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Effect of a New Thermal Treatment in Combination with Saprobic Fungal Incubation on the Phytotoxicity Level of Alperujo

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ABSTRACT: Byproducts generated from food industries, such as olive oil mills, have been studied to decrease harmful pollution and their environmental consequences. In this work, a new thermal pretreatment and saprobic fungal incubation to detoxify alperujo (two-phase olive mill waste) have been evaluated in view of its use as fertilizer in agriculture. The sequential use of both methods simplifies the thermal conditions and incubation times of the fungal treatment. Optimization of the thermal treatment from 150 to 170 °C for 45 and 15 min, respectively, reduced the incubation time with *Coriolpsis rigida* from 20 to 10 weeks needed to reduce phytotoxic effects on tomato plants. Therefore, the combination of thermal and biological treatments will allow the development of the potential benefits of alperujo to improve nutrients in agricultural soil.

KEYWORDS: saprobic fungi, thermal treatment, alperujo, fertilizer, detoxification, phenols

INTRODUCTION

Spain is the largest producer of olive oil in the world (30% of the world's production) and, thus, the biggest producer of olive mill waste. The two-phase system used for olive oil extraction generates a liquid phase (oil) and a semisolid waste with a high percentage of moisture (65−70%) called alperujo. Approximately 4−5 million tons of alperujo per year are produced in Spain as hazardous waste,[1] which necessitates waste reduction and environmentally friendly ways to dispose of it. Currently, alperujo is used as biomass for energy generation, where the major technical problems are high moisture and organic contents.[2] However, alperujo could be used as fertilizer due to its high organic and mineral contents, but, like the majority of plant byproducts, alperujo is phytotoxic.[3] One of the most promising studies on alperujo technologies is its biological degradation with white rot fungi. However, the effectiveness of this treatment is not always satisfactory, particularly because it is time-consuming.[4]

Considerable efforts to decrease the phytotoxicity of dry olive mill residue by different biological, chemical, physical, and physicochemical treatment methods have been made.[5−7] Nevertheless, none of these individual approaches appears to be a general solution for reducing these contaminants.[8] However, with a combination of physical and biological treatments, the use of this agrowaste as an organic amendment in agricultural soil may be possible.[9] The possibility of enhancing the removal of biorecalcitrant phenols from olive mill wastewaters by pretreatment has been described.[10,11] No studies to diminish the phenol content to enhance further biological treatments have been performed. It would be useful to develop a pretreatment for alperujo to extract components present in the residue that can potentially be exploited and to generate a residue that is more accessible to biological methods.

It is known that bioactive molecules, such as antioxidant phenols and oligosaccharides, and interesting compounds such as sugars, functional oil, proteins or cellulose make alperujo a rich source of valuable components.[12] Alperujo must be pretreated to separate the phases (liquid and solid) and solubilize the main bioactive compounds into the liquid phases (oil and aqueous). Several treatments have been developed for bioactive molecule extraction, including extraction using static−dynamic superheated liquids[13] or a hot-water reactor at high pressure.[14] Despite these studies, only a new thermal treatment with direct steam has recently been scaled up to the industrial sector.[15] After the steam treatment, an easy solid−liquid separation is obtained, producing a liquid rich in phenols and sugars extracted from the solid and a final solid rich in cellulose and proteins but poor in phenols.[16] The advantages for the final solid are the concentration of cellulose and protein and the partial detoxification due to the removal of the phenols. Furthermore, concentrated cellulose is more accessible to enzymatic processes than untreated cellulose.[17] Bioprocessing allows the use of the final solid as a natural source of fertilizer for agronomic purposes.

The aim of this study is to decrease the phytotoxicity of alperujo residue by the combination of steam treatment as a physical treatment and incubation with saprobic fungi as a biological treatment. The combination of steam treatment with specific fungi could lead to reduced incubation times and reduced severity of the thermal treatment and provide an industrially viable sequential processing method.

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MATERIALS AND METHODS

Materials. Two samples of alperujo, a wet solid waste from two-phase decanters (sample I from the 'Arbequina' variety and sample II from the 'Marteña' variety), were collected directly from a virgin oil mill (Almazara Experimental, Instituto de la Grasa, Sevilla, Spain). Samples were immediately treated in the steam reactor, and aliquots of treated and untreated alperujo were stored at −20 °C until analysis.

Alperujo samples were dried and treated with solvent for oil extraction. Their initial moisture content was increased by adding up to 25% deionized water before fungal incubation. The two saprophytic fungi were *Coriolopsis rigida* LPSC strain 232 (CECT 20449) and *Fusarium oxysporum* BAFC culture 738, which were obtained from the culture collection of the Centro de Investigaciones Biológicas (CIB, Madrid) and the University of Buenos Aires (Argentina), respectively. Stock cultures were kept at 4 °C on 2% malt extract agar.

The soil was a gray loam obtained from the field of the Estación Experimental del Zaidín (Granada, Spain). The soil had a pH of 8.1 in a 1:1 soil/water ratio. The P, N, K, Fe, Mn, Cu, and Zn concentrations were determined according to methods from Lachica et al.[18] using H_2SO_4/H_2O_2 as extractants. $NaHCO_3$-extractable P was 6.2 mg/kg, N was 2.5 mg/kg, K was 132 mg/kg, Fe was 9.6 mg/kg, Mn was 110 mg/kg, Cu was 5.8 mg/kg, and Zn was 5.7 mg/kg. The soil texture was 358 g/kg sand, 436 g/kg silt, 205 g/kg clay, and 18 g/kg organic matter. Tomato (*Lycopersicon esculentum* L.) was used as a test plant.

Steam Treatment and Separation. The hydrothermal treatments were performed in a newly patented pilot reactor[15] (Spanish patent request P201031236) on a pilot plant unit. The system had a 100 L capacity and was equipped with a stainless steel reactor chamber that can operate at temperatures between 50 and 190 °C and a maximum pressure of 1.2 MPa. Approximately 20−30 kg of alperujo at 65−70% humidity was loaded in each treatment. Temperatures of 150 and 170 °C for 15−90 min were used for the thermal treatment. The treated material was centrifuged at 4700*g* in a semi-industrial machine with a maximal operating capacity of 20 kg (RTL-4BD, Comteifa, S.L., Barcelona, Spain).

The solid−liquid separation after the thermal treatment was evaluated as easy, medium, or difficult depending on the total time required for the filtration or centrifugation phases, and easy and medium separations were considered to be industrially viable. Some of the treated samples were washed with water during centrifugation to evaluate the effect of washing. The treated samples (with or without washing) were subjected to the incubation assays.

Chemical Composition. The solid loss after treatment was quantified as the final solid compared to the initial solid dry matter. Solid samples were dried in an oven at 60 °C until a constant weight was reached and then overnight on a vacuum desiccator. The crude fat content was determined using the traditional method of extraction in a Soxhlet with *n*-hexane for 6 h.

The neutral sugar composition was determined with (total) or without (monomeric) hydrolysis with 2 N trifluoroacetic acid (TFA) at 121 °C for 1 h[19] and measured by HPLC analysis. The method was described by Gomis et al.[20] for the simultaneous determination of aldoses and uronic acids that were previously derivatized with *p*-aminobenzoic ethyl ester (ABEE). HPLC analysis was performed using a Shimadzu SCL-10A (Shimadzu Corp., Kyoto, Japan) with an ultraviolet−visible detector. An Ultrabase C8 column (250 × 4.6 mm i.d.; particle size = 5 μm) (Análisis Vínico S.L., Spain) was used at 40 °C. The elution flow rate was 0.5 mL min^{-1}, and 88% tetrahydrofuran in sodium citrate buffer (0.1 M, pH 5.5) and acetonitrile were used as the mobile phases. Chromatograms were recorded at 307 nm.

The quantities of representative phenols hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4-β-D-glucoside (HTG), and tyrosol (Ty) were determined by HPLC analysis. Previously, two analytical thermal treatments were also used for phenol quantification. A "steam explosion" treatment in a flash-hydrolysis laboratory pilot unit designed and installed in the Instituto de la Grasa (Seville, Spain), equipped with a 2 L stainless steel reactor with a maximum operating pressure of 4.2 MPa at 230 °C for 5 min with sulfuric acid (2.5% v/v), was used for the determination of the maximum concentration of HT and Ty, as in previous studies.[12] The samples were also treated at 90 °C for 2 h to obtain the maximum concentration of soluble DHPG.[21]

An aliquot from the water-soluble fraction was filtered with a 0.45 μm membrane for the direct HPLC determination of HT, DHPG, HTG, and Ty. HPLC analysis was carried out using a Hewlett-Packard series 1100 liquid chromatography system equipped with an ultraviolet−visible detector and a Rheodyne injection valve (20 μL loop). A Spherisorb ODS-2 column (250 × 4.6 mm i.d.; particle size = 5 μm) (Teknokroma, Barcelona, Spain) was used at room temperature. Elution was performed at a flow rate of 1.0 mL/min for 30 min, using TFA in water (pH 2.5) and acetonitrile as the mobile phases with a gradient of 5−25% acetonitrile. The chromatograms were recorded at 280 nm. The phenol concentrations were calculated by linear least-squares regression analysis of the calibration curves of each compound using commercially available standards of DHPG and Ty and naturally purified HT and HTG.

Phytotoxicity Experiments. Barley seeds were inoculated with a thin slice of potato dextrose agar (PDA) (1 × 1 cm) with the mycelia of a 14-day-old culture of saprobic fungi grown at 28 °C. Three barley seeds that had been previously colonized by the mycelium for 7 days were inoculated in Erlenmeyer flasks containing 125 g of alperujo or alperujo that had been thermally treated and steam-sterilized three times. The flasks were covered with sterile cotton plugs and incubated under static conditions at 28 °C for 0, 2, 10, or 20 weeks. After the incubation with the saprobic fungi, the alperujo and the thermally treated alperujo were sterilized and added to the soil in pots.

The phytotoxicity experiments were carried out in pots with 300 g of soil that was steam-sterilized and mixed with sterilized quartz sand (1:1 by volume). Tomato seeds were pregerminated and selected for uniformity prior to transplantation. Plants (one per pot) were grown in a greenhouse with supplementary light provided by Sylvania incandescent cold-white lamps (400 nmol m^{-2} s^{-1} at 400−700 nm), with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity. Plants were watered from below and fed with 10 mL of nutrient solution per week.[22] Plants were harvested after 6 weeks.

A dose of 4 kg m^{-2} (20 g/kg) is recommended for farmyard manure applications.[23] In our experiment, the alperujo and the thermally treated alperujo were applied to the 300 g soil/sand mixtures in mixture pots at concentrations of 0, 12.5, and 25 g/kg.

Four experiments were designed to test the effect of the new steam treatment and saprobe incubation on the phytotoxicity level of alperujo:

Experiment 1. The first experiment was designed to test the effect of the amended treatment on alperujo (sample I), which was treated first with the saprobic fungi and then with a steam treatment at 170 °C for 30 min. The saprobic fungi were *C. rigida* and *F. oxysporum*, and the residue doses were 0, 12.5, and 25 g/kg. The following variables were tested in the experiment: four treatments, two doses of alperujo, and four incubation times (0, 2, 10, and 20 weeks).

Experiment 2. The second experiment was performed to test the effect of the amended treatment on alperujo (sample I), which was treated first with a steam treatment at 170 °C for 30 min and then with the saprobic fungi. The saprobic fungi were *C. rigida* and *F. oxysporum*, and the residue doses were 0, 12.5, and 25 g/kg. The following variables were tested in the experiment: four treatments, two doses, and four incubation times (0, 2, 10, and 20 weeks).

Experiment 3. The third experiment selected the most appropriate conditions for the steam treatment of alperujo (sample II) at a dose of 12.5 g/kg (as amended). The following variables were tested in the experiment: three treatments, five steam-treatment conditions, and three incubation times (0, 2, and 10 weeks).

Table 1. Compositions of Two Samples of Untreated Alperujo and the Liquid Phase Obtained from Both Samples Treated under Different Thermal Conditions by the New Steam Reactor

		treated alperujo I			treated alperujo II				
	raw alperujo I	150 °C, 90 min	170 °C, 30 min	raw alperujo II	150 °C, 45 min	150 °C, 60 min	150 °C, 90 min	170 °C, 15 min	170 °C, 45 min
moisture (%)	68.5			65.5					
phase separation		easy	easy		difficult	medium	easy	easy	easy
stone (%)	49.3	61.7	71.1	48.3	63.4	66.4	66.6	62.9	72.1
fat in solid (g/100 g of dry matter)	11.4	14.2	18.3	8.5	11.6	11.9	12.2	11.4	12.7
increase of fat in solid[a] (%)		19.7	37.7		24.6	26.5	28.2	23.1	30.9
solid loss (%)		20.1	30.7		23.8	27.2	27.5	23.2	33.0
phenolic components (g/100 g of dry, defatted, and deseeded solid)									
HT[b]	1.00[c]	1.04	1.19	2.11	0.61	0.91	1.65	1.28	1.57
DHPG[b]	0.06[d]	0.04	0.02	0.27	0.09	0.06	0.10	0.18	0.09
Ty[b]	0.08[d]	0.18	0.40	0.27	0.12	0.18	0.31	0.22	0.29
HTG[b]				0.63 [d]	0.79	0.90	1.17	0.59	0.85

[a] Percentage at which the fat is concentrated in the solid after thermal treatment due the solid loss. [b] Phenolic components: hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol 4-β-D-glucoside (HTG), and tyrosol (Ty). [c] Concentrations were determined by HPLC after steam-explosion extraction at 230 °C for 5 min with sulfuric acid 2.5% (v/v). [d] Concentrations were determined by HPLC after extraction treatment at 90 °C for 120 min.

Experiment 4. In the fourth experiment, the effect of washing the solid after the alperujo (sample II) steam treatment at a dose of 12.5 g/kg (as amended) was tested. The following variables were tested in the experiment: three treatments and three incubation times (0, 2, and 10 weeks).

Statistical Treatment of Data. Data obtained were subjected to ANOVA, and multiple pairwise comparisons were performed by Duncan's test.

RESULTS AND DISCUSSION

Composition. The compositions (on a dry weight basis) of the two untreated alperujo samples and both liquid phases obtained after steam treatment are shown in Table 1. The phenols are expressed with respect to the destoned and defatted dry matter. The solid used for the incubation tests was defatted but not destoned, because stones do not affect the experiments. After the treatment, the liquid−solid separation was effective when the temperature was above 150 °C for 60 min. The percentage of fat in the final solid increased by 20−30% in average, the same range as the solid loss and the solubilization of alperujo. The oil was not altered by the thermal treatment and was proportionally concentrated in the final solid, which is easier to extract than the untreated matter.

The phenol concentrations in the two raw material columns were determined by thermal analytical methods to extract their maximum quantities. The phenol concentrations in treated alperujo were measured in the liquid phase obtained after the thermal treatment. Therefore, the high phenol concentration values, which are close to the raw alperujo values, indicate that the phenols have been successfully removed from the solid phase. The final concentrations of DHPG provide information not only about the solubilization but also about the degradation by thermal oxidation.[21]

The concentrations of HT and Ty in liquid phase increased depending on the severity of treatment. Consequently, their major parts were removed from final solid. HTG was present only in sample II, and its concentration increased in the 150 °C treatment, whereas the higher temperatures seemed to enhance the hydrolysis into HT. In general, the steam treatment decreased the phenol content in the solid phase, but remnants of the liquid phase remained after the separation. This fact could be considered to be a partial detoxification, because the phenol compounds had a high level of toxicity in further bioprocesses.

High solubilization of the treated solid is also supported by the hydrolysis of the hemicelluloses (TFA-hydrolyzable carbohydrate) from the cell material, which renders the cellulose more accessible to further enzymatic treatment. The sugar composition is shown in Table 2. The final values of the mono- and oligosaccharides were the result of the balance between the solubilization and the thermal degradation of the sugars. The compositions of the neutral and acid sugars indicate that the treatment increases oligosaccharide content, and xylose, glucose, galactose, and galacturonic acid are the main components. Monosaccharides were also increased depending on the severity of the treatment, except for Glc in sample II. A high concentration of glucose in the raw material makes the balance between thermal degradation and slightly cellulosic solubilization negative. The remaining sugars mainly come from the hydrolyzed hemicellulose.[17]

The results show that the final solid obtained after thermal treatment has low phenolic content and high cellulose accessibility to hemicellulosic solubilization.[17] It is suggested that these reduce the final toxicity of the thermally treated solid for further fungal treatment. The treatment at 170 °C for 30 min provided better solubilization of phenols in sample I and equal solubilization of sugars compared to the 150 °C treatment for alperujo. Hence, this condition was chosen to assess the optimal order of operation (saprobe fungal incubation followed by thermal treatment or vice versa).

Phytotoxicity Experiment. In the present study, toxicity was determined on the basis of the reduction of shoot biomass in *L. esculentum* plants grown in soil in the presence of alperujo that was either untreated, thermally treated, or incubated with saprobic fungi compared to plants grown in the absence of the waste. The application of 12.5 and 25 g/kg of alperujo to the soil decreased

Table 2. Neutral and Acid Sugar Compositions [Mono- (M) and Oligosaccharide (O) Forms] of Two Samples of Untreated Alperujo and the Liquid Phase Obtained from Both Samples Treated under Different Thermal Conditions by the New Steam Reactor[a]

			treated alperujo I			treated alperujo II				
		raw alperujo I	150 °C, 90 min	170 °C, 30 min	raw alperujo II	150 °C, 45 min	150 °C, 60 min	150 °C, 90 min	170 °C, 15 min	170 °C, 45 min
composition of neutral sugar (g/100 g of dry, defatted and deseeded solid)										
rhamnose (Rha)	M	0.12 (0.01)	0.09 (0.01)	0.08 (0.01)			0.12 (0.01)	0.18 (0.01)		2.10 (0.10)
	O	0.38 (0.03)	0.55 (0.03)	0.40 (0.07)	0.36 (0.02)	0.33 (0.02)	0.32 (0.02)	0.70 (0.02)	0.60 (0.03)	0.58 (0.02)
arabinose (Ara)	M		0.12 (0.01)	0.13 (0.02)		0.10 (0.01)	0.35 (0.03)	0.26 (0.02)	0.24 (0.03)	0.32 (0.03)
	O	0.86 (0.05)	1.96 (0.12)	1.81 (0.13)	0.36 (0.03)	0.59 (0.02)	0.96 (0.09)	1.10 (0.04)	1.14 (0.05)	0.92 (0.09)
xylose (Xyl)	M		0.08 (0.01)	0.12 (0.03)		0.13 (0.03)	0.22 (0.01)	0.14 (0.02)		0.13 (0.01)
	O	0.15 (0.02)	2.38 (0.16)	4.88 (0.33)	0.33 (0.02)	2.10 (0.15)	0.87 (0.07)	1.06 (0.09)	0.95 (0.08)	1.97 (0.07)
galactose (Gal)	M		0.08 (0.01)	0.08 (0.03)				0.58 (0.02)		0.14 (0.01)
	O	0.59 (0.04)	0.83 (0.03)	0.79 (0.06)		1.98 (0.10)	2.09 (0.14)	0.65 (0.05)	0.45 (0.05)	0.54 (0.05)
glucose (Glc)	M		0.08 (0.01)	0.09 (0.01)	17.41 (0.03)	7.51 (0.19)	6.11 (0.11)	2.66 (0.24)	0.47 (0.03)	0.64 (0.06)
	O	0.97 (0.08)	1.09 (0.08)	0.85 (0.01)	0.64 (0.05)	0.58 (0.04)	1.31 (0.15)	6.12 (0.32)	2.69 (0.17)	3.60 (0.16)
composition of acid sugar (g/100 g of dry, defatted, and deseeded solid)										
glucuronic acid	M	0.19 (0.04)			0.15 (0.02)					
	O		0.24 (0.05)							
galacturonic acid	M	0.36 (0.06)		0.15 (0.02)	0.33 (0.03)	0.19 (0.04)	0.18 (0.02)	0.27 (0.02)		0.21 (0.01)
	O	0.91 (0.09)	0.69 (0.07)	0.72 (0.09)	0.49 (0.06)	0.44 (0.03)	0.54 (0.05)	0.67 (0.03)	0.54 (0.02)	0.53 (0.02)

[a] Values in parentheses correspond to standard deviation.

Table 3. Shoot Dry Weight (Milligrams) of Tomato (*Lycopersicon esculentum* L.) Plants Grown either in Soil without Alperujo (Sample I) or in Soil Amended with Alperujo That Was Left Untreated (Control), Thermally Treated at 170 °C for 30 min (S-Treatment), or Sequentially Processed with Fungal Incubation Followed by Steam Treatment[a]

				incubation time of alperujo							
alperujo treatment	soil without alperujo		alperujo concn (g kg^{-1})	0 weeks		2 weeks		10 weeks		20 weeks	
control	740	a2	12.5	16	b1	18	b1	24	b1	22	b1
	728	a2	25	4	a1	5	a1	7	a1	9	a1
S-treatment	731	a2	12.5	76	d1	81	d1	83	d1	78	c1
	745	a2	25	46	c1	52	c1	48	c1	53	c1
C. rigida + S-treatment	741	a4	12.5	78	d1	336	g2	493	g3	370	f2
	735	a4	25	53	c1	170	e3	186	e3	106	d2
F. oxysporum + S-treatment	739	a3	12.5	67	d1	280	f2	296	f2	260	e2
	725	a4	25	51	c1	80	d2	133	de3	111	d3

[a] Column values followed by the same letter and row values followed by the same number are not significantly different as assessed by Duncan's multiple-range test ($P = 0.05$). Data are the means of four replicate samples.

the shoot dry weight of the tomato plants (Tables 3−6). Chlorotic symptoms were also observed in the plants that had been grown with untreated alperujo (data not shown). The shoot dry weight of the tomato was lower in the presence of 25 g/kg of alperujo than in the presence of 12.5 g/kg of alperujo. The detrimental effects of alperujo and other olive residues in the soil on the root dry weight of the tomato and other plants have been observed previously.[24−26]

Steam treatment of alperujo reduces its phytotoxicity (Tables 3−6). Thus, the shoot dry weight of plants grown in the presence of thermally treated alperujo was higher than that of plants grown with the untreated waste. In addition, steam treatment strongly decreased the phenolic content (Table 1). It seems that there was a relationship between the removal of the phenol from the alperujo and the decrease in phytotoxicity. These findings confirmed that phenols could be the main determinants of olive mill waste phytotoxicity.[4,27] However, the present study shows that thermal treatment of alperujo did not totally eliminate phytotoxicity for tomato. In fact, a single-stage biological, chemical, or physical treatment is unlikely to achieve complete detoxification at a reasonable cost, due to the complexity and heavy pollution load of alperujo. On the other hand, a well-designed sequential treatment consisting of various physical, chemical, or biological processes with well-defined treatment objectives may be the optimum solution.[28−30] It is known that the bioremediation of olive residues is improved by

Table 4. Shoot Dry Weight (Milligrams) of Tomato (*Lycopersicon esculentum* L.) Plants Grown either in Soil without Alperujo (Sample I) or in Soil Amended with Alperujo That Was Left Untreated (Control), Treated with Steam Treatment at 170 °C for 30 min (S-Treatment), or Sequentially Processed with Steam Treatment Followed by Fungal Incubation[a]

				incubation time of alperujo							
alperujo treatment	soil without alperujo		alperujo concn (g kg^{-1})	0 weeks		2 weeks		10 weeks		20 weeks	
control	760	a2	12.5	18	b1	22	b1	21	b1	25	b1
	750	a2	25	4	a1	5	a1	8	a1	10	a1
S-treatment	753	a2	12.5	69	d1	76	d1	78	d1	66	c1
	741	a2	25	45	c1	47	c1	50	c1	53	c1
S-treatment + *C. rigida*	739	a4	12.5	74	d1	540	g2	716	g4	680	f3
	758	a5	25	50	c1	300	e2	676	f4	546	e3
S-treatment + *F. oxysporum*	765	a4	12.5	70	d1	486	f2	623	f3	476	d2
	747	a4	25	47	c1	313	e2	460	e3	433	d3

[a] Column values followed by the same letter and row values followed by the same number are not significantly different as assessed by Duncan's multiple-range test ($P = 0.05$). Data are the means of four replicate samples.

the physical pretreatment of the residues and seems to considerably decrease their phytotoxicity.[5,25] The thermal treatment of alperujo can potentially be a source of commercially valuable products, particularly hydroxytyrosol.[13,14] In addition, microorganisms are able to decrease soil contamination caused by toxic residues from plants.[31,32] The bioremediation of olive residues using specific strains of fungi, some of which are isolated directly from olive mill wastes (primarily filamentous fungi, white rot fungi, and yeasts), has been extensively investigated. Several studies have reported a reduction in phytotoxicity following the treatment of the olive mill waste with fungi.[33−37] However, to date there have been no studies that used a dual treatment with thermal and biological processes.

Inoculation of alperujo with the saprobic fungi *C. rigida* and *F. oxysporum* before thermal treatment decreased its phytotoxic effects on the tomato plant (Table 3). The effect of reduced alperujo phytotoxicity on tomato shoot dry weight increased with time until 10 weeks of incubation. However, the effect of alperujo phytotoxicity on tomato shoot dry weight after 20 weeks was similar to that after 2 weeks. The doses of alperujo incubated with the saprobic fungi before thermal treatment increased the shoot dry weight compared to that of the plants grown in the presence of untreated or thermally treated alperujo (Table 3). Biological treatment with *F. oxysporum* before the steam treatment decreased the negative effect of alperujo on the tomato less than biological treatment with *C. rigida*. Both saprobic fungi decreased the phytotoxicity of alperujo but to different extents. These differences can be attributed to differences in their enzymatic machineries that degrade phenolic compounds.[3] Nevertheless, neither fungal treatment prior to the thermal treatment eliminated the negative effects of this residue on plant growth.

The incubation of thermally treated alperujo with the saprophytic fungi *C. rigida* and *F. oxysporum* was also able to decrease phytotoxicity for tomato (Table 4). However, our data indicate that thermal treatment prior to saprobe incubation led to a higher phytotoxicity reduction than sequential processing with fungal incubation followed by steam treatment. This study confirmed that saprobic fungi decrease the phytotoxicity for tomato of thermally treated alperujo after 2, 10, and 20 weeks of incubation, but this effect was more pronounced when alperujo was incubated with *C. rigida* for 10 weeks. On the basis of our results, we selected *C. rigida* as the most effective saprobic fungus. Furthermore, in the presence of alperujo that was first treated thermally and then incubated with *C. rigida* for 10 weeks, the tomato shoot dry weight was similar to that grown without alperujo (Table 4). Pretreatment prior to biological digestion has been proven to be one of simplest and most effective methods to improve the biodegradability of lignocellulosic materials.[38] The thermal treatment of alperujo can produce a phenol-poor and cellulose-rich and protein-rich solid,[17] which is more efficiently degraded by the saprobic fungi and their ligninolytic and hydrolytic enzymes.

To improve the biovalorization of alperujo with a thermal pretreatment, different temperatures and times for the steam treatment were studied. Table 5 shows that the alperujo treated at 150 °C for 45 min and at 170 °C for 15 min had a smaller phytotoxic effect on tomato shoot dry weight than untreated alperujo. Thermal treatment of alperujo at 150 °C for 60 min, at 150 °C for 90 min, and at 170 °C for 45 min did not decrease its phytotoxicity to tomato roots. By contrast, the phytotoxicity of alperujo was significantly reduced by sequential processing with all of the thermal treatments and *C. rigida* incubation for 2 and 20 weeks, as indicated by the increases in tomato dry weight. Tomato shoots in soil in the presence of alperujo thermally treated at 150 °C for 45 min and incubated with *C. rigida* for 10 weeks had a weight similar to that of plants grown without alperujo, which indicates that a combination of thermal and biological treatments may facilitate the use of these wastes as organic amendments for agricultural soils. Several studies have reported that the reduction of alperujo phytotoxicity by fungal treatment requires long colonization times, regardless of the species employed and their growth rates in the waste.[39] One of the objectives of this study was to assess whether the addition of thermal treatment to the incubation might improve colonization rates and, thus, reduce the time necessary for detoxification and bioconversion. The physical structures and chemical compositions of lignocellulosic materials could be altered through various pretreatments that render the compositions of lignocellulosic materials more accessible to anaerobic microorganisms and readily biodegradable.[40] Ten weeks of incubation of thermally treated alperujo seemed to be enough to reduce its phenol content (data not shown) and, therefore, its toxic effects on plants, but a 2 week period was not sufficient to achieve the same results.

Table 5. Shoot Dry Weight (Milligrams) of Tomato (*Lycopersicon esculentum* L.) Plants Grown either in Soil without Alperujo (Sample II) or in Soil Amended with Alperujo (12.5 g kg^{-1}) That Was Left Untreated (Control), Steam-Treated (S-Treatment), or Sequentially Processed with Steam Treatment at Different Temperatures and Times and Incubation with *C. rigida*[a]

				incubation time of alperujo					
treatment	soil without alperujo		steam treatment[b]	0 weeks		2 weeks		10 weeks	
control	673	a2		14	a1	18	a1	20	a1
S-treatment	700	a2	A	65	b1	68	b1	73	b1
	672	a2	B	52	ab1	58	ab1	68	b1
	683	a2	C	47	ab1	47	ab1	44	ab1
	705	a2	D	98	c1	104	c1	120	c1
	675	a2	E	50	ab1	48	ab1	54	ab1
S-treatment + *C. rigida*	701	a3	A	68	b1	360	d2	660	f3
	708	a4	B	50	ab1	210	c2	400	d3
	675	a4	C	45	ab1	330	d2	460	de3
	710	a4	D	99	c1	380	d2	520	e3
	670	a4	E	47	ab1	280	cd2	410	d3

[a] Column values followed by the same letter and row values followed by the same number are not significantly different as assessed by Duncan's multiple-range test ($P = 0.05$). Data are the means of four replicate samples. [b] A, 150 °C, 45 min; B, 150 °C, 60 min; C, 150 °C, 90 min; D, 170 °C, 15 min; E, 170 °C, 45 min.

Table 6. Shoot Dry Weight (Milligrams) of Tomato (*Lycopersicon esculentum* L.) Plants Grown either in Soil without Alperujo (Sample II) or in Soil Amended with Alperujo (12.5 g kg^{-1}) That Was Left Untreated (Control) or Sequentially Processed with Steam Treatment at 150 °C for 45 min (S-Treatment) with or (W) or without (NW) Washing and Incubation with *C. rigida*[a]

				incubation time of alperujo					
alperujo treatment	soil without alperujo		post-treatment	0 weeks		2 weeks		10 weeks	
control	670	a2		14	a1	13	a1	18	a1
S-treatment	658	a2	W	69	b1	70	b1	72	c1
	663	a3	NW	55	b1	50	b1	53	b1
S-treatment + *C. rigida*	672	a4	W	63	b1	313	c2	490	d3
	661	a3	NW	56	b1	526	e2	626	f3

[a] Column values followed by the same letter and row values followed by the same number are not significantly different as assessed by Duncan's multiple-range test ($P = 0.05$). Data are the means of four replicate samples.

The high phytotoxicity of alperujo was even evident when tomato plants were grown in soil with 12.5 g/kg alperujo and did not decrease with time in the alperujo abiotic control (Table 6). In contrast, alperujo that had been colonized by *C. rigida* for 10 weeks after the thermal treatment and without washing did not exhibit any toxicity. In our experiments, a smaller increase in the tomato shoot dry weight was observed when the plants were grown in pots with alperujo that was treated with sequential processing consisting of steam treatment, washing with water, and incubation with *C. rigida* than with alperujo that was thermally treated, not washed with water, and incubated with *C. rigida*. It seems that washing with water after the thermal treatment can eliminate the sugar that is necessary for fungal growth.

These results suggests that a combination of thermal and biological treatments allows the reduction of the time required to eliminate phytotoxic olive residues and opens the way for guidelines for the management of these wastes and their use as organic amendments in agricultural soil.

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