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Fungal Strain as Biological Tool to Remove Genotoxicity Effect of Phenolic Compounds from Olive Mill Wastewater

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Abstract: This study aims to select fungi isolates to reduce olive mill wastewater (OMWW) chemical oxygen demand (COD) and phenolic compounds (PC), as well as their genotoxicity effect. Treatment with mold, isolated by an innovative technique using phenolic compound-selective media, showed a reduction rate of about 4% for COD and 2% for PC during one month of incubation without optimization of the treatment conditions. Whereas this percentage reached 98% and 96% for COD and PC, respectively, after only 12 days of treatment, when the C:N ratio was adjusted to 30 by adding urea as a nitrogen source at 150 rpm agitation speed. Genetic sequence homology of the most efficient mold isolate showed 100% similarity to *Penicillium chrysogenum*. High-performance liquid chromatography analysis of phenolic extracts of untreated OMWW showed the presence of five compounds—hydroxytyrosol at 1.22 g.L^{-1}, tyrosol at 0.05 g.L^{-1}, caffeic acid at 0.16 g.L^{-1}, p-coumaric acid at 0.05 g.L^{-1} and oleuropein at 0.04 g.L^{-1}—that were eliminated during the degradation process at 88.82%. Genotoxicity, assessed by the *Vicia-faba* root cell, showed a significant decrease in micronucleus frequency of about 96% after fungal treatment. These results confirm the positive role of fungal treatment of OMWW to eliminate genotoxicity and their ability to improve the agronomic potential.

Keywords: olive mill wastewater; biological treatment; *Penicillium chrysogenum*; phenolic compounds; genotoxicity test; *Vicia faba*

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1. Introduction

Olive oil extraction from olive fruits is a growing industry in many Mediterranean countries, and over 30 million tons by-products are generated annually from olive mill wastewater, the liquid by-product generated during olive oil production process [1]. In Morocco, a discharge of about 250,000 tons of olive mill wastewater containing high contents of toxic organic chemical substances material, mainly the phenolic compounds, are produced by olive oil production units [2]. The olive mill wastewaters are discharged into the sewerage network and/or stored in evaporation ponds to reduce their volume and/or are spread directly on the soil and result in adverse environmental impacts [3–5]. Their high pollution load is due to their high organic matter content such as sugars, tannins, phenolic compounds, polyalcohols, pectins and lipids compounds [3].

Soils in arid and semi-arid regions are relatively poor in organic matter, and this impoverishment is accelerated by the cultivation intensification, the light texture of the soils and the non-recovery of crop residues in the soil [6,7]. The organic matter depletion of these soils accentuates the degradation and the decrease of their fertility, thus favouring the processes of erosion and desertification [8,9]. To preserve these soils and maintain their

productivity, the contribution of organic amendments becomes essential [10]. However, these amendments are not always available, and their quality often leaves something to be desired. Among these amendments, OMWW are sources of organic matter, available phosphorus (P) and potassium (K), and bioactive molecules for plant growth and confer protection against pathogen attacks [11–14]. OMWW can constitute a possible amendment for soils in arid regions poor in this element. Furthermore, untreated olive mill wastewater spread in soils increases the soil electrical conductivity, impairs salinity and balances the equilibrium of useful soil microorganisms [12,13,15]. However, less costly detoxification and treatment of OMWW is needed to enable farmers to take full advantage of their use on agricultural land, which leads to the development of a circular economy, which should lead to the future development of more sustainable agriculture [16].

Treatment techniques have been developed at the international and/or national level, including physicochemical technologies, oxidation processes and biological treatment [17]. Nevertheless, the viability of these technologies is questionable as they are costly (i.e., large quantities of chemicals are required), generate large volumes of sludge and/or do not produce high-quality effluents [18]. Therefore, a search for alternative treatment methods, such as a biological method that integrate the use of microorganisms, is emerging as new technology becomes affordable and rapidly adoptable by small producers [19].

Previous studies have demonstrated the benefits of fungi to degrade phenolic compounds from the olive mill wastewater [20–22]. These fungi produce highly oxidative enzymes, such as ligninase and laccase, to degrade recalcitrant organic molecules, in particular the phenolic bound molecules [23]. Eventually, various toxic molecules present in olive mill wastewater may not completely be degraded by the microorganisms, but transform into secondary end products or metabolic intermediates and become highly toxic compared to the initial compounds [24,25]. Hence, the treatment efficiency of olive mill wastewater products must also focus on the reduction of toxic effects [26]. This has led to conducting genotoxicity bioassays to assess the potential hazard and risk of olive mill wastewater products prior to use as agricultural inputs products [26,27]. The genotoxicity approach consisting of elimination the toxic effect using a biological agents could be more useful and cost-effective for estimating the risks of several organic and inorganic compounds in addition to physicochemical analyses [25,28,29]. Evidence from biotesting with plant roots has provided reliable, cost effective and valuable alternative methods for determining the negative effects of environmental contaminants compared to other biological test systems [25,30,31].

The objectives of the present study were (a) to screen and investigate of the ability of several strains of fungi to decrease COD and phenolic compounds from OMWW under controlled laboratory conditions, (b) to optimize the treatment conditions using *Penicillium chrysogenum* cultures and (c) to investigate the effect of bioremediation on the genotoxicity removal of olive mill wastewater.

2. Materials and Methods

2.1. Characterization of OMWW

OMWW used in this work was obtained from an olive oil production plant located in Chichaoua, Marrakech, which uses a 3 phases process for the extraction of olive oil. The samples were stored at −20 °C until required for analysis.

OMWW samples were analyzed for the following parameters: pH was measured by a pH-meter (HANNA instruments, HI 2210-02, Portugal). Electrical conductivity (EC) was measured by a conductometer (HANNA instruments, HI 9033, Portugal). Total suspended solids (TSS) were determined after filtering a sample through a filter (Whatman membrane filters nylon pore, size 0.45 µm, diameter 47 mm) and drying the retained residue at 105 °C until constant weight. Total solids (TS) were determined by oven-drying of 10 mL of the sample at 105 °C for 24 h, according to the APHA standard method. Total ash content was determined by the incineration of TS at 600 °C for 6 h. Total Kjeldahl nitrogen (TKN) was determined after mineralization and distillation. During the TKN analysis, 5 mL of OMWW

were mixed with a Kjeldahl catalyst tablet (0.5 g) and 10 mL of H2SO4 (96%). Digestion was carried out at 400 °C during 2 h. After cooling, 100 mL of distilled water and 50 mL of NaOH solution (40 g/L) were added before distillation according to the AOAC analytical methods [32]. Chemical oxygen demand (COD) was determined by the Standard Methods for the Examination of Water and Wastewater [33]. The appropriate amount of wastewater samples was diluted up to 1000 times, and two milliliters of diluted OMWW was introduced into a lab-prepared digestion solution containing sulfuric acid, potassium dichromate and mercuric sulphate. The mixture was then incubated for 120 min at 150 °C in a COD reactor, and the COD concentration was measured calorimetrically at 600 nm. Total organic carbon (TOC) was determined after oxidation using dichromate of potassium according to the Anne's method as described by Aubert [34]. The total lipid concentration was determined using the method of Folch et al. [35]. This involved the OMWW being macerated with 60 mL of a 2/1 v/v chloroform/methanol mixture for 24 h at 4 °C. The supernatant was then recovered and filtered through a Durieux filter. This was repeated twice more to ensure maximum recovery of the lipid. The pooled supernatants were then shaken with 60 mL of 1% NaCl to separate the methanol from the chloroform phase containing the lipid. The chloroform phase was then dried over anhydrous sodium sulphate (Na_2SO_4), and the total lipid content was determined after evaporation of the chloroform phase at 40 °C. The amount of total sugars was determined spectrophotometrically according to the method described by Dubois et al. [36]. Three replicates were used, and the mean values of the parameters recorded. The main features of OMWW are presented in Table 1 [37].

Table 1. Physicochemical characteristics of raw olive mill wastewater.

Parameters	Unit	OMWW
pH		4.80 ± 0.04
EC	(mS/cm)	13.9 ± 0.1
TSS	($g.L^{-1}$)	10.0 ± 0.3
TS	($g.L^{-1}$)	122.90 ± 2.62
AshC	($g.L^{-1}$)	40.7 ± 1.0
COD	($g.L^{-1}$)	187.6 ± 19.1
BOD	($g.L^{-1}$)	60.1 ± 1.42
TOC	($g.L^{-1}$)	73.26 ± 1.80
TKN	($g.L^{-1}$)	0.160 ± 0.001
Proteins	($g.L^{-1}$)	1.00 ± 0.02
Lipids	($g.L^{-1}$)	4.51 ± 0.40
Sugars	($g.L^{-1}$)	21.45 ± 0.40
PC	($g.L^{-1}$)	4.3 ± 0.1

Values are the average of three measurements ± standard error. EC: electrical conductivity; TSS: total suspended solids; TS: total solids; AshC: ash contents; COD: Chemical oxygen demand; BOD: biological oxygen demand, TOC: total organic carbon; TKN: total Kjeldahl nitrogen; PC: phenolic compounds; OMWW: olive mill wastewater.

2.2. Extraction and Quantification of Phenolic Compounds

The extraction technique of phenolic compounds was described by Macheix et al. [38]. To 5 mL of OMWW previously filtered on filter paper (Watman N°1), we added a 40% (v/v) ammonium sulfate (ACS reagent, ≥99.0%, Sigma-Aldrich, St. Louis, MO, USA) solution to increase the medium's ionic strength and a 20% (1/10 v/v) metaphosphoric acid (ACS reagent, ≥33.5%, Sigma-Aldrich) solution to prevent the oxidation of phenolic compounds. Afterward, we proceeded with delipidation and depigmentation by petroleum ether (ACS reagent, ≥ 90%, Sigma-Aldrich) (1/2 v/v) 3 times. The extraction was continued with ethyl acetate (ACS reagent, ≥99.5% Sigma-Aldrich) (v/v) 3 times, the extract was evaporated to dryness at 35 °C and the residue was recovered in 5 mL of methanol (ACS reagent,

≥99.8%, Sigma-Aldrich). The determination of total phenolic compounds was performed by the Folin-Cioccalteu reagent [39]. Then, 50 µL of the phenolic extract was placed in a test tube, to which we added 1.35 mL of distilled water and 200 µL of Folin-Cioccalteu reagent. After 3 min, 400 µL of a 20 % sodium carbonate (Sigma-Aldrich) solution was added, and the tubes were shaken and incubated at 40 °C for 30 min. The absorbance of the blue solution was then measured with a spectrophotometer at 760 nm. The phenolic compounds contents are expressed in $g.L^{-1}$ by reference to a standard range based on caffeic acid (Sigma-Aldrich) prepared at 60 mg/100 mL in 36% methanol (Sigma-Aldrich).

The analysis of extracted phenolic compounds was carried out by high-performance liquid chromatography (HPLC). The system consisted of a Knauer-type device with a Knauer diode array PDA detector for the detection of compounds at wavelengths and software for data processing. The C18 reversed-phase column (Eurospher II 100–5 C18, 250 × 4.6 mm) and precolumn was used. The column temperature was set at 25 °C. The mobile phase consisted of a gradient of acetonitrile and bi-distilled water acidified to pH 2.6 with o-phosphoric acid and then filtered on Millipore (0.45 µm). The mobile phase composition was acetonitrile/water 5:95 (v/v). The flow rate was 1 mL/min, and the injection volume was 10 µL. The separation was performed on a gradient of 5% to 95% acetonitrile for 60 min. PC were simultaneously detected at a wavelength of 280 nm and identified by comparison with controls for their retention time and UV spectrum. Co-injections with commercial standards (hydroxytyrosol, tyrosol, catechol, caffeic acid, acid p-coumaric, oleuropein, gallic acid and syringic acid) were carried out to confirm the identity of the compounds. Calibration curves of the analyzed compounds were constructed by injecting 10 µL of standard solutions at five different concentrations [19,37].

2.3. Fungal Isolates and Culture Conditions

The fungal isolates used in this study were selected after screening from several extreme mediums which are known by their high load of pollutants, such as sludge from OMWW evaporating ponds and soil irrigated with OMWW. The samples were collected from an 8 to 10 cm depth using a sterile spatula and transferred to pre-autoclaved sterile glass bottles with rubber stoppers. The samples were brought to the laboratory and stored under refrigeration temperature. Then, 1 g of each sample was suspended in 9 mL sterilized physiological water. The suspension was incubated at 28 °C, 150 rpm for 2 h, and a serial dilution (1:10) was prepared in sterilized physiological water. From each dilution, 0.1 mL was spread on potato dextrose agar (PDA) containing 50 µg/mL of chloramphenicol to inhibit bacterial growth. The Petri dishes were incubated for 72 h at 28 °C. Fungal isolates were isolated and purified based on their morphological characteristics, including texture, color, shape, size and mycelial type.

To examine the ability of fungal isolates to grow in OMWW, the isolated fungi were cultivated using an OMWW-based medium. In fact, to prepare this culture medium, the OMWW was diluted to 25%, 50%, 75% and 100% v/v in distilled water. Next, 15 $g.L^{-1}$ of agar-agar was added to each dilution before being sterilized at 120 °C for 30 min and then dispensed into 90 mm diameter Petri dishes. After solidification of the culture media, they were inoculated in the center with agar plugs (6 mm) of pure fungal cultures obtained from the edges of the 7-day-old culture of fungal isolates grown on potato dextrose agar (PDA), then incubated at 30 °C until growth (15 d). The ability of fungal isolates to grow using only phenolic compounds as a sole source of carbon and energy was tested on a minimal medium (composition: 2 $g.L^{-1}$ sodium nitrate, 1 $g.L^{-1}$ potassium phosphate and 0.5 $g.L^{-1}$ magnesium sulphate) with different concentrations of phenolic compounds (4000 $mg.L^{-1}$, 2000 $mg.L^{-1}$, 1000 $mg.L^{-1}$ and 500 $mg.L^{-1}$) extracts from the OMWW.

The Petri dishes were kept in an incubator at 30 °C for 15 days. The mycelium of fungi exhibited a radial growth, so its growth rate could be estimated by measuring the radius of

the mycelium throughout the growth period. The radial growth was calculated using the formula growth (Gr) according to Dubey and Maheshwari [40]:

$$Gr = \frac{(R_1 - R_0)}{(t_1 - t_0)}$$

where R_0 and R_1 are the colony radii at time t_0 and t_1, respectively.

2.4. OMWW Fungal Treatments

Fungal inocula (plugs of 6 mm diameter) were obtained from the peripheral part of 7-day-old mycelia growing on solidified OMWW medium (25% *v*/*v* OMWW supplemented with 1.5% *w*/*v* agar) and were then transferred into 500 mL bioreactor containing 400 mL of raw OMWW. The 500 mL bioreactors were made of autoclavable borosilicate glass. Each bioreactor was equipped with a magnetic stirrer, which was activated sequentially and designed to ensure sufficient mixing of the media (substrate/inoculum) by counteracting the phenomena of decantation/flotation. Cultures were incubated at 30 °C in batch cultures, and 3 agitation speeds were tested: 100, 150 and 200 rpm. For the investigation of the effect of the nitrogen supplementation, a range of different nitrogen sources at a final concentration of 3 $g.L^{-1}$ were tested, i.e., urea, ammonium nitrate and ammonium sulphate. The COD and phenolic compounds evolutions were followed during 12 days of treatment. Non-inoculated controls were incubated in parallel under the same conditions.

2.5. Identification of the Fungal Isolate

2.5.1. Preliminary Identification

Preliminary identification of fungal isolate was performed based on their morphological characteristics, including colony texture, colony color, shape, size, mycelial type and radial growth rate on Czapek and PDA medium. The mold was seeded on Czapek-agar medium and on PDA and incubated at 25 °C for one week. Microscopic examination of fungal spores by the slide culture technique was performed using lactophenol cotton blue for staining then compared with the identification key established in the "A Manual of Soil Fungi" [41].

2.5.2. Molecular Identification of Fungal Isolate

DNA Extraction

The DNA extraction procedure was adopted with slight modifications from the method of Aamir et al. [42]. Genomic DNA was extracted from 7–10-day-old fungal cultures grown in liquid medium (potato dextrose broth). The fungal mass was removed from the culture medium by filtration through four layers of sterile cheesecloth that allow the broth to pass through and retain the fungal mass. The resulting fungal mycelium mass was placed in 1.5 mL Eppendorf tubes containing 60–80 mg of sterile glass beads and 800 µL of lysis buffer (100 mM Tris HCl (pH 8.0), 50 mM EDTA, 3% SDS). The tubes were closed, mixed well to resuspend the glass beads and incubated in a water bath at 65 °C for 10 min. The resulting fungal tissue was centrifuged for 10 min at 13,000 rpm twice. The supernatant was then thoroughly transferred to a new tube and placed on ice for about 30 s to allow the fungal tissue to settle to the bottom of the tube. Then, 2 mL of Rnase solution (10 mg/mL) was added to the supernatant. This was incubated at 37 °C for 15 min. After incubation and Rnase treatment, an equal volume (500 µL) of phenol:chloroform:Isoamyl alcohol (25:24:1) was added to the reaction mixture, mixed well and centrifuged at 13,000 rpm for 10 min. The upper aqueous phase was carefully removed into a new tube, and then an equal volume (800 µL) of 100% ethanol was added. The tubes were gently mixed and incubated in a refrigerator for 2 h at −20 °C to allow the DNA to precipitate. The contents were centrifuged at 12,000 rpm for 10 min to pellet the DNA. The supernatant was discarded, and the DNA pellet was then washed twice with 800 µL of chilled 70% ethanol and centrifuged at 12,000 rpm for 5 min. The supernatant was removed, and the pellet

was air-dried at room temperature overnight until no ethanol was visible. The pellet was dissolved in 50 µL of sterile 1× TE buffer and stored at −20 °C for future use.

PCR Amplification

Extracted fungal genomic DNA was subjected to PCR amplification identification of the ITS1-5.8S-ITS2 region using the universal primers ITS1 (5′-TCCGTAGGTGA ACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [43] according to the following protocol: The PCR reaction for the amplification of ITS1-5.8S-ITS2 rDNA was performed in a total volume of 22 µL. A volume of 0.3 µL of fungal DNA sample was added to the PCR mixture prepared in a microtube. PCR mixture contained 2.5 µL of $MgCl_2$ (25 mM), 5 µL of 5X PCR buffers, 1 µL of a deoxynucleoside triphosphate mixture (2.5 mM each of dNTPs), 0.625 µL of each primer (20 µM), 0.5 µL of BSA (10 mg/mL) and 0.3 µL of Go Taq buffer (Promega, Madison, WI, USA), and the total volume was supplemented with distilled water. Amplification was performed in the programmed thermal cycler with the following thermal profile: initial denaturation at 95 °C for 3 min, followed by 35 cycles, each cycle consisting of 5 min of denaturation at 95 °C, and 1 min of hybridization at 58 °C. Reactions were completed with an initial 10 min elongation at 72 °C, 1 min elongation at 72 °C, followed by a final elongation at 72 °C for 10 min. The PCR product was subjected to electrophoresis on a 1% agarose gel for 15 min sat 100 v. The gel was then stained with ethidium bromide (ETB) solution, rinsed in sterilized water and observed under UV light on a Gel Doc 1000/2000 system.

DNA Sequencing

Direct sequence determinations of PCR-amplified DNAs were performed with an ABI PRISM dye terminator, cycle sequencing ready-reaction kit (Perkin-Elmer, Waltham, MA, USA) and an ABI PRISM 377 sequencer (Perkin-Elmer, Waltham, MA, USA) according to the manufacturer's instructions, and the resultant nucleotide sequences were compared to reference sequences available in the GenBank databases from the National Centre of Biotechnology Information database using the BLAST search. Phylogenetic analyses were performed using MEGA X after multiple alignments of the data by Clustal W. Distances and clustering were determined using the neighbor-joining, maximum likelihood principle and maximum parsimony algorithms. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications).

2.6. OMWW Toxicity Analysis

2.6.1. *Vicia faba* Micronucleus (MN) Test

OMWW innocuity to plant health has been evaluated. We used the Vicia faba micronucleus test to reveal genotoxic and cytotoxic effects of untreated and treated OMWW. The MN frequency assay was carried out according to El Hajjouji et al. [26,27], El Fels et al. [28] and Zegzouti et al. [25]. The seeds of *Vicia faba* were prepared according to the normalized method AFNOR standard (NF T90-327) [44]. *V. faba* seeds were soaked in demineralized water for 24 h, then removed from the seed coat, disinfected with calcium hypochlorite $Ca(ClO)_2$ 1% for 10 min and germinated between two layers of moist absorbent cotton in a germination incubator at 25 ± 1 °C to avoid the degradation of some photosensitive molecules.

After 72 h, the primary roots of *V. faba* seeds, having reached 3–5 cm in length, were selected for the MN assay. Before transplanting the *V. faba* seedlings to soil conditions, the primary root tip was cut off (2 mm) to stimulate the emergence of secondary roots.

The LUFA soil wetted with distilled water represents the negative control (NC), and the maleic hydrazide (MH) at a concentration of 10^{-5} M was used as positive control. Five different dilutions of untreated raw OMWW (1, 2.5, 5, 10 and 20% (v/v)) and eight dilutions of treated OMWW (1, 2.5, 5, 10, 25, 50%, 75 and 100% (v/v)) were tested. For each experiment, 3 seeds were used per dilution. The moisture content of each test was maintained at 2/3 of the field capacity. After 72h of exposure, the secondary roots of the three replicates were collected separately.

2.6.2. Observation and Quantification of Micronuclei

The secondary root tips were harvested, rinsed with distilled water and fixed in Carnoy solution (glacial acetic acid/ethanol 1:3, *v*/*v*) at 4 °C overnight, rinsed again with deionized water for 10 min and then transferred to 70% ethanol before storage. Subsequently, the root tips were hydrolyzed with 1N HCl for 7 min at 58 °C. After staining the root tips with 1% aceto-orcein for 3 min, the roots were crushed to evenly distribute dividing cells. Interphase cells, as described by Ma et al. [45], El Fels et al. [28] and Cotelle et al. [46], were counted on a slide with a cover plate at 400× magnification. The MN frequencies were expressed in per 1000 cells (‰), while the mitotic index (MI) was expressed in per 100 cells %. The micronucleus test was only carried out on root tips with MI more than 2% to prevent underestimated micronucleus frequency due to altered cell proliferation rates.

2.7. Statistical Analyses

The statistical analysis was performed with repeated measures analysis of variance (ANOVA) to evaluate the significant differences between treatments. In the first approach, one-way ANOVA was conducted to test the significant difference in the radial growth rate, COD, PC removal rate of *P. chrysogenom* and genotoxicity effect at 0.05 of significant level ($p < 0.05$). Furthermore, the study also used Tukey's Honest Significance Test to evaluate whether there were any statistically significant differences from the means of the parameters under consideration. Principal Component Analysis (PCA) was performed using XLStat software V. 2021. To obtain an effective data analysis with PCA, only the dependent variables that showed significant differences between the treatments were selected for these analyses.

3. Results and Discussion

3.1. Screening and Selection of Fungi

Radial growth rates of all fungal isolates showed different peak growth rates depending on the dilution of the OMWW (Figure 1). Two-way analysis of variance (ANOVA) showed significant difference in the radial growth rates of fungi and diluted OMWW. Indeed, the radial growth rates were higher for fungal isolates for the OMWW diluted to 25%, with values of 11.42, 10, 10.57, 8 and 8.57 mm.d^{-1} for isolates M1, M2, M3, M4 and M5, respectively (Figure 1). Conversely, the results were shown for OMWW diluted to 50, 75, and 100% (raw OMWW). The effects of OMWW dilutions on fungal growth rates have a significant impact on the selection of potential fungal isolates for the biological treatment of OMWW. It can be seen that the M1 isolate was able to grow better on all OMWW dilutions tested during day 15, with growth rates of 11.42, 10.71, 7.17 and 6.57 mm.d^{-1} for OMWW diluted to 25%, 50%, 75% and raw OMWW (100%), respectively. These differences are related to the concentrations of phenolic compounds in the OMWW. Indeed, dilution is considered a mechanism by which the concentration of toxic compounds can be reduced [47]. The similar effect of dilution on the growth of fungal strains was observed by [48]; according to these authors, the highest growth rate of *Lentinula edodes* was recorded for OMWW diluted to 10% with a value of 10.72 mm.d^{-1}, while this value was 4.97 mm.d^{-1} for OMWW diluted to 20% for the same strain.

The Isolate M1 strain has the ability to grow best on the media with phenolic concentration up to 4000 mg.L^{-1}. The results suggest that M1 could be used for bioremediation of OMWW. Fungi, due to their tolerance to high loads of phenolic compounds and acidic pH, are designated as the most microorganisms used to treat OMWW [49,50].

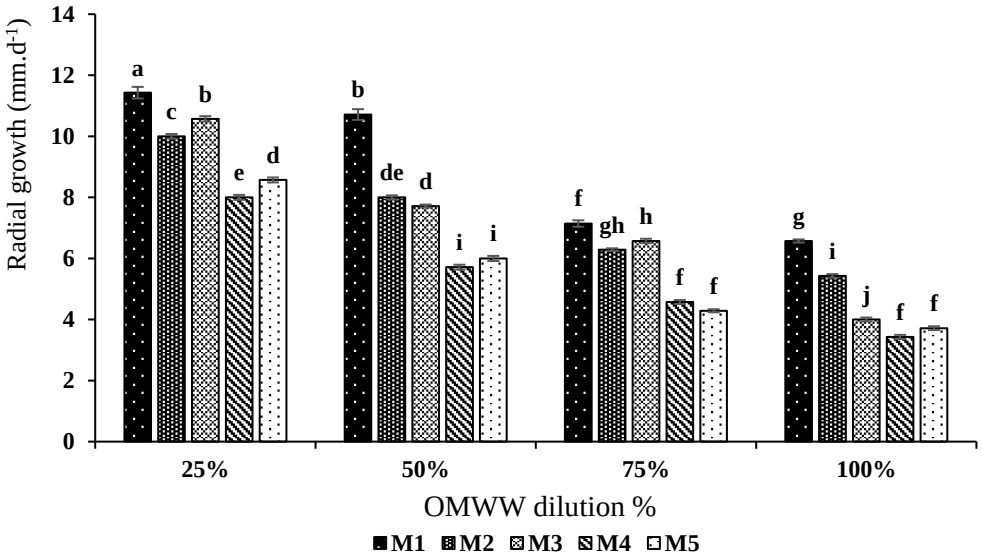

Figure 1. Radial growth of fungal isolates on different OMWW dilutions and raw OMWW. Data in the same bar graph followed by the same letter(s) were not significantly different according to Tukey HSD test ($p \leq 0.05$). (M1–M5 = fungal isolates).

3.2. Identification of Fungal Isolate

The fungal isolate M1 showed different characteristics in terms of colony morphology on both culture media (Czapek and PDA mediums) after incubation for 7 days. The colonies of the isolate had a compact yellow suede-like surface with radical streaks and transparent exudate on Czapek medium; a dense green felt-like surface with a prominent white margin on PDA medium; and flat, loose colonies with mostly white cottony mycelia and a light green spot on the medium. A similar description of the colony morphology of *P. chrysogenum* has been reported by Bandh et al. [51] and Xia et al. [52]. Microscopic morphology was observed and showed that isolate M1 had branched conidiophores and branched metules, giving brush-like clusters, which is the typical characteristic of *Penicillium* species and well-developed hyphae, as well as a large amount of spherical dispersing spores.

Genetic sequence homology of fungal isolate M1 showed 100% similarity with *P. chrysogenum* (Figure 2). Based on morphological and molecular identification, the strain was identified as *Penicillum chrysogenum.*

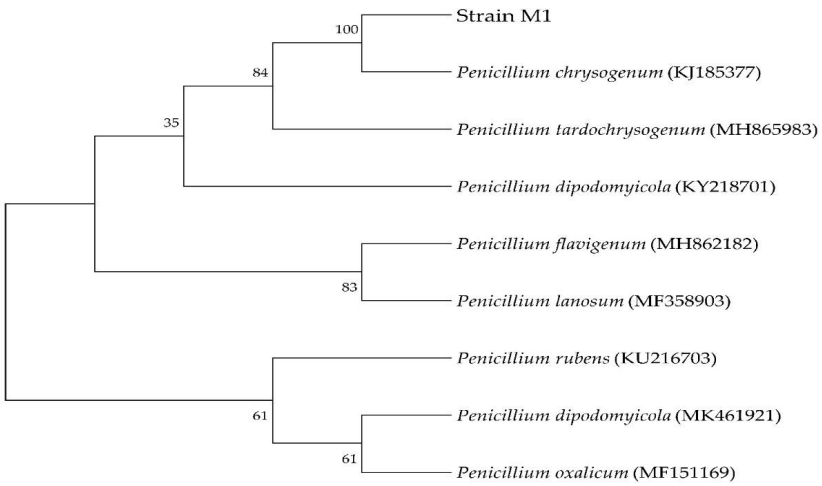

Figure 2. Phylogenetic tree of the fungal isolate M1 and other fungal species relatives based on a neighbor-joining analysis of ITS sequences. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets.

3.3. Bioremediation of OMWW by Penicillium Chrysogenum

3.3.1. Chemical Oxygen Demand Removal

The effect of agitation and nitrogen sources on the biodegradation of OMWW was investigated for raw OMWW by *P. chrysogenum* (Figure 3). Without agitation, the initial COD (187.6 g.L^{-1}) was removed by only ~4%. However, with agitation, the COD reduction increased significantly, reaching 48% and 76% at 100 rpm and 150 rpm, respectively. Nevertheless, COD abatement was decreased when increasing the agitation to 200 rpm, where the maximum reduction observed was 46%. The percentage of COD removed was significantly different depending on the agitation speeds. This can be attributed to more effective aeration of the medium to supply more dissolved oxygen for *P. chrysogenum* cultivation at agitation rates (150 rpm), but excessive agitation (200 rpm) could lead to mycelium shearing and, consequently, the biomass yield would decrease [53]. The most promising results of COD removal were reported by García García et al. [54] when cultivating *P. chrysosporium* aerobically and with agitation. Aissam et al. [55] evaluated several fungi to reduce OMWW COD, and the fungal strains reduced about 42 and 77% in the diluted OMWW at 25%, and 25–65% removal was observed for the media containing 50% of OMWW and 10–20% for OMWW diluted to 75%. In the study of Salgado et al. [56] in undiluted OMWW, the percentage of COD reduction was 1.4%, 7% and 10.9% using *A. uvarum, A. niger* and *A. ibericus,* respectively.

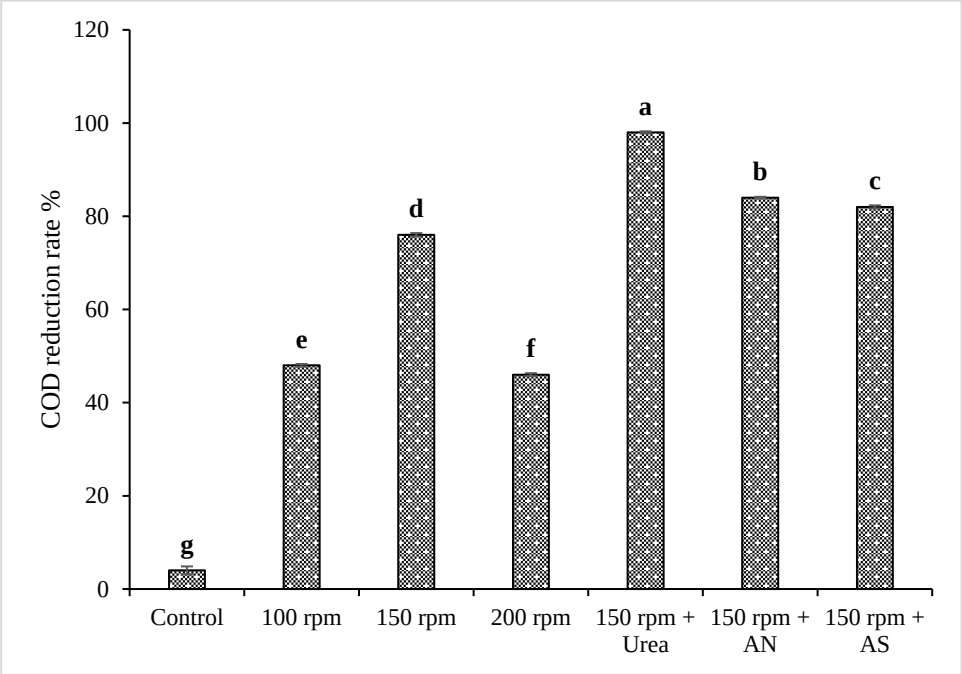

Figure 3. Effect of different agitation speeds and nitrogen source on the undiluted olive mill wastewater COD removal by *P. chrysogenum.* Data in the same bar graph followed by the same letter(s) were not significantly different according to Tukey HSD test ($p \leq 0.05$). (AN: ammonium nitrate; AS: ammonium sulphate).

When urea was added as a nitrogen source at a concentration of 3 g.L^{-1} to adjust the C/N ratio to 30, the COD removal rate at 150 rpm (76%) was significantly increased to 98% in only 12 days of incubation, with a significant difference ($p < 0.05$) compared to other treatment conditions. The addition of a nutrient supplement is performed to prevent a lack of essential nutrients such as nitrogen, which should be in agreement with high C/N ratios for an efficient microbial growth [57,58]. One strain of *A. flavus* was able to achieve 46% removal of the organic load of OMWW without dilution, while it significantly improved its degradation capabilities when the OMWW was diluted to 10% and supplemented with adequate levels of N and P and was able to remove 93% of the

initial COD values. [59]. Aissam et al. [55] confirmed the importance of adding nutrients to correct OMWW deficiencies; in fact, when raw supplemented OMWW were treated with *Candida boidinii* and *A. niger*, the COD removal values were about 45% for *C. boidinii* and 78% for *A. niger* after 15 days of incubation. Whereas when they were cultivated directly on an undiluted OMWW medium containing 82 $g.L^{-1}$ of COD, these strains eliminated only 4% of the COD.

3.3.2. Phenolic Compounds Removal

The PC removal rate by *Penicillium chrysogenum* differed with different agitation speeds, with rates of 41%, 73% and 42% for 100, 150 and 200 rpm, respectively (Figure 4). These removal rates are much higher than those recorded in the control (2%) without agitation. This suggests that agitation at 150 rpm has a positive effect on PC degradation by *P. chrysogenum*. This can be explained by the fact that the lower agitation speed could lead to insufficient oxygen concentration, while the higher agitation speed develops shear forces among the suspended fungal cells in the culture medium, and the production drops due to cell damages which results from cell collision [60]. Shear forces also can have several effects on the fungal cell. It can cause morphological changes in the fungus by damaging external and internal cell structures, variation in fungal growth and yield formation [61]; therefore; the synthesis of target product is affected [62], particularly ligninolytic enzymes [53]. Similar results to the present work were reported by Yesilada et al. [63], where reduction rates of 88% and 72% were recorded for *Coriolus versicolor* and *Funalia trogii*, respectively, at a 150 rpm agitation rate. Ergül et al. [64] also reported the positive effect of agitation on the removal rate of PCs by comparing the degradation of PCs in two culture experiments, static and 200 rpm shaking flask, and showed that there was a different removal rate of PCs from raw OMWW of about 39% and 78% under static and 200 rpm conditions, respectively, during treatment by *T. versicolor* for 20 days.

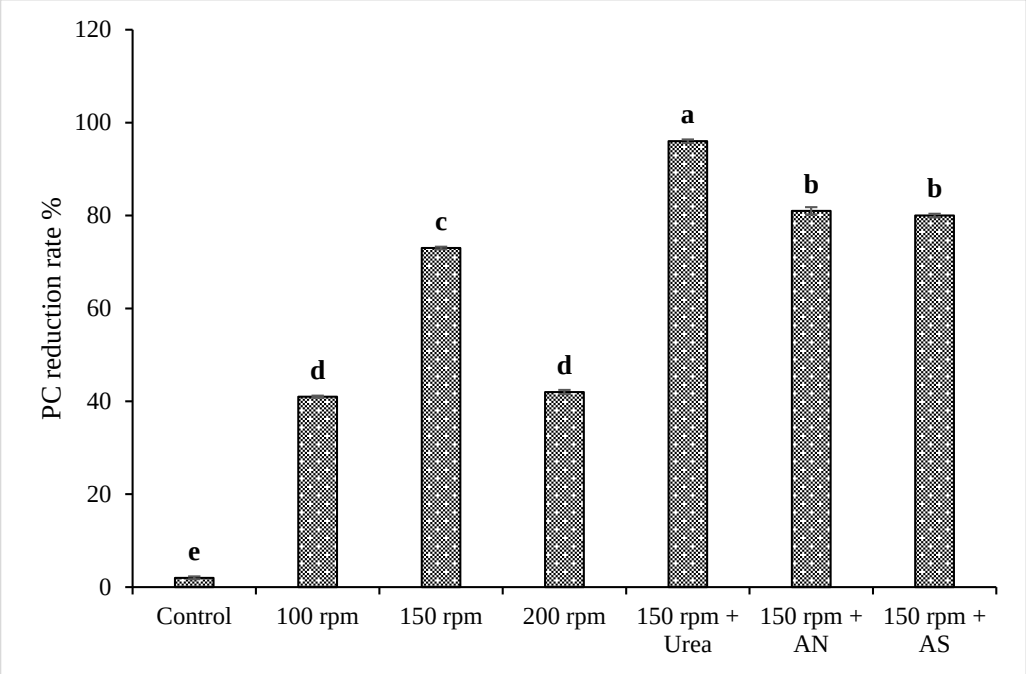

Figure 4. Effect of different agitation speeds and nitrogen source on the undiluted olive mill wastewater PC removal by *P. chrysogenum*. Data in the same bar graph followed by the same letter(s) were not significantly different according to Tukey HSD test ($p \leq 0.05$). (AN: ammonium nitrate; AS: ammonium sulphate).

OMWW supplemented with urea at 3 g.L^{-1} showed a degradation rate by *P. chrysogenum* that exceeded 96% at 150 rpm, higher than the previous screening study of Zerva et al. [53], where 86.4% and 86.1% was achieved after nitrogen supplementation for *P. citrinopileatus* when yeast extract and corn steep liquor were used, respectively. On the other hand, limited research results reported a high degree of PC removal when undiluted OMWW without additional nutrients was used.

3.4. HPLC Tool to Characterize the Phenolic Compound Profile in OMWW Extracts

The identification of phenolic compounds was carried out by comparing their retention times with those of standards (Figure 5). HPLC analysis of the phenolic extract of untreated OMW showed the presence of five compounds: hydroxytyrosol at 1.22 g.L^{-1}, tyrosol at 0.05 g.L^{-1}, caffeic acid at 0.16 g.L^{-1}, p-coumaric acid at 0.05 g.L^{-1} and oleuropein at 0.04 g.L^{-1}. The identified phenolic compounds have been previously reported in OMWW from the Marrakech region (Morocco) by Fakharedine et al. [65], Boutafda et al. [37] and El Fels et al. [66].

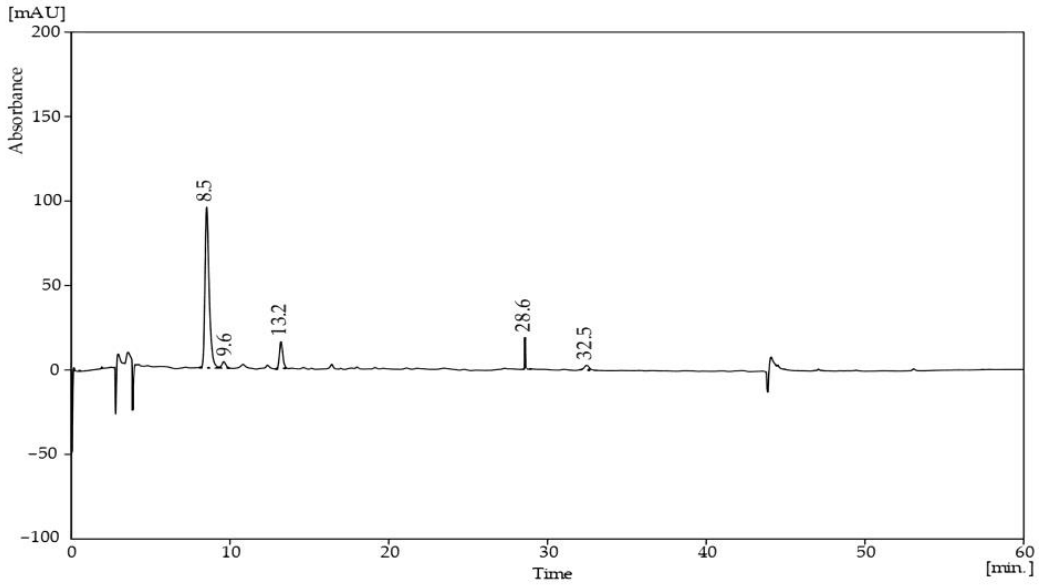

Figure 5. HPLC chromatogram of the phenolic profile of raw OMWW.

Previous work has indicated that the OMWW phenolic fraction is characterized by great complexity [67,68]. All compounds identified in the raw OMWW phenolic extracts without treatment disappeared after only 12 days of treatment by *P. chrysogenum* (Figure 6a,b) with urea at 150 rpm, except the compounds hydroxytyrosol, caffeic acid and p-coumaric acid, which persisted with a low concentration of 0.06 g.L^{-1} for hydroxytyrosol, 0.07 g.L^{-1} for caffeic acid and 0.04 g.L^{-1} for p-coumaric acid (Figure 6b). This can be explained by the biodegradation of the phenolic compounds by *P. chrysogenum* as well as by the bioconversion of the oleuropein to hydroxytyrosol as reported by Santos et al. [69].

3.5. Micronucleus Test

3.5.1. Olive Mill Wastewater Genotoxicity

The micronucleus test results of the negative control, positive control and diluted and untreated OMWW are presented in Figure 7. In fact, low micronucleus frequencies with 0.58‰ were recorded in the root tips of control plants (negative control) and the mitotic index of about 13.54%, whereas MH as a positive control significantly increased the frequency of micronuclei to a value of 28.52‰ and decreased the mitotic index to a value of 6.3%. Figure 7 shows that OMWW significantly increased the frequency of micronuclei formation in *V. faba* root cells. The highest frequency of micronuclei (27.17‰) was detected for the 10% dilution, whereas the lowest value (5.27‰) was noticed in the 1%

dilution, which was 9-fold higher than the negative control. For dilutions above 20%, roots appeared necrotic and blackened at the end of exposure, revealing acute toxicity. The high percentage of induced micronuclei may indicate the mutagenic effect of the pollutants [70]. El Hajjouji et al. [27] showed that raw OMWW was toxic at 10% concentration, and the higher concentrations (20%) were responsible for blackening of root tips and inhibition of mitosis. Inhibition of DNA synthesis is affected by OMWW toxicity, leading to the interruption of protein synthesis on *Triticum aestivum* root tips, which is probably related to the content of phenolic compounds [71].

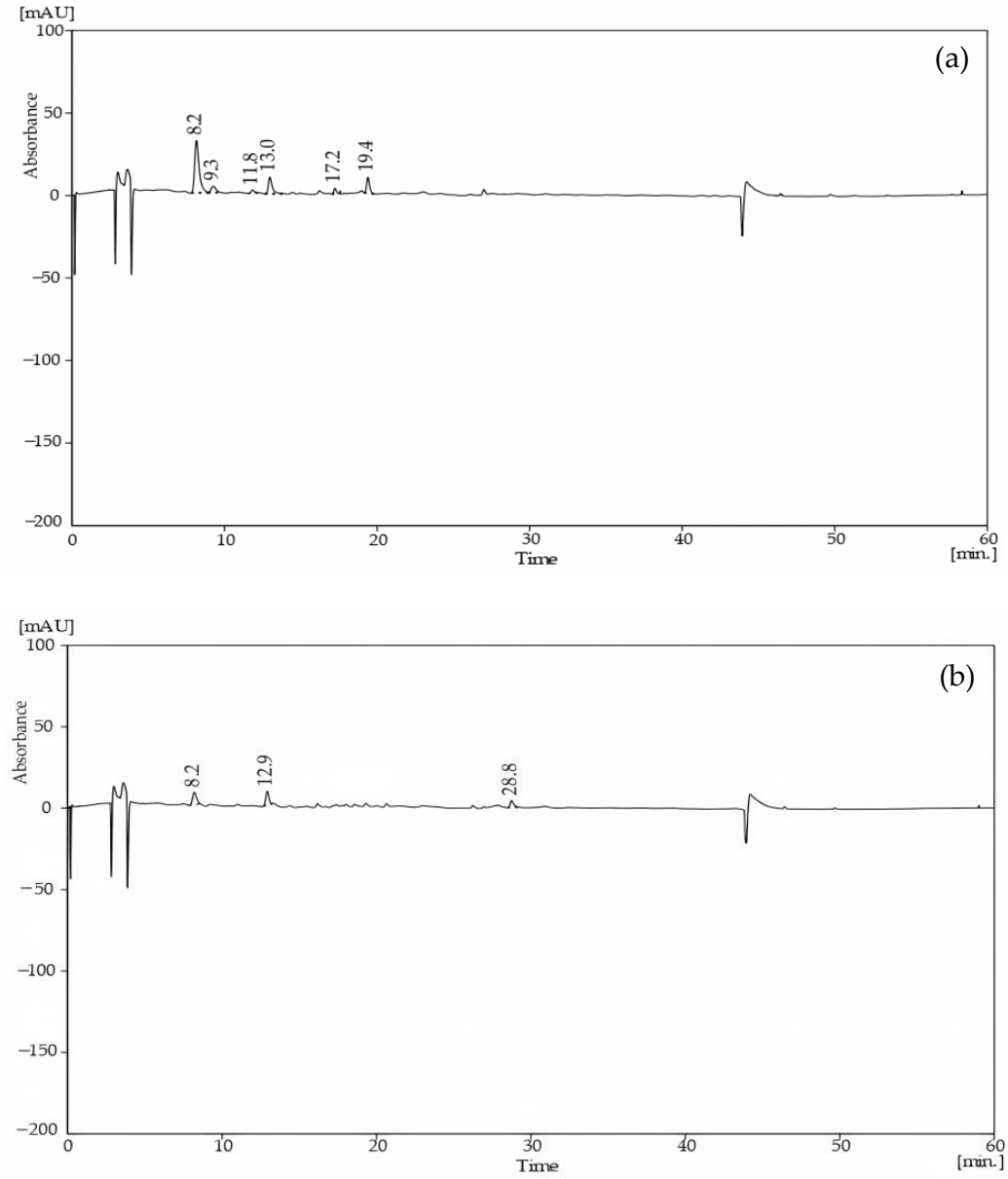

Figure 6. HPLC chromatogram of phenolic extract from olive mill wastewater after *P. chrysogenum* treatment, (**a**) agitation speed (150 rpm) and (**b**) agitation 150 rpm + nitrogen source (urea).

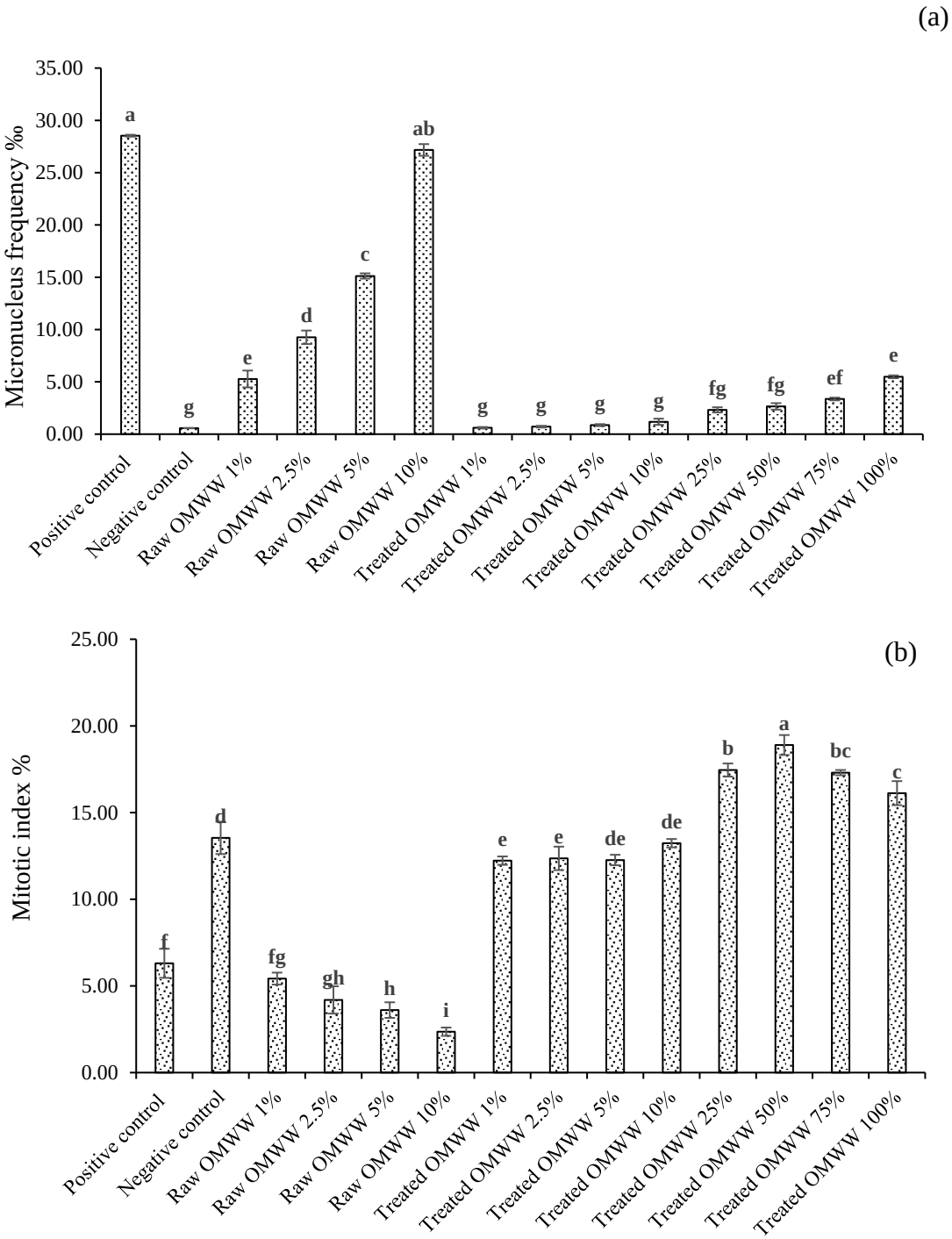

Figure 7. Micronucleus frequency (**a**) and mitotic index (**b**) values in *Vicia faba* roots exposed to different dilutions of treated and untreated OMWW. Data in the same bar graph followed by the same letter(s) were not significantly different according to Tukey HSD test ($p \leq 0.05$).

Regarding the mitotic index, a progressive decrease was noticed from 5.42% to 2.36% for dilutions from 1% to 10%, respectively. The reduction in the mitotic index in our study may have been due to the blockage of the G2 phase of the cell cycle or the inhibition of DNA synthesis [72]. This reduction in the mitotic index may have also been caused by altered nucleoprotein synthesis and reduced ATP levels to provide energy for spindle assembly and chromosome movement [73].

3.5.2. OMWW Genotoxicity Assessment after Fungal Treatment

The results of MN frequency and MI in *V. faba* cells roots exposed to different dilutions of treated OMWW (1%, 2.5%, 5% 10%, 25% 50% 75% and 100% (raw OMWW)) are presented in Figure 7a,b. Raw OMWW is highly toxic and cannot induce neither MI nor MN. However, 10% of raw OMWW induced high genotoxicity with 27.17‰ MN (no significantly different to positive control = 28.53‰) and 50-fold higher than the negative control. After 12 days of treatment with *P. chrysogenum*, the OMWW showed a noticeable decrease of the genotoxic effect and enhancement of the mitotic index in all dilutions compared to the untreated OMWW, even for the raw treated OMWW ($p < 0.05$). The biological treatment strongly reduced the micronucleus frequency by 86.34%, 92.22%, 94.25% and 95.66% for the treated OMWW (1, 2.5, 5, 10%), respectively, and enhanced the mitotic index by 55.64%, 66.06%, 70.59% and 82.17% for the treated OMWW (1, 2.5, 5, 10%), respectively (Figure 7a,b). This strong decrease in MN frequencies proved the biodegradation activity of *P. chrysogenom* and its ability to eliminate genotoxic compounds, such as phenolic compounds (96% of reduction). The PCA analysis showed a high positive correlation rate between phenolic compounds and micronucleus frequency in *Vicia faba* cell roots in raw OMWW. A significant decrease of MN with the abatement of PC after 12 days of aerobic treatment with *P. chysogenum* was also shown (Figure 8). This could explain the efficiency of fungi biological treatment to remove the toxicity of phenolic compounds. Indeed, fungi are capable of producing a wide variety of enzymes that can degrade recalcitrant organic compounds such as phenolic compounds and mineralize other substances [74]. According to Aggelis et al. [75], the toxicity of OMWW on *Lepidium sativum* seeds decreased significantly after biological treatment with *P. ostreatus*. These authors showed the ability of some strains of *P. ostreatus* to eliminate phenolic compounds (86%) from a diluted OMWW (50%) and, consequently, the decrease in toxicity.

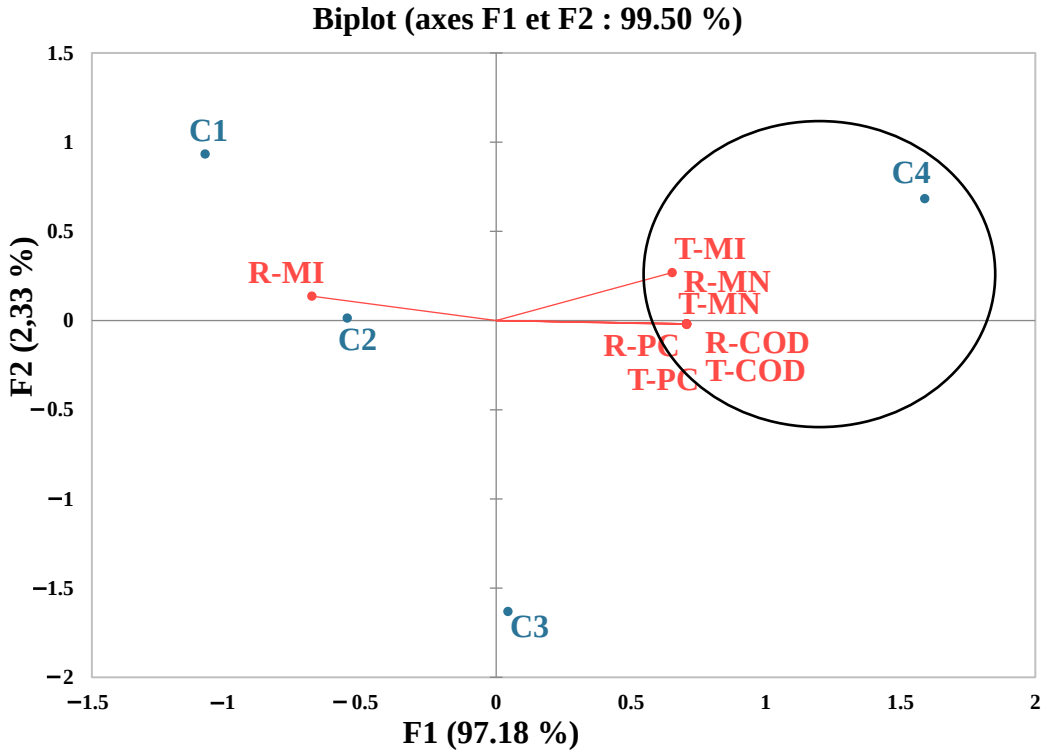

Figure 8. The principal component analysis (PCA) of the studied parameters. COD: chemical oxygen demand; PC: phenolic compounds; MN: micronucleus frequency; MI: mitotic index; C1: OMWW 1%; C2: OMWW 2.5%; C3: OMWW 5%; C4: OMWW 10%; R: raw OMWW; T: treated OMWW.

4. Conclusions

Fungal strain as a biological treatment was able to remove 98% and 96% of the initial COD and PC concentrations, respectively, under optimum conditions at an agitation speed of 150 rpm and with urea as a nitrogen source. The most important phenolic compounds in raw OMWW identified by HPLC were eliminated after 12 days of treatment. Morphological and molecular identification revealed that the fungal isolate belonged to the species *Penicillium chrysogenum*. The efficiency of this fungal strain to reduce the genotoxicity of OMWW was demonstrated by a significant reduction in the micronucleus frequency of *V. faba* roots during treatment up to 95.66%. Overall, these results show that the biological treatment of OMWW, using *P. chrysogenum*, provided very promising results in terms of reduction the pollutant load and elimination of genotoxicity, and therefore in terms of the importance of testing the feasibility of this bioprocess on a larger scale and serving as a model for a socio-economic evaluation of the establishment of a semi-industrial plant. In this way, the effluents obtained after treatment can be used as a liquid biofertilizer for certain plants such as maize, wheat and tomato.

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