during the drying process. To overcome this issue, the whole drying process has been studied but solutions increase costs because of the energy required [1]. In fact, 2P-OMSW requires a preliminary dryness, so most of the energy obtained by combustion would be used in that drying step, so the total energy recovery is low. One alternative is to use de-oiled 2P-OMSW for biomass gasification which originates synthetic gas ("syngas"). In contrast, after the second oil extraction of 3P-OMSW, the exhausted olive cake can be used as fuel to obtain thermal or electric energy through combustion, since it has relatively high calorific power (400 kcal/kg) [1].

Composting is perhaps the most used management option of OMW. As shown in Table 3.3, through a composting process it is possible to manage both OMWW and OMSW, and produce a compost without phytotoxic properties. In the case of composting, even 2P-OMSW can be easily processed with a few adjustments. Due to its semi-solid consistence, it needs to be mixed with bulking agents before composting [1].

2P-OMSW can be used as a low-cost substrate for the production of valuable compounds, not only phenolic compounds but also pectins. The extraction of phenols is a promising way of detoxify OMSW, since it originates a new liquid phase were phenols are concentrated for further utilizations, such as in pharmaceutics, food industries and cosmetics, preventing the utilization of synthetic antioxidants [1, 7].

OMSW can be used in anaerobic digestion for biogas production that, although the high levels of phenols constitute a limiting factor [1].

Fenton's oxidation process is recognized for detoxifying these type of agro-industrial wastes. However, its effectiveness on reducing phytotoxicity is more associated with the treatment of liquid effluents than solid wastes. In fact, there are only few studies involving the application of this detoxification system for solid wastes. Chemical remediation seemed to be a good approach for soil remediation, either using the traditional Fenton's process or new and more suitable approaches like nanoremediation. However, studies addressing its impact on terrestrial organisms are still scarce. Rede et al concluded that both green nZVIs and Fenton oxidation treatments are effective on the removal of the pollutant but their eco-toxicological impact could be more prejudicial for non-target organisms than the contaminant [50]. Thus, bearing in mind the interesting pollutant abatement capacity of this chemical system, it is worthy to perform further studies to understand the role of phenolic composition over OMW phytotoxicity character and the ability of Fenton's process to decrease such impact.

4 MATERIAL AND METHODS

To accomplish the aim of the present work, two types of olive mill solid wastes were characterized (2P-OMSW and 3P-OMSW). Every test was made at least in triplicate using blank experiments as control.

4.1 SAMPLING AND STORAGE

2P-OMSW was collected from a 2-phase olive mill in the Spanish region of Estremadura (Fig. 4.1 a)). It had a wet appearance due to its high moisture, so in the lab it was frozen in small portions and defrosted whenever necessary for the analyses.

3P-OMSW was collected from a 3-phase olive mill in the center of Portugal (Fig. 4.1 b)**Erro! A origem da referência não foi encontrada.**). It had a dry appearance and was kept in an opaque plastic bag at room temperature.

While 2P- looked like a sludge, 3P-OMSW contain a number of small portions (similar to slices) of dry and hard pomace.

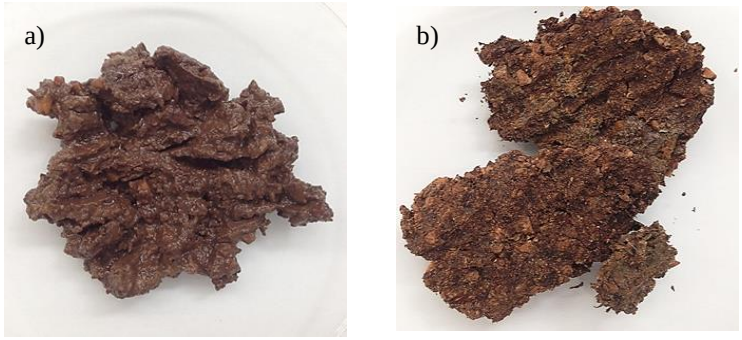


Fig. 4.1 Samples of a) 2P-OMSW and b) 3P-OMSW.

4.2 CHARACTERIZATION

4.2.1 Moisture and total solids

A sample of both fresh wastes with known weight was dried in an oven at 105 °C until constant weight (approximately 24 hours). Moisture content, H, corresponds to the difference between initial and final weights (Eq. (4.1)),

$$H\ (\%) = \frac{m_{fresh\ sample} - m_{dried\ sample\ at\ 105\,℃}}{m_{fresh\ sample}} \times 100 \qquad (4.1)$$

where $m_{fresh\ sample}$ is the mass of fresh sample (g) and $m_{dried\ sample\ at\ 105\ ℃}$ is the mass of dried sample at 105 °C (g).

Total solids, TS, is the percentage of dried solids (Eq. (4.2)).

$$TS\ (\%) = 100 - H\ (\%) \tag{4.2}$$

4.2.2 Volatile solids and total organic carbon

Volatile solids, VS, are determined using crucibles previously calcined. After drying a sample at 105 °C, it was calcined at 550 °C, during 1 h. VS is calculated through Eq. (4.3),

$$VS\ (\%) = \frac{m_{dried\ sample\ at\ 105\ ℃} - m_{calcined\ sample\ at\ 550\ ℃}}{m_{dried\ sample\ at\ 105\ ℃}} \times 100 \tag{4.3}$$

where $m_{calcined\ sample\ at\ 550\ ℃}$ is the sample weight after calcination at 550 °C (g).

The percentage of total organic carbon, TOC, was calculated using an empirical correlation referred in [51], shown in Eq. (4.4),

$$TOC\ (\%) = \frac{VS\ (\%)}{1.8} \tag{4.4}$$

4.2.3 Water holding capacity

Water holding capacity, WHC, reflects the capacity to hold water. The experimental method requires small containers perforated in the bottom and nets with different mesh sizes. Those nets were placed inside the containers, to retain solid but allowing water diffusion. After closing the containers, they were soaked in water during 24 h. After this period, water was drained off by gravity for 24 h. WHC is computed by Eq. (4.5),

$$WHC\ (\%) = \frac{m_{soaked\ and\ drained\ sample} - m_{dried\ sample\ at\ 105\ ℃}}{m_{soaked\ and\ drained\ sample}} \times 100 \tag{4.5}$$

where $m_{soaked\ and\ drained\ sample}$ corresponds to the samples weight after being soaked and drained (g).

4.2.4 pH and EC

Wastes were also characterized in terms of pH using the CRISON micro pH 2002. The calibration of the sensor was based on two standard solutions with pH of 4 and 7. pH of each fresh waste was measure in aqueous extracts, which were agitated during 1 h to allow contact of the suspension with a L/S ratio of 10 L/kg (dry basis). pH was measured through the supernatant of the extracts, which were resting for approximately 10 min after agitation to avoid contact between solids and the sensor.

The electrical conductivity (EC) was measured using the Multiparameter analyzer Consort C863, in the same extract used to measure pH.

4.2.5 Chemical oxygen demand

Chemical oxygen demand (COD) was assessed through the procedure of [52]. The adopted methodology involved preparation of sample test vials and calibration vials, which were digested in the ECO25 thermoreactor (VELP Scientifica) for 2 h at 150 °C. Then, after cool until room temperature, absorbance of each solution in the vials was read in the photometer PhotoLab S6 (WTW) at 605 nm.

Test vials had the following content:

- 1 mg of waste (dried at 105 °C);
- 399 mg of distilled water;
- 3.6 mL of acid solution;
- 3.6 mL of digestion solution.

In Table 4.1 is presented the content of calibration vials.

Table 4.1 Calibration solutions for COD.

	$m_{\text{potassium hydrogen phthalate}}$ (mg)	$m_{\text{distilled water}}$ (mg)	$V_{\text{acid solution}}$ (mL)	$V_{\text{digestion solution}}$ (mL)
0	0	400.0	3.6	3.6
1	0.5	399.5	3.6	3.6
2	1.0	399.0	3.6	3.6
3	1.5	398.5	3.6	3.6
4	2.0	398.0	3.6	3.6
5	2.5	397.5	3.6	3.6
6	5.0	395.0	3.6	3.6

Acid solution was prepared diluting 9.6 g of silver sulfate in 1 L of concentrated sulfuric acid. This solution has to rest for 2 days before being used. In what regards the digestion solution, this one consists in an aqueous solution of potassium dichromate (0.25 M).

In this test, every time a new solution (acid or digestion) is prepared it requires a new calibration curve to minimize inconsistencies in the results interpretation. The relation between COD and absorbance is obtained from a calibration curve (0.7 g/L potassium hydrogen phthalate corresponds to 200 mg O_2/ L), therefor calibration is a critical step.

4.2.6 Total phenolic content

Total phenolic content (TPH) was determined by Folin-Ciocalteau Method, according to the methodology described in [18] with a few modifications. Calibration curve was developed using gallic acid dissolved in methanol/water solution (80/20 v/v%), in a linear range of 10 to 100 μg/mL.

To prepare the calibration solutions, it was added 0.3 mL of Folin-Ciocalteau reagent and 2.4 mL of concentrated Na_2CO_3 to 0.3 mL of each standard solution. In the case of the sample vessels, Folin-Ciocalteau and Na_2CO_3 were added to an OMSW extract, prepared with 1 g of dried sample and 12 mL of the methanol/water solution. These extracts were magnetically agitated for 1 h and then filtrated through a 0.45 μm filter.

Before analysis standard and sample solution last for 1 h in the dark to allow reaction. TPH was measured by reading absorbance at 760 nm in the UV/Vis Spectrophotometer T60.

4.2.7 Total kjeldahl nitrogen

The determination of total kejeldahl nitrogen (TKN) consists of three steps: digestion, distillation and titration.

The digestion requires adding 0.5 g of dry waste, a Kjeldahl catalyst tablet and 10 mL of H_2SO_4 (96%) to a digestion tube. Two blank tubes were prepared with the same composition but without waste sample. These tubes were digested in the DKL Fully Automatic Digestion Unit (VELP Scientifica), at 420 °C during, approximately 2 h, until white vapors appeared. Then, solutions were left cooling until room temperature.

Before the second step, it was added 100 mL of distilled water to the tubes. Then, distillation was performed in the UDK Distillation Unit (VELP Scientifica), for 7 min, where 50 mL of NaOH (400 g/L) were added. The distillate (100 mL) was collected, 10 mL of a pH indicator solution were added and then it was finally titrated with HCl 0.1 M. The indicator solution contains boric acid with bromocresol green and methyl red.

4.2.8 Total nitrogen

Total nitrogen (TN) of both 2P- and 3P-OMSW was assessed using the DKL Fully Automatic Digestion Unit (VELP Scientifica). Digestion tubes with 0.5 g of dried

samples, 0.5 g of chromium 100 mesh and 20 mL of hydrochloric acid 7% were intermittent agitated for 5 min and then heated until boiling for 4 min. After cooling, it was added 7 g of potassium sulfate anhydrous, 100 mg of HgO and 100 mL of sulfuric acid. These mixtures were digested at 420 °C for 60 min.

4.2.9 Total phosphorous

Total phosphorus (TP) was measured by taking 37.5 mg of dry waste sample and dissolve it in 50 mL of distilled water. Then, it was added 1 mL of sulfuric acid 11 N and 0.4 g of ammonium persulfate. This mixture was agitated manually and then digested in digestion tubes in the DKL Fully Automatic Digestion Unit (VELP Scientifica), for 30 min at 100 °C.

After cooling during 10 min, 5 mL of a sodium dissulphite solution (5.2 g of $NaHSO_3$ in 100 mL of H_2SO_4 1N) was added to the tubes, and digested for 30 min at 95 °C. Tubes were left cooling for 10 min. Then, 4 mL of a solution of ammonium molybdate and potassium tartrate antimony and 2 mL of an ascorbic acid solution were added. After 5 min, absorbance of each sample was read at 650 nm in the UV/Vis Spectrophotometer T60.

TP concentration was assessed through a calibration curve, prepared with a solution of 0.75 g/L of potassium dihydrogen phosphate. Standard solutions were prepared diluting 0.1, 0.2, 0.3, 0.5 and 0.6 mL in 50 mL of distilled water. Each standard solution was put in digestion tubes and digested in the UDK Distillation Unit (VELP Scientifica). Subsequent steps were similar to those performed for the waste samples.

4.3 PHYTOTOXICITY ANALYSIS

4.3.1 Cress bioassays

Germination tests were conducted using *Lepidium sativum* L. (garden cress), by preparing aqueous extracts. The extracts of 2P- and 3P-OMSW were prepared using L/S ratios of 5, 10, 25, 50, 100, 250 and 500 L/kg (volume of water/dry mass of waste). After magnetic agitation for 1 h and centrifugation at 3500 rpm for 20 min, 5 mL of supernatant was taken for moisten filter papers placed in the Petri dishes (9 cm diameter), over which 10 seeds of cress were placed in.

For testing the influence of different concentrations of phenolic compounds on cress germination, solutions of 5, 10, 25, 50, 100, 250 and 500 ppm were prepared. This assessment was made for gallic acid, protocatechuic acid, cinnamic acid, syringic acid, 3,4,5-trimethoxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, *p*-coumaric acid, caffeic acid and phenol.

Besides, a synthetic effluent that simulated agro-industrial wastewaters was also considered. This OMWW artificial effluent was prepared as described in [32], containing six phenolic compounds (3,4,5-trimethoxybenzoic, 4-hydroxybenzoic, protocatechuic, syringic, vanillic and veratric acid). This effluent was tested at 5, 10, 25, 50, 75 and 100 ppm.

To assess the impact of Fenton's peroxidation on the effluent phytotoxicity, treated synthetic OMWW and real OMSW were tested. In what concerns the treated solid wastes, after performing the chemical treatment, samples were left for a week evaporating all remaining hydrogen peroxide in open containers at room temperature. Then, the sample was dried at 60 °C and extracts were prepared and processed as previously described.

In all the cases, petri dishes were placed in dark conditions, for 48 h, at 25 °C. A blank experiment with distilled water was always included. After that, the number of germinated seeds and their root length were registered and compared with the blank. So it was possible to calculate GI of each study case by Eqs. (2.1), (2.2) and (2.3) (see chapter 2).

4.3.2 Growth tests

Growth tests with *Lepidium sativum* were held in plastic pots of 100 mL with 4 drain holes (3 mm diameter) in the bottom. Pots were filled with soil:waste mixtures in the following quantities (v/v%): 100:0 (control), 95:5; 90:10; 75:25; 50:50 and 0:100 [45].

Soil used in this assay was collected in Coimbra (Escola Superior Agrária). The soil was dried at 38 °C, milled and sieved with a 2 mm mesh.

These experiments were carried out with 2P- and 3P-OMSW both air dried. Each solid mixture was moistened with tap water until their consistency looked like appropriate for plants growth. The final moisture content of each treatment was between 15 and 40%. Pots were watered as needed.

In each pot, 7 seeds were sown uniformly to a depth of about 0.2 cm [44]. Pots were placed in the dark for 24 h and then experiments were conducted in a well-lit location.

The test lasted two weeks and the analysis followed the procedure described in [43] (see chapter 3). Results were compared with the control experiments and expressed in terms of:

$$Relative\ germination\ (\%) = \frac{N_S}{N_{S,control}} \times 100 \quad (4.6)$$

$$Relative\ growth\ (\%) = \frac{SL}{SL_{control}} \times 100 \quad (4.7)$$

$$Dry\ biomass\ (\%) = \frac{m_{after\ dryness}}{m_{before\ dryness}} \times 100 \quad (4.8)$$

where, N_S and $N_{S,control}$ are the mean number of shoots in the treatment and in the control, respectively; SL and $SL_{control}$ are the mean shoot lengths in the treatment and in the control, respectively. Dry biomass of treatments and control was also assessed by knowing the shoots weights before and after the drying process ($m_{before\ dryness}$ and $m_{after\ dryness}$, respectively). The number of leaves in the pots containing wastes samples and in the control (NOL and $NOL_{control}$, respectively) was also counted.

4.4 CHEMICAL STABILIZATION – FENTON'S OXIDATION PROCESS

Fenton's process was implemented for 2P- and 3P-OMSW, considering the optimal conditions described in [52]. Thus, an extract of 5 L/kg of L/S ratio was continuously magnetically agitated. At the beginning, pH was set at 3 with a solution of H_2SO_4 (2 M). Then, it was added the catalyst (Fe^{2+}) in the form of an iron salt, $FeSO_4{\cdot}7H_2O$. After 15 min, hydrogen peroxide (33%) was carefully introduced in the previous mixture because of the formation of vapors. After 30 min, pH was adjusted to 7 with NaOH solutions (from 0.5 to 9 M) to stop the reaction and promote iron precipitation. The resulting product was dried in an oven at 60 ºC until the supernatant was evaporated for 48 h. The final treated and dried material was used to prepare an aqueous extract with a L/S ratio of 10 L/kg, which was used to assess phytotoxicity through cress bioassays as already described in this chapter.

For the synthetic effluent it was followed an identical procedure, but for the best conditions achieved in [32], which were $FeSO_4{\cdot}7H_2O$ mass corresponding to 271 mg of Fe^{2+} for 300 mL of effluent, a concentration of H_2O_2 of 488 mM and 6 h of Fenton's reaction. In this case, germination assays were performed only a week after finishing the reaction, to guarantee that some remaining hydrogen peroxide would not interfere in the subsequent tests.

4.5 STATISTICAL ANALYSIS OF THE RESULTS

The experiments were performed at least in triplicate to guaranty reproducibility. An outlier analysis was performed whenever appropriate so that these values would not influence final results. Thus, the number of germinated seeds (N_{GS} and $N_{GS,control}$) and their root length (RL_{GS} and $RL_{GS,control}$), resulting from cress bioassays were verified in order to eliminate any inappropriate seedling or replicate, i.e. outliers. To help this process, it was made box plots in the software *SigmaPlot*. The outlier analysis is summarized in Appendix A.

Considering growth experiments, these were statistically processed through an analysis of variance (ANOVA) in the software *Microsoft Excel*. This tool allowed to determine if *n* samples or treatments could be considered statistically identical. This procedure is indicated in Appendix B.

In the present work, 2P- and 3P-OMSW growth tests were analyzed through one-way ANOVA, in which the factor is the volumetric percentage of waste in the pots in the different treatments and the effects are the number of shoots, shoot length and percentage of dry biomass.

5 RESULTS AND DISCUSSION

5.1 MATERIALS CHARACTERIZATION

The physic-chemical characteristics of the selected wastes (2P- and 3P-OMSW) are summarized in Table 5.1. All results obtained in this work are expressed in dry basis.

Table 5.1 Characterization of 2P- and 3P-OMSW.

Parameter	**2P-OMSW**			**3P-OMSW**		
	This work	[37]	[53]	This work	[29]	[54]
H (%)	67.1 ± 0.5	61.8 [(a)]	48.20	17.6 ± 0.6	42 – 49	n.d.
TS (%)	32.9 ± 0.5	38.2 [(a)]	51.80	82.4 ± 0.6	n.d.	n.d.
VS (%)	95.2 ± 0.2	97.4 [(a)]	n.d.	94.2 ± 1.8	n.d.	94.3
TOC (%)	52.9 ± 0.1	55.9	53.50	52.4 ± 1.0	n.d.	n.d.
WHC (%)	97.8 ± 6.9	n.d.	n.d.	91.4 ± 16.8	n.d.	n.d.
pH	4.82 ± 0.00	4.9	5.70	4.96 ± 0.02	6.3 – 7.2	6.7
EC (mS/cm)	2.77 ± 0.03	1.78	5.02	0.92 ± 0.05	n.d.	0.9
COD (g O_2/g d.w.)	2.40 ± 0.19	n.d.	n.d.	2.48 ± 0.05	n.d.	n.d.
TPH (mg GAE/g d.w.)	0.99 ± 0.03	0.54 [(b)]	0.65 [(d)]	0.93 ± 0.03	0.20 [(e)]	0.551 - 0.950 [(g)]
TKN (mg/g d.w.)	10.51 ± 4.69	10.5	16.00	11.08 ± 1.75	n.d.	n.d.
Total N (mg/g d.w.)	11.74 ± 0.05	n.d.	n.d.	13.95 ± 0.93	12 – 14	n.d.
P (mg/g d.w.)	0.26 ± 0.01	0.15 [(c)]	2.75	0.03 ± 0.01	1.30-1.70 [(f)]	n.d.

mean ± std; d.w.: dry weight; n.d.: not determined; GAE: gallic acid equivalents; [(a)] % fresh matter; [(b)] Phenolic compounds (% d.w.); [(c)] [55] ;[(d)] mg/g fresh matter, [48]; [(e)] % (caffeic acid equivalents); [(f)] [56];[(g)] [8].

Globally, the characteristics of 2P- and 3P-OMSW used in this work are consistent with the literature. It was proven that 2P-OMSW has definitely higher moisture content that 3P-OMSW. Concerning to VS, TOC and COD, there is not great differences between both residues. The same conclusion is obtained when the results are compared to the values of literature. It was not possible to find typical values for WHC for the wastes in the literature, but this study showed that 2P- has higher WHC than 3P-OMSW. In terms of EC, 2P-OMSW shows certainly higher values than the other waste, which is also coherent with literature. In this work, quantification of the phenolic fraction of both wastes, TPH, was reported as grams of gallic acid equivalents per mg of dry matter. Between both residues, no significant differences were obtained, but for 2P-OMSW, TPH is a little higher than 3P-OMSW, as literature also shows. Quantification of N is also consistent with literature, and values of both wastes are also in the same order of magnitude. At last,

phosphorous assessment led to very different values between both residues. In the literature few studies included this parameter and there are also some discrepancies.

5.2 PHYTOTOXICITY ASSESSMENTS OF OMSW

Several extracts of both wastes prepared with different L/S ratios were tested for GI, in order to investigate their phytotoxicity. Results are shown in Fig. 5.1. Note that they are represented in logarithmic scale to ease interpretation.

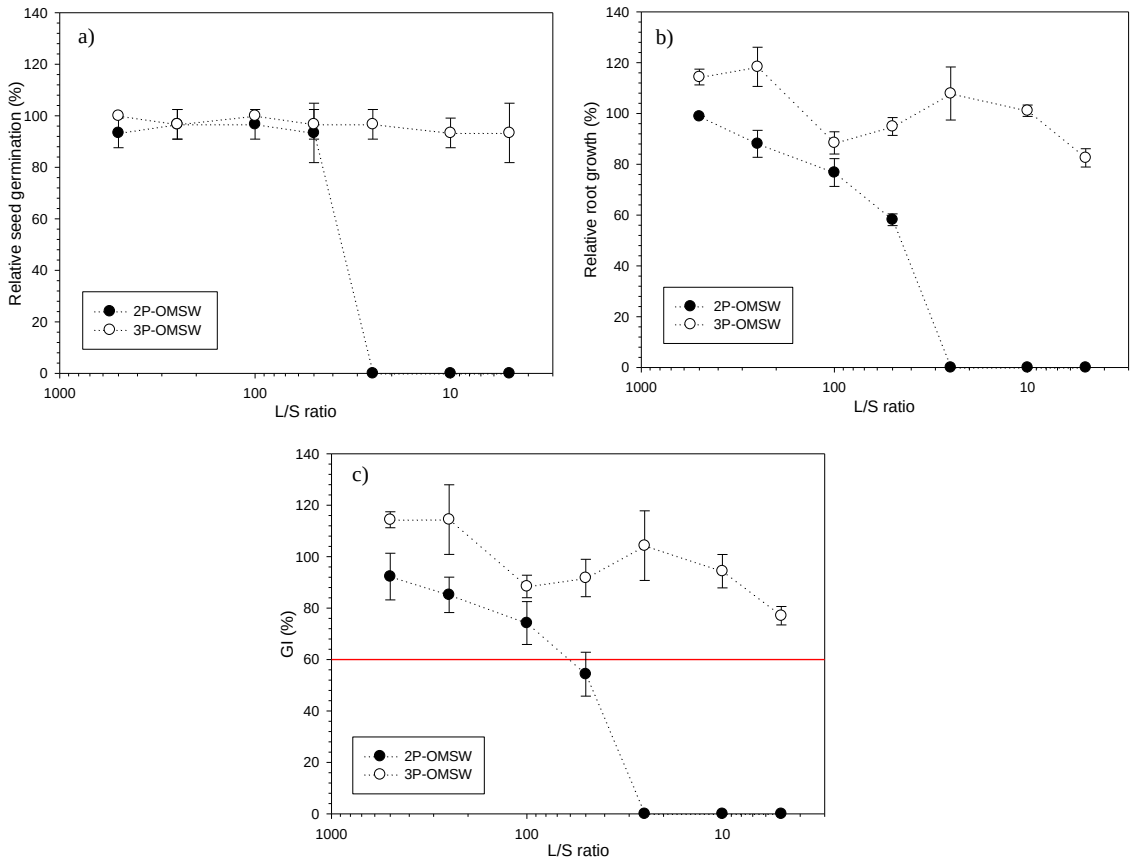


Fig. 5.1 Results of cress bioassays with 2P- and 3P-OMSW extracts of different L/S ratios. a) Relative seed germination (RSG); b) Relative root growth (RRG); c) Germination index (GI).

From Fig. 5.1 it is possible to conclude that 2P-OMSW, unequivocally, caused more harmful effects on cress than 3P-OMSW. Concerning to seed germination (Fig. 5.1 a)), 3P-OMSW did not affect this step, because for every tested L/S ratio, the percentage of germinated seeds rounded 100%, which means that it was identical to the control experiment with distilled water. However, for 2P-OMSW, 25 L/kg of waste were enough to completely inhibit cress germination. Until this concentration, RSG was also near 100%. However, as shown in Fig. 5.1 b), the root length of the germinated seeds tended to decrease as the L/S ratios decreased (i.e. as the amount of waste in the extracts

increased). In contrast, Fig. 5.1 b) also reveals that 3P-OMSW extracts may promote cress root growth. When this waste is highly diluted, i.e. L/S ratio higher than 250 L/kg, RRG is higher than 100%, which means that the grown roots are longer than those obtained in distilled water. This can be related with the nutrients available in the wastes that, at low concentrations, may promote the growth. For L/S ratios lower than this, it was not noticed a reduction of less than 20% of the blank.

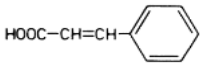
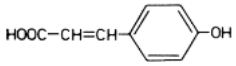
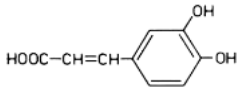
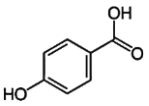
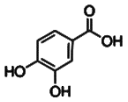
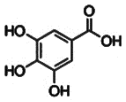
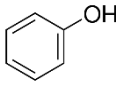
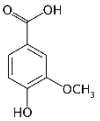
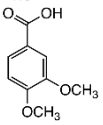
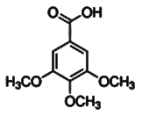
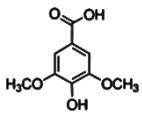
GI was calculated to conclude about the phytotoxic character of the wastes extracts. Fig. 5.1 c) shows that for L/S ratios of approximately 25 L/kg, 2P-OMSW is considered phytotoxic, because GI is equal or lower than 60% (Table 2.4). Nevertheless, 3P-OMSW appeared to be no phytotoxic, since GI is higher than 60% in the entire test range.

OMSW are complex matrixes so it is their phytotoxic character. Literature attributes to polyphenols present in OMSW the main responsibility for their phytotoxicity [1, 4, 5, 30]. In this work, results of total phenolic content of both 2P- and 3P-OMSW showed similar results, although cress bioassays demonstrated that 2P-OMSW is much more phytotoxic than 3P-OMSW, as reported in literature. This may indicate that there is a synergistic action between the phenolic fraction of OMSW and the matrix itself, in which other factors may contribute to its phytotoxicity, such as the presence of fatty acids, lipids and organic acids, pH, some nutrients concentration, salinity, and others [26]. This may be influenced by the different olive oil extraction process (i.e. partition coefficient, temperature, solubility, etc.), which has consequences in the composition of the wastes [4, 9]. In the following section, it is assessed the relation between some phenolic compounds and their individual phytotoxicity.

5.3 INFLUENCE OF PHENOLIC COMPOUNDS IN THE GERMINATION INDEX

To understand how the phenolic compounds identified in OMSW influence phytotoxicity (i.e. GI), they were tested individually through cress bioassays. Due to logistics issues and lack of time, only the compounds in stock in the lab were tested. These were grouped according to their chemical structure, in order to facilitate interpretation of the results, as presented in Table 5.2.

Table 5.2 Phenolic compounds tested in cress bioassays: group and chemical structure.

Group	Compound	Chemical structure
Cinnamic acids	Cinnamic acid	
	p-Coumaric acid	
	Caffeic acid	
Benzoic acids (-OH groups) and Phenol	4-Hydroxybenzoic acid	
	Protocatechuic acid	
	Gallic acid	
	Phenol	
Benzoic acids (-OH and -OCH_3 groups)	Vanillic acid	
	Veratric acid	
	3,4,5-Trimethoxybenzoic acid	
	Syringic acid	

From the ten studied compounds, three are derived from cinnamic acid, so belong to the category of cinnamic acids. In the second group, there are the tested benzoic acids (i.e. those derived from benzoic acid) only encompassing -OH groups, as well as phenol. At last, there is a group composed of the benzoic acids with one or more methoxy group.
The following Fig. 5.2 includes the results of cress bioassays with the cinnamic acids of Table 5.2 in a concentration range of 5-500 ppm.

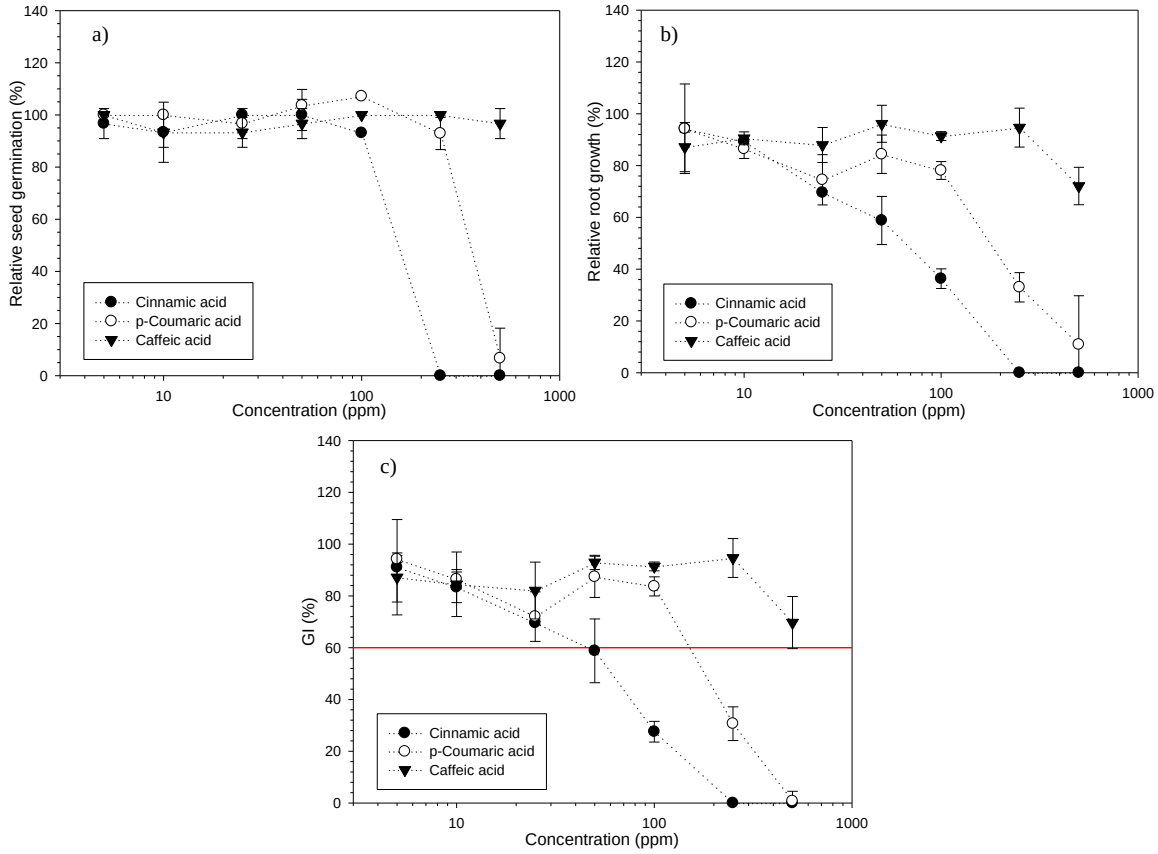


Fig. 5.2 Results of cress bioassays with solutions of three cinnamic acids at different concentrations: a) Relative seed germination (RSG); b) Relative root growth (RRG); c) Germination index (GI).

After performing cress bioassays with three cinnamic acids – cinnamic, *p*-coumaric and caffeic acid – the number of germinated seeds and their root lengths were measured, so it was possible to compute RSG and RRG, which are represented in Fig. 5.2 a) and b), respectively. Through Fig. 5.2 a), it is possible to conclude that within a concentration range of 5 to 500 ppm, caffeic acid was the compound with less influence on cress germination. The germination in caffeic acid solutions was always about 90%, which means that there was a reduction of 10% by comparison with the germination in the control experiments. It would be necessary to test this compound in a larger range of concentrations to see the concentration at which this compound begins to affect germination. In turn, *p*-coumaric acid showed a behavior similar to caffeic acid until 250 ppm. However, when it was tested a solution of 500 ppm, RSG fell to values near zero. Concerning to cinnamic acid, it was only possible to observe seed germination until 100 ppm.

Regarding Fig. 5.2 b), until 10 ppm, root length of the three compounds was identical between them, but around 10% lower than the control. From concentrations of 25 ppm, cinnamic, *p*-coumaric and caffeic acid started to exhibit different behaviors. Cinnamic

acid led to the worst results, as RRG had the sharpest decline of the three acids. For this one, at 100 ppm, RRG was only 40%, while for *p*-coumaric acid it was the double and for caffeic acid it was about 90%. Caffeic acid presented the most satisfactory results for cress root growth, since even at 500 ppm, the difference between the assays using this compound and distilled water (i.e. control) was less than 30%, approximately.

Finally, GI for these cinnamic acids at the studied concentrations are illustrated in Fig. 5.2 c). Caffeic acid revealed to be non-phytotoxic in the whole concentrations range studied. Cinnamic and *p*-coumaric acid are already phytotoxic at 50 and 250 ppm, respectively, because for this concentrations they led to values of GI lower than 60%.

Relating these results with the chemical structure of the tested compounds, it is evident that increasing the number of -OH substituents in the molecules is traduced in lower values of phytotoxicity.

Following the same approach, three benzoic acids (4-hydroxybenzoic, gallic and protocatechuic acid) were also individually studied – Fig. 5.3. For comparative purposes, phenol was also analyzed. In fact, this compound is largely studied in literature when simulating OMWW.

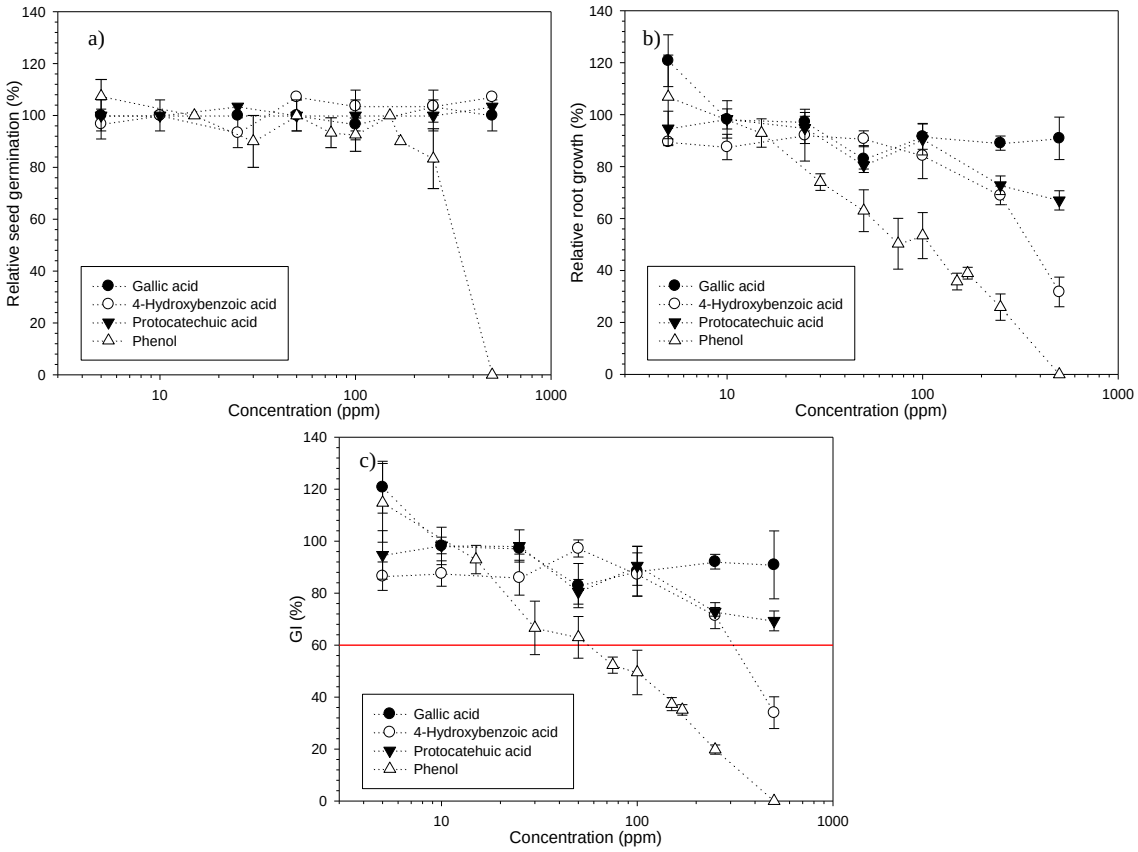


Fig. 5.3 Results of cress bioassays with solutions of three benzoic acids (-OH groups) and phenol at different concentrations: a) Relative seed germination (RSG); b) Relative root growth (RRG); c) Germination index (GI).

Concerning to the amount of germinated seeds, Fig. 5.3 a) shows that, excluding phenol, there are not significant differences between the other three tested compounds, since the percentage of germination compared to the distilled water control was around 100% in the whole concentration range. However, for concentrations higher than 150 ppm, phenol reduced RSG from 100% to approximately 80% and, for 500 ppm it was verified that none of the seeds had germinated in the phenol extract.

Moreover, in some cases, the tested substance seemed to benefit seed germination. In these cases, RSG is sometimes higher than 100% (control value), which means that there were more seeds germinating in the tested extracts than in the control experiments with water.

In Fig. 5.3 a), it is possible to conclude that, besides phenol at high concentrations, these compounds had not negative influence on cress germination. However, they influenced root length as Fig. 5.3 b) proves. It demonstrates that for these four compounds, the higher their concentration, the shorter their roots. The substance whose effects on root length were more visible was phenol. A solution of 75 ppm is enough to reduce cress roots in 50% compared to the control. While at this concentration, RRG remains between 80 and 90%. In the second position is 4-hydroxybenzoic acid which at 500 ppm exhibit a RRG percentage of about 30%. In contrast, protocatechuic acid improved root growth when applied at concentrations lower than 25 ppm and its lowest value of RRG was about 60% and it was achieved for 500 ppm. At last, gallic acid was the substance with less influence in cress root length, i.e. between 5 and 500 ppm, RRG floated between approximately 120 and 85%.

Fig. 5.3 c) shows the results of GI of these 4 phenolic compounds. It is possible to conclude that phenol was the most phytotoxic substance. Bearing in the mind that GI values lower than 60% traduce a phytotoxic behavior, for phenol this limit is achieved at concentration around 50 ppm. This limit was only achieved by phenol and 4-hydroxybenzoic acid, but for the second one it was necessary a solution with a concentration higher than 250 ppm, so it is only harmful for cress in limit situations of really high concentrations.

Regarding to gallic and protocatechuic acid, although none of them leads to values of GI lower than 60%, the second one seemed to reveal a mild inhibition to plant development (Table 2.4), for concentrations upper than 100 ppm. Through these experiments, gallic

acid was considered no phytotoxic because GI was always higher than 80% in the tested concentration range.

These results may be related with the chemical structure of these compounds. Linking information from Table 5.2 and Fig. 5.3, it can be concluded that phytotoxicity of benzoic acids are influenced by the number of -OH groups, i.e. the higher the number of -OH groups in the molecules structure, the higher its phytotoxicity. So, the ascending order of phytotoxicity of the three tested benzoic acids is gallic, protocatechuic and 4-hydroxybenzoic acid.

At last, phenol has no carboxyl group (-COOH), but only a hydroxyl group (-OH) connected to the benzoic ring. The lack of a carboxyl functional groups is what differs from all of the other tested compounds, specially comparing phenol with 4-hydroxybenzoic acid, so it may be related to its high phytotoxicity, since this substance was the most phytotoxic of these tests.

Finally, results of cress bioassays with four benzoic acids containing hydroxyl and methoxy groups (-OH and -OCH_3, respectively) are presented in Fig. 5.4.

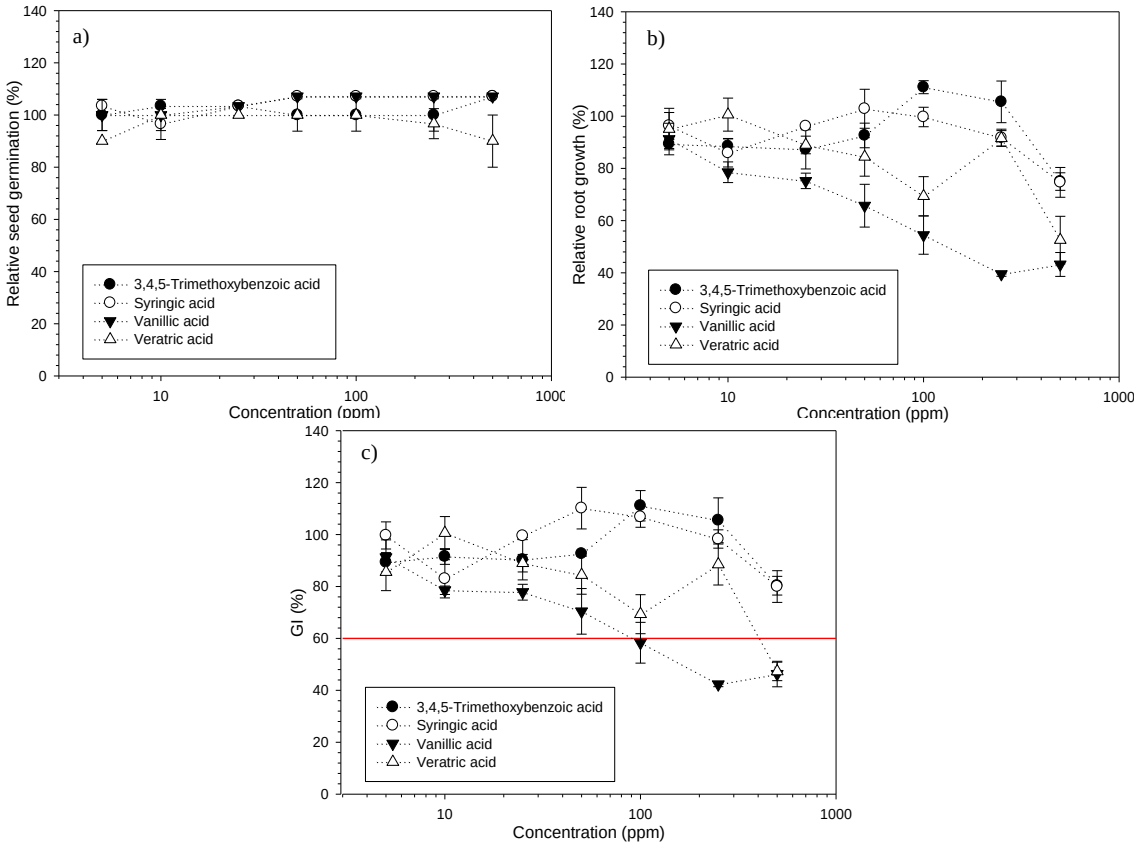


Fig. 5.4 Results of cress bioassays with solutions of four benzoic acids (-OH and -OCH_3 groups) at different concentrations: a) Relative seed germination (RSG); b) Relative root growth (RRG); c) Germination index (GI).

The tested -OCH_3 phenolic acids were: 3,4,5-trymethoxybenzoic, syringic, vanillic and veratric acid.

Through Fig. 5.4 a), one can verify that cress seeds had similar responses in terms of germination for all the four tested phenolic compounds. Syringic, vanillic and 3,4,5-trimethoxybenzoic acid led to RSG values equal or higher than 100% for the whole concentration range. For veratric acid, RSG varied between 80 and 100%.

In contrast, vanillic acid led to the worst results in terms of root growth. As shown in Fig. 5.4 b), RRG dropped to 50% when a 100 ppm solution was tested. This was also achieved for veratric acid but at a higher concentration (500 ppm). Syringic and 3,4,5-trimethoxybenzoic acid revealed more favorable results of root growth. Until 50 ppm, the first one exhibited higher values of RRG (between 100 and 90%), and after this concentration, the second one showed the higher values (upper 100%). At the highest concentration of 500 ppm, both showed similar results (approximately 75%).

The results of GI exhibited the same tendency. For a concentration of 100 ppm, vanillic acid was already phytotoxic because GI was lower than 60%. Concerning to veratric acid, it was necessary 500 ppm to achieve a phytotoxic effect. Syringic and 3,4,5-trimethoxybenzoic acid did not reach a phytotoxic level.

These four compounds have a carboxyl group linked to their benzoic ring as well as methoxy and hydroxyl groups. The most phytotoxic compound was vanillic acid which has one methoxy and one hydroxyl group. Then there is veratric acid which has two methoxy groups and none hydroxyl. Between 3,4,5-trimethoxybenzoic and syringic acid it is more difficult to stablish a relation between their phytotoxicity and their functional groups. However, at higher concentrations syringic acid revealed lower GI. This has one -OH group between two -OCH_3, while 3,4,5-trimethoxybenzoic acid has three -OCH_3 groups with no -OH, which is consistent with the results of vanillic and veratric acid. Thus, perhaps the connection is that the less -OCH_3 groups in these molecules, higher their phytotoxicity.

Polyphenols accumulation in plants arises from highly regulated processes, including cell, tissue, development and environment specific controls. In literature, phytotoxicity effect of phenolic compounds is related to their lipophilic character (i.e. solubility in a lipid medium). Thus, according to [57], it is possible to stablish a relation between the structure and phytotoxicity of these molecules, through their hydrophobicity. Lipophilic substances tend to be more phytotoxic because they have an easier passage through cell membranes. This pattern is shown in Fig. 5.2, Fig. 5.3 and Fig. 5.4, since phytotoxicity of hydroxyl

derivatives was lower than the more lipophilic compounds. A hydroxyl group is polar and therefore hydrophilic, while a carbon chain is nonpolar and hydrophobic (i.e. more lipophilic). This explains why the group of cinnamic acids was globally the most phytotoxic. Moreover, within this group, as the hydroxyl groups promote water affinity, it is consistent with the fact that cinnamic acid revealed to be the most phytotoxic tested compound, then *p*-coumaric and, at last, caffeic acid, since they have none, one and two -OH groups, respectively.

The same conclusion can be obtained for benzoic acids. The more hydroxyl groups they have, the less phytotoxic they showed to be, since 4-hydroxybenzoic acid with only one group -OH was the most phytotoxic and, in contrast, gallic acid, having three -OH groups, was the less.

Concerning to benzoic acids with -OH and -OCH_3 groups, to find a relation between their chemical structure and phytotoxicity is more difficult, because some of the tested compounds have both -OH and -OCH_3 substituents. In general, hydroxyl and methoxy groups have promoting and inhibitory effects, respectively, on plants development, although phytotoxicity is also influenced by the number and position of these substituents in the aromatic ring [58]. In general, a substituent in ortho position in phenolic molecules decreases its toxicity and meta substitutions increase toxic action [59]. Although, in this study, linking their chemical structure (Table 5.2) with the results of GI, it is visible that the compounds with less substituent groups (vanillic and veratric acid) are more phytotoxic than those with more -OCH_3 groups.

Finnaly, phenol was the second more phytotoxic compound (after cinnamic acid). As it has been discussed, the extent and nature of the hydroxylation pattern of the aromatic ring are main responsible. In this case, phenol has only one -OH group connected to the benzene ring and, in contrast with the other tested compounds, no other substituent group, while cinnamic acid has no -OH group but a carbon chain, which as it has been discussed induces higher phytotoxicity.

In conclusion, phenolic compounds toxicity is mainly related with the hydrophobicity of the individual compounds. It affects the solubility, enhancing the interaction of the compound with specific plant structures. The strength of phytotoxicity influence of a certain phenolic compound also stems from position of the substituent [59].

Cress bioassays with individual phenolic compounds present in OMSW allowed identifying which induce higher inhibition to plant germination. However, their single effect may be different from when they are together. So that, it was tested a solution

consisting of six of the ten tested compounds to evaluate if there were synergistic effects. This solution was composed of 3,4,5-trimethoxybenzoic, 4-hydroxybenzoic, protocatechuic, syringic, vanillic and veratric acid and it was tested at 5, 10, 25, 50, 75 and 100 ppm. This solution was selected since several works were developed using it to simulate the phenolic character of OMWW [32, 60, 61]. In addition, for each concentration, it was estimated the expected values of RSG, RRG and GI from the results obtained for this six substances individually, and then compared with those of a solution that combined them. Results are presented in Fig. 5.5.

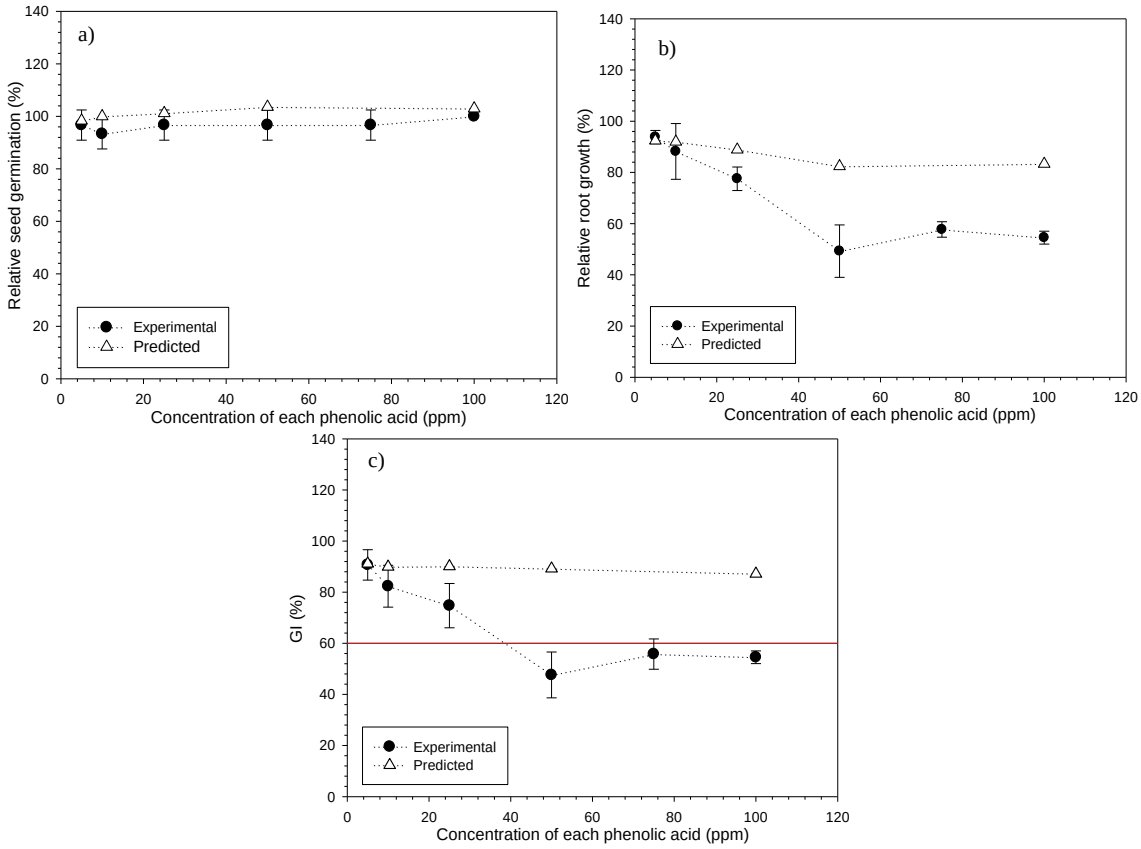


Fig. 5.5 Results of cress bioassays with a synthetic effluent at different concentrations and prediction based on the results of cress bioassays with individual compounds: a) Relative seed germination (RSG); b) Relative root growth (RRG); c) Germination index (GI).

Analyzing Fig. 5.5, it is unmistakable that a combination of phenolic compounds causes synergistic effects. If their individual effects remained when they were brought together, the results would be much more favorable for cress germination and root growth.

Concerning to the percentage of germinated seeds by comparison with the control (Fig. 5.5 a)), there was not a great difference between the tested solution and the expected results if the compounds kept their individual phytotoxic properties when put together.

On the other side, in Fig. 5.5 b) the expected values of RRG were significantly higher than the predicted ones. This means that, as there were synergistic interactions between the phenolic compounds, their combined effect is much harmful to root growth than if there was no interaction. If no synergistic effect existed, root growth would be around 90% in this concentration range. However, a solution combining these six compounds led to much lower values, truly influenced by its concentration. The higher the concentration, the smaller is cress root length at the end of the experiments. However, it barely reaches a decrease of 50% of the control contrasting to what happened for some of the compounds.

Also GI is highly influenced by synergistic relations between the compounds. As it is shown in Fig. 5.5 c), the synthetic effluent revealed to be phytotoxic for concentrations higher than 25 ppm, while the prediction would be no phytotoxicity in the whole concentration range, since, as it is shown in Fig. 5.2 c), Fig. 5.3 c) and Fig. 5.4 c), at 100 ppm only cinnamic acid, vanillic acid and phenol were toxic, and only vanillic was used in the synthetic effluent.

These synergistic effect has already been reported by various authors, mostly related with plants allelopathy or with phenolic compounds antioxidant activity [6, 15, 57, 60–62]. These experiments allow concluding that it is difficult to find the main responsible phenolic compound for OMW phytotoxicity, since their action is a synergy, in which other parameters should be considered. Bioactive intermediate compounds derived from the transformation of phenolics may also be toxic, as well as organic compounds like fatty acids, lipids, or even pH and the osmotic stress caused by the presence of high Na^+ and Cl^- concentrations may play an important role in OMW acute phytotoxicity [6].

5.4 GROWTH TESTS

Growth tests were performed during two weeks using garden cress to infer about long term effect of OMSW. Both 2P- and 3P-OMSW were tested in different volumetric proportions of waste/soil. The properties of the soil are in Fig. C.1.

These experiments were recorded in pictures, as shown in Fig. 5.6.

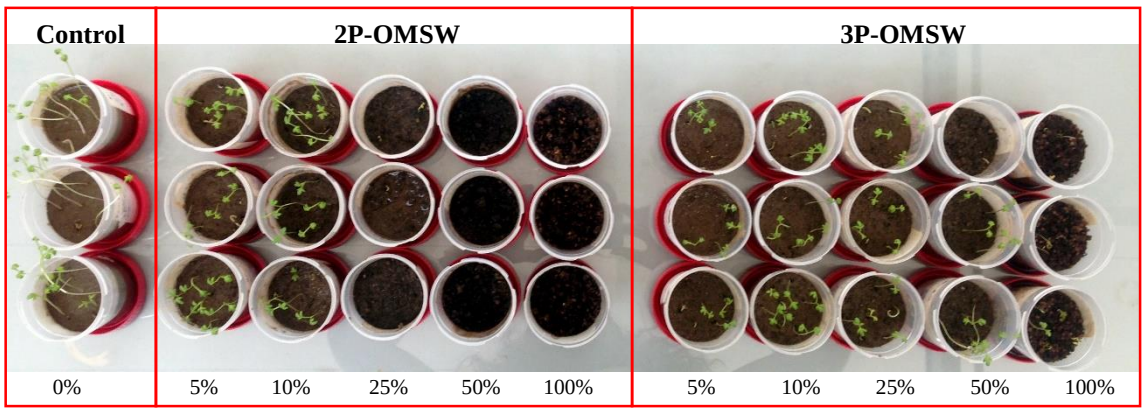


Fig. 5.6 Design of growth test experiments.

At the end of the experiments, number of survival seedlings, height, number of leaves and biomass dry weight were measured. Results are represented in Fig. 5.7 and they were also subjected to an ANOVA analysis.

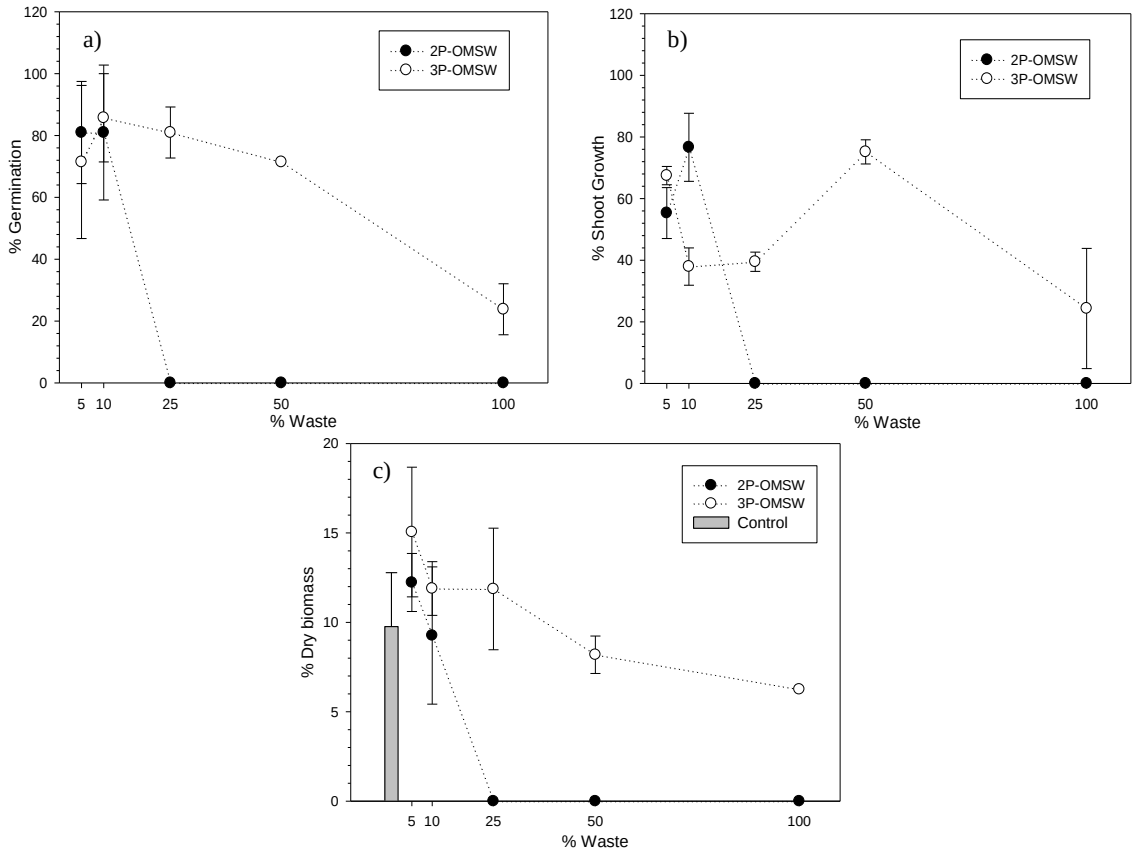


Fig. 5.7 Results of growth tests in mixtures of waste and proper soil: a) percentage of germinated seeds; b) percentage of shoots growth; c) percentage of dry biomass.

The results of germination and growth (Fig. 5.7 a) and b)) are given in percentage of the control (trial with only soil). Thus, it is evident that cress seeds responded more negatively to 2P-OMSW than 3P-OMSW. In terms of germination, even at small volumetric percentage of 2P-OMSW in the pots (5%), germination was 20% lower than the control. In the pots of 25% of waste, or more, no germination occurred. However, cress showed to be capable of germinating in every trial with 3P-OMSW. In this residue, the higher the percentage of waste in the pots, the lower the cress germination. The results obtained for the wastes showed to be statistically different (*p*-value of 6.58×10^{-8} and 4.21×10^{-4} for 2P- and 3P-OMSW, respectively). It should be referred that every shoot in this test had two leaves.

For 2P-OMSW, 10% of waste led to the higher heights of the shoots, while in the 3P-OMSW experiments, the same was recorded at 50% of waste. In both cases the maximum shoot growth was about 75%, which means that it was 25% lower than the control. In the blank experiments, the mean shoot length was 46 ± 14 mm. Regarding these results, null hypothesis had to be rejected and results were considered statistically different since *p* was lower than 0.05 for 2P- and 3P-OMSW (5.68×10^{-11} and 5.43×10^{-6}, respectively).

Measurements of shoots dry weight showed to be reverse of percentage of growth. Comparing Fig. 5.7 b) and c), for taller shoots, percentage of dry biomass tended to be lower. Although the results of percentage of dry weight in the experiments within 2P-OMSW showed to be statistically different (*p* equal to 9.56×10^{-6}), 3P-OMSW trials revealed to be identical, because *p* was 0.12, so higher than 0.05.

These results support the fact that 2P-OMSW is more suitable of causing harmful effects to plants than 3P-OMSW. Besides the results of shoots dry weight, this test may also indicate that even germination and growth be possible, at higher concentrations of waste, shoots seemed to have an abnormal development, since they started to look more fragile and also changes in color were evident. If the test had been extended, they would probably not survive.

Literature usually refers to OMSW application in soils of olive trees cultivation. In [53] it is described the used of 2P-OMSW (without any treatment) as soil amendment for olive trees, during five years. In contrast to the present work, it was verified that the direct application of 2P-OMSW, raw and de-oiled, improved the properties of the soil, by increasing, for example, the aggregate stability and organic matter content, total N and available K and P and had a positive effect on olive yield. Thus, direct application of raw

or de-oiled 2P-OMSW can be a way to add organic C to typical Mediterranean soils, which have been continually degraded and, consequently, improve plant production.

Concerning to direct application of 3P-OMSW in soils, also positive effects were registered in literature. It is reported to increase moisture retention in the amended soils by increasing amount of organic matter, and thus minimizing water deficit effects in Mediterranean soils [65].

Therefore, the non-satisfactory results for the direct application of 2P- and 3P-OMSW may be related with several aspects. Experiments were performed using seeds, while in the referred studies, wastes are disposed in high plants cultivation fields, so in the present work phytotoxicity may have influenced seed germination. In addition, it was used a very sensitive species, whose germination process was perhaps influenced not only by wastes phytotoxicity but also by other factors, such as, light, temperature, moisten, soil nutrients, etc. Nevertheless, each species has different behavior when subjected to toxic stress. Thus, soil application of wastes must be carefully controlled.

5.5 DETOXIFICATION OF OMSW

In order to test a method of detoxification of OMSW, Fenton's peroxidation process was applied to 2P-, 3P-OMSW and synthetic effluent.

The results showed that the used system was not appropriate for this aim. Concerning to 2P- and 3P-OMSW, it did not lead to the reducing of phytotoxicity. Instead, it originated a more phytotoxic product at the end of the treatment, in which none of the seeds germinated at all (i.e. GI was zero). Fig. 5.8 illustrate the appearance of cress bioassays on both residues after treatment.

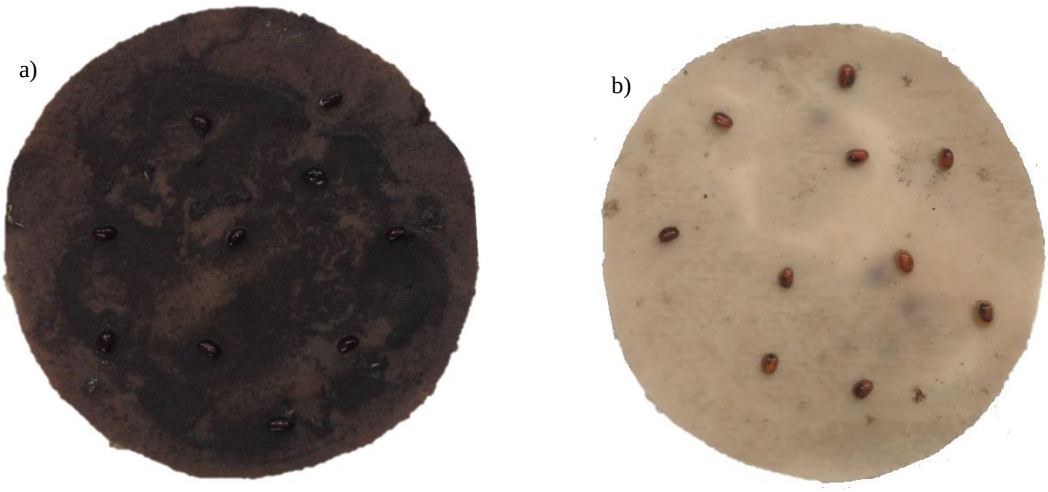


Fig. 5.8 Cress bioassays after chemical treatment: a) 2P-OMSW; b) 3P-OMSW.

As shown in Fig. 5.8, after the 48 h incubation of cress bioassays, none of the seeds had germinated. It was also verified that they assumed a dark color.

This chemical treatment was only effective on the liquid effluent, as results in Table 5.3 show.

Table 5.3 Results of cress bioassays for the synthetic effluent at 100 ppm, before and after Fenton's process.

	Before Fenton's	**After Fenton's**
RSG (%)	100.0 ± 0.0	93.3 ± 5.8
RRG (%)	54.5 ± 2.5	68.7 ± 0.8
GI (%)	54.5 ± 2.5	64.1 ± 4.2

For the synthetic effluent, Fenton's process revealed to be a good alternative of detoxification, since GI increased about 10%, although the final result indicated that the material continued phytotoxic (i.e. GI higher than 60%).

This results are consistent with literature. The possibility of using Fenton's process has an effective option of reducing OMWW phytotoxicity is been widely studied. However, this methodology needs to be combined with other treatment options to be capable of reducing OMWW phytotoxicity, since these effluents are characterized by high pollutant load. Fenton's has been claimed as part of integrated treatment for degradation of these agro-industrial effluents. There are several options: Fenton oxidation under low pH followed by coagulation under high pH for decolourization of OMWW [4]; it can be applied as a pretreatment before a biological process [2]; photo-Fenton after a pretreatment to remove suspended solids that would obstruct light from entering the liquid [66]; or even the combination of chemical oxidation process (Fenton's and ozonation) and their consecutive treatment with aerobic microorganisms [4].

Regarding the application of Fenton's chemical treatment to 2P- and 3P-OMSW, the negative results are also consistence with literature. The addition of chemicals to the initial matrix led to a final material whose properties were not appropriate for seed germination. Chemical remediation of soils contaminated with organic pollutants is being pointed out as a good solution, however, the knowledge on its ecotoxicological impact on plants is still scarce [50]. According to [50], after both Fenton's and nanoremediation processes, the treated mediums were more phytotoxic than initially. Concerning to Fenton's oxidation, the phytotoxic effects may be related with various factors: the production of free radicals (e.g. $HO^{\bullet}$ and other oxygen species) and oxidizing species (ferryl ions) that could promote oxidative stress in crop plant organs; the precipitation of

iron oxides; and/or the formation of intermediates of contaminants. Also the deposition of iron species can inhibit water and nutrients uptake, which causes the decrease in seed germination. Moreover, the reactants themselves showed toxic effects that can be justified by the combination of different factors, such as the production of free radicals ($HO^{\bullet}$) and the precipitation of iron oxides [50].

As it has been referred in this work, extraction of residual oil from 3P-OMSW and consecutive energy recovery still the most used management option for this solid waste [31]. In [67], six alternatives of OMSW management were found and compared using a multi-criteria decision making process. The criteria used for comparison were environmental effects, economical benefits and the technology involved. Thus, co-combustion was the winner option.

In turn, to find an environmentally friendly and economic management option for 2P-OMSW is still a challenging task. Nowadays, this residue is mostly disposed on soil as amendment, both raw and after composting. Indeed, composting or co-composting of 2P-OMSW is a very suitable option because it allows producing mature compost free from phytotoxicity and with high organic matter and nutrient content and with a positive economic impact. Olive mill owners can compost their season's waste production and produce a high quality soil amendment that can then be sold [34].

In fact, its use as soil amendment is Mediterranean areas is a valid alternative, because in this region, soils have frequently problem of organic matter lack and active desertification processes. Thus, this is a useful solution for both sustainable utilization of olive mill by-products and soil fertility conservation [31].

Diluting until no phytotoxic effect is noticed is also an option, although it requires large amounts of water [5].

6 CONCLUSIONS AND FUTURE WORK

The main objective of this work was to evaluate phytotoxicity of olive mill solid wastes from 2- and 3-phase centrifugation processes. The role of phenolic compounds in their phytotoxicity was also analyzed.

Phytotoxicity was assessed through cress bioassays and expressed as germination index (GI).

Cress bioassays revealed that 2P-OMSW was clearly more phytotoxic than 3P-OMSW.

Ten phenolic compounds were individually tested in cress bioassays. Results showed that their chemical structure influence their phytotoxic character, showing that their hydrophobicity is the main responsible factor. It was also verified that the combination of different phenolic compounds promoted synergistic effects which led to a increasing phytotoxicity. Due to this synergy, it is difficult to identify the principal phytotoxic compound present in OMW.

Growth test allowed testing 2P- and 3P-OMSW as soil amendments without any detoxification treatment. Globally, 3P-OMSW had more positive results than 2P-OMSW in the three selected parameters (germination, growth and dry weight). Within each waste trials, ANOVA revealed that the results were statistically different (p-value lower than 0.05), except the results of percentage of dry biomass in 3P-OMSW trials. In general, these results were worse than the results obtained in the control, revealing that cress germination and early shoots growth did not react well to these wastes. Some external conditions may also had influenced cress responses, so a different approach or design should be tested, because in literature some studies obtain really satisfactory results in crops growth and nutrients content by adding these wastes to cultivation soils. Nevertheless, different species were used probably less sensitive than *Lepidium sativum*.

As the phenolic content of OMSW was the main subject in this work, a detoxification treatment was performed to both wastes, in order to reduce their phytotoxicity. The chosen method was Fenton's oxidation process, which showed not to be a good option, since it originated an even more phytotoxic matrix. The same treatment was applied to a synthetic effluent composed of six phenolic compounds in an aqueous extract. In this case, Fenton's peroxidation reduced the effluent phytotoxicity, although for values of GI higher than 60% (phytotoxicity limit).

Globally, this work confirms that OMSW are complex matrixes, whose properties cause phytotoxic effects. As reported in literature, phenolic compounds play an important role

as phytotoxins, although other synergistic factors may also be involved. Nevertheless, the lipophilic character of these wastes is highly correlated with their phytotoxicity.

Using the 3P-OMSW as solid fuel after de-oiling may be the best option, which cannot be applied to the 2P-OMSW, because of its physic and chemical properties. For this residue, composting seems a good environmental friendly and economic option or simply diluting until no phytotoxic effects are noticed.

The suggestions for future works are testing different detoxification methods capable of reducing phytotoxicity and/or improving Fenton's process for semi-solid basis; and changing the experiments for testing the possibility of using OMSW as soil amendments, for example, to use a more resistant, but still sensitive, species and/or applying the wastes after the plant achieve a certain level of robustness.

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APPENDIX A – OUTLIERS ANALYSIS

The verification of the existence of outliers in the results of cress bioassays was made through box plots, which are represented in Fig. A.1. These diagrams show data central tendency and the variability of the observation around the minimum and maximum factors and the values of the 1st, 2nd and 3rd quartiles (i.e. Q_1, Q_2 and Q_3, respectively). Thus, they allow to identify the values that can be statistically considered outliers or extremes. As it is possible to evaluate in Fig. A.1, in a box plot the length of the box is L, which is equal to $Q_3 - Q_1$. It is considered an outlier, any nonconforming result that is significantly distant from the other observations ($Q_1 \pm 1.5L$). When there is an even more extreme situation, it is considered an extreme value ($Q_1 \pm 3L$) [68].

The outliers and extremes identified in this work were eliminated from the calculations, because they would have affected the statistical parameters and final conclusions.

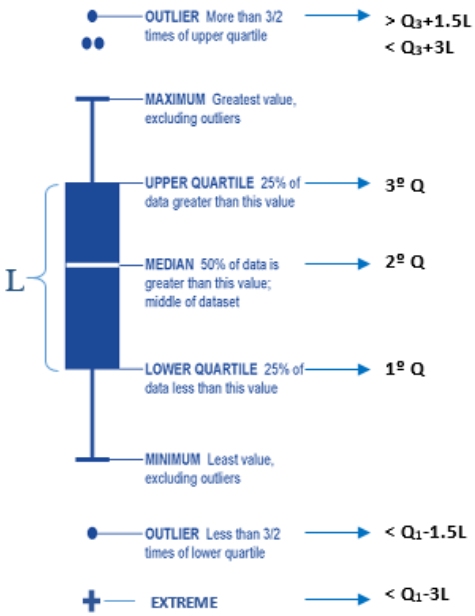

Fig. A.1 Schematic description of a box plot.

APPENDIX B – ANOVA ANALYSIS

The analysis of variance performed in this work aimed to evaluate the results of different treatments with one factor – one-way ANOVA, so it was possible to compare their mean values, as showed in Table B.1.

This statistical process uses a hypothesis test methodology that admits a null hypothesis (H_0) and an alternative hypothesis (H_1):

$$H_0: \quad \bar{y}_1 - \mu_1 = \bar{y}_2 - \mu_2 = \cdots = \bar{y}_M - \mu_M = 0 \tag{B.1}$$

$$H_1: \quad \bar{y}_M - \mu_M \neq 0, \text{ for at least one } M \tag{B.2}$$

According to Eqs. (B.*1)* and (B.*2)*, M is the number of levels with only one factor (number of samples or treatments), $\bar{y}$ is the mean of the observations and μ is the real mean with a confidence of $(100-\propto)\%$ [68].

Table B.1 Empiric results with one factor [68].

Treatment	**Observations**				**Mean**
	1	2	…	n	
1	y_{11}	y_{12}	…	y_{1n}	$\bar{y}_1$
2	y_{21}	y_{22}	…	y_{2n}	$\bar{y}_2$
…	…	…	…	…	…
M	y_{M1}	y_{M2}	…	y_{Mn}	$\bar{y}_M$

Therefore, it is possible to obtain a p-value to the required significance level, $\propto$. In this work, it was established a confidence level of 95%, so $\propto$ is 0.05. It is also necessary to assume that each group of observations comes from a population with a normal distribution. Thus, if it is obtained a p higher than $\propto$, it must be concluded that H_0 is true and the results are equal (with 95% of confidence). In contrast, if p is lower than $\propto$, they are statistically different [68].

ANOVA can be examined through a summary table as Table B.2, where it is presented the results of the sum of squares (SS), degrees of freedom (df), mean square (MS) and F_0, that are necessary to obtain p-value, and also F_{critic}, which is a table value (F-Fischer distribution).

Table B.2 One-way ANOVA summary table.

Variation source	**Sum of squares (SS)**	**Degrees of freedom (df)**	**Mean square (MS)**	F_0	p	F_{critic}
Between treatments	$SS_{Treatment}$	$M-1$	$MS_{Treatment}$	$MS_{Treatment}/MS_{Error}$	p value	F_{critic} value
Between observations	SS_{Error}	$N-M$	MS_{Error}			
Total	SS_{Total}	$N-1$				

In turn, SS values can be computed by Eqs. (B.3), (B.4) and (B.5),

$$SS_{Total} = \sum_{i=1}^{M}\sum_{j=1}^{n} {y_{ij}}^2 - \left[\sum_{i=1}^{M}\sum_{j=1}^{n} y_{ij}\right]^2 \Big/ N \tag{B.3}$$

$$SS_{Treatment} = \sum_{i=1}^{M}\left[\sum_{j=1}^{n} y_{ij}\right]^2 \Big/ n - \left[\sum_{i=1}^{M}\sum_{j=1}^{n} y_{ij}\right]^2 \Big/ N \tag{B.4}$$

$$SS_{Error} = SS_{Total} - SS_{Treatment} \tag{B.5}$$

where y_{ij} is the empiric value of treatment i and observation j; n is the number of observations per treatment and N is the total number of samples ($N = M \times n$) [68].

APPENDIX C – SOIL CHARACTERIZATION

INSTITUTO POLITÉCNICO DE COIMBRA
ESCOLA SUPERIOR AGRÁRIA

Laboratório de Solos e Fertilidade

Serviço / Nome do Interessado:	Escola Superior Agrária de Coimbra/ Laboratório de Solos e Fertilidade		
Morada:	Bencanta		
Localidade:	Coimbra	**Código Postal:**	3045-601
Propriedade:	Caldeirão - AB	**Área (ha):**	
Cultura:	Hortícolas	**Prof. (cm)**	0-40

Relatório de Análise de Solo

Data de Entrada: 20-10-2015 **Data de Saída:** 25-11-2015

		Nº Laboratório	**50094**			
Parâmetros			Caldeirão			
Textura de campo			**Média**			
Terra fina (ø< 2mm)	%	80,78				
Mat. orgânica	%	2,02	**Baixa**			
pH (H_2O)		6,4	**Pouco Ácido**			
pH (KCl)		-				
Condutividade Eléct	mS cm^{-1}	0,18	**Baixo**			
P_2O_5	mg P_2O_5 $100g^{-1}$	0,56	**Baixo**			
K_2O	mg K_2O $100g^{-1}$	2,10	**Médio**			
CaO	mg CaO $100g^{-1}$	6,02	**Baixo**			
MgO	mg MgO $100g^{-1}$	0,66	**Baixo**			
Na	mg Na 1000g	-				
Calcário Activo	%	-				
Cloretos	me Cl^- $100g^{-1}$	-				
Potássio	me K^+ $100g^{-1}$	-				
Sódio	me Na^+ $100g^{-1}$	-				
Cálcio	me Ca^{2+} $100g^{-1}$	-				
Magnésio	me Mg^{2+} $100g^{-1}$	-				
Cobre extraível	mg Cu kg^{-1}	1,17	**Médio**			
Zinco extraível	mg Zn kg^{-1}	2,90	**Médio**			
Ferro extraível	mg Fe kg^{-1}	88,30	**Muito Alto**			
Manganês extraível	mg Mn kg^{-1}	13,48	**Baixo**			
Azoto mineral	mg $N\text{-}NO_3^-$ kg^{-1}	-				
	mg $N\text{-}NH_4^+$ kg^{-1}	-				
Azoto Kjeldahl	%					
			Valores-limite *	**Valores-limite ***	**Valores-limite ***	**Valores-limite ***
Cádmio total	mg Cd kg^{-1}	-	1	-	-	-
Chumbo total	mg Pb kg^{-1}	-	70	-	-	-
Cobre total	mg Cu kg^{-1}	-	50	-	-	-
Crómio total	mg Cr kg^{-1}	-	60	-	-	-
Mercúrio total	mg Hg kg^{-1}	-	0,5	-	-	-
Níquel total	mg Ni kg^{-1}	-	50	-	-	-
Zinco total	mg Zn kg^{-1}	-	150	-	-	-

Observações: * Valores-limite da concentração de metais pesados nos solos segundo Decreto-Lei n.º 103/2015

O Responsável

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Fig. C.1 Characterization report of the soil used in the growth tests.