# Assessment of the genotoxicity of olive mill waste water (OMWW) with the *Vicia faba* micronucleus test

H. El Hajjouji [a,b], E. Pinelli [b,*], M. Guiresse [b],
G. Merlina [b], J.-C. Revel [b], M. Hafidi [a]

[a] *Laboratoire d'Ecologie Végétale, Sol et Environnement, Département de Biologie, Faculté des Sciences*
*Semlalia, Université Cadi Ayyad, BP 2390, Marrakech, Maroc*
[b] *Ecole Nationale Supérieure Agronomique de Toulouse, EcoLab, UMR 5245 CNRS-UPS-INPT,*
*Avenue de l'Agrobiopole – BP 32607, Auzeville Tolosane, 31326 Castanet-Tolosan, France*

**Abstract**

The present study concerns the genotoxicity of olive mill waste water (OMWW) generated in mills producing olive oil in Morocco. The *Vicia faba* micronucleus test was used to evaluate the genotoxicity of OMWW and the six major phenolic compounds identified by HPLC in this effluent. Five dilutions of OMWW were tested: 0.1, 1, 5, 10 and 20%. Maleic hydrazide was used as a positive control. The results showed that OMWW was genotoxic at 10% dilution. In order to investigate the components involved in this genotoxicity, the six major phenols present in this effluent, oleuropein, gallic acid, 4-hydroxyphenyl acetic acid, caffeic acid, paracoumaric acid and veratric acid, were studied at concentrations corresponding to the genotoxic concentration of the OMWW itself. Two phenols, gallic acid and oleuropein induced a significant increase in micronucleus frequency in *Vicia faba*; the four other phenols had no significant genotoxic effect. These results suggest that under the experimental conditions of our assay, OMWW genotoxicity was associated with gallic acid and oleuropein.

*Keywords:* Olive mill waste water; Genotoxicity; Micronucleus assay; *Vicia faba*; Phenolic compounds

## 1. Introduction

Olive mill waste water (OMWW) can cause serious environmental hazards in olive-producing countries, especially around the Mediterranean basin. It has been estimated that $30 \times 10^6 \, m^3$ of waste water are produced per year [1]. In Morocco, olive mills belong to the foremost polluters: the volume of OMWW produced annually is estimated at $180\,000 \, m^3$ during the production season [2]. Most frequently, OMWW is pumped and discharged into evaporation ponds or directly dumped in rivers or spread on soil [3,4]. The effect on the environment is negative, leading to a saturation of the soil, causing pollution of superficial groundwater and of the water table itself. This unfavourable effect of OMWW on the environment is exacerbated by its acidity and high phenol content. Only few studies deal with analysis and identification of phenols in olive waste water.

It is well known that phenolic compounds are major contributors to the toxicity and the antibacterial activity of OMWW. They limit its microbial degradability [4,5].

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* Corresponding author. Tel.: +33 5 62 19 39 45;
fax: +33 5 62 19 39 01.

*E-mail address:* pinelli@ensat.fr (E. Pinelli).

The application of dry olive mill residues decreased the dry weight of tomato and soybean plants due to its phenolic content [6]. Phenols in seeds have also been proposed as germination inhibitors [7]. The presence of phenols causes the inhibition of germination of *Atriplex Triangularis* and *Pinus laricio* seeds [8]. One of the phenolic compounds, gallic acid significantly reduced larval growth of *S. frugiperda* neonates [9]

In addition, phenols exert other toxic and genotoxic effects on animal and human cells. For example, the exposure of Syrian hamster embryo cells to phenol and catechol induced cell transformation, gene mutations, unscheduled DNA synthesis, chromosomal aberrations and sister chromatid exchange [10]. *In vitro* studies with human lymphocytes showed that catechol and phenol significantly induced the formation of micronuclei and also increased the number of kinetochore-positive micronucleated cells [11].

However, phenolic compounds possess strong antioxidant properties [12], which may turn the olive-oil residues into an affordable source of natural antioxidant. They may also play a major role in preventing chronic human diseases [13]. Many phenolic compounds, e.g. caffeic acid, tyrosol and 4-hydroxybenzoic acid are well-known precursors in the pharmaceutical, chemical and food industries [14].

*Vicia faba* has been used for evaluating chromosomal aberrations since the early 1920s [15,16]. In addition, the detection of micronuclei in *Vicia* root tips has been more recently developed to evaluate the genotoxicity in some different matrices, including water [17], soils [16–18], waste water and industrial effluent [19]. No study, however, has been conducted to test potentially polluted OMWW with this method. In the present work, the *Vicia* root-micronucleus assay was chosen to assess genotoxicity induced by different concentrations of raw OMWW and of the six major phenols found in this effluent, in order to identify the genotoxic compounds.

## 2. Materials and methods

### 2.1. Origin of olive mill waste water

The waste water was taken from a modern three-phase centrifugation olive mill in Marrakech (Morocco). It was transported in 5-l bottles and refrigerated at 4 °C until required for analysis and exposure experiments.

### 2.2. Physico-chemical analysis

The main physico-chemical characteristics of raw OMWW are reported in Table 1. The pH and the electrical conductivity were measured at ambient temperature following the recom-

Table 1
Composition of raw OMWW

| Parameters | Values |
|---|---|
| pH | $4.8 \pm 0.01$ |
| Electrical conductivity (ms/cm) | $44.3 \pm 0.2$ |
| Dry matter (g/L) | $168.93 \pm 7.38$ |
| Organic matter (g/L) | $129.93 \pm 6.72$ |
| Ashes (g/L) | $39 \pm 0.71$ |
| Total phenols (mg/L) | $970 \pm 8.64$ |
| Chemical oxygen demand (COD) (g/L) | $135 \pm 4$ |
| $Na^+$ (g/L) | 2.10 |
| $Cl^-$ (mg/L) | 954.55 |

mendations of Rodier [20]. The dry matter was measured after drying the OMWW at 105 °C for 24 h and the organic matter was weighed by determining the loss-on ignition at 550 °C for 24 h. The phenolic compounds were extracted using the method of Macheix et al. [21]. The extraction was carried out with methanol (80%) in the presence of phosphoric acid and ammonium sulphate. The phenols were assayed with the Folin-Ciocalteu reagent [22]. The chemical oxygen demand (COD) was determined according to Rodier [23]. Concentrations of sodium and chloride were determined by ion chromatography with a Dionex high-pressure liquid ionic chromatograph DX-100 (Ionpac CS-12A and AS4A-SC, Dionex Co., Sunnyvale, CA, USA).

### 2.3. Characterization of phenols by HPLC

Analytical HPLC was conducted on a Hewlett-Packard (HP) 1100 liquid chromatograph, fitted with a PRP-1 reverse-phase column (Hamilton). Phenolic compounds were detected with a UV diode-array detector (HP 1110) set at 280, 320 and 350 nm. One μL of phenolic compounds extracts of OMWW were injected into the HPLC. The best resolution was obtained with an elution gradient with a constant flow rate of 1 mL/min and a mobile phase consisting of a mixture of acetonitrile 5% (A) and distilled water 90% (B) at pH 2.6 adjusted with orthophosphoric acid. The mobile phase composition started at 5% of solvent A and 95% of solvent B for 5 min, followed by a linear increase of solvent A to 80% and a decrease of solvent B to 20% over the next 65 min; the initial conditions were reached again 70 min after the start. The phenolic compounds were identified by their retention times in comparison with commercial standards (oleuropein, gallic acid, 4-hydroxyphenyl acetic acid, caffeic acid, paracoumaric acid and veratric acid). The chemical structures of these phenols are presented in Fig. 1.

### 2.4. Micronucleus test

Seeds of *Vicia faba* that had been stored at 4 °C were used for this study. The Vicia test was carried out according to Knassmüller et al. [18], Ma et al. [24], and in a normalized method [25]. Dry seeds of *Vicia faba* were soaked for 24 h in deionised water, the seed coats were removed and the seedlings
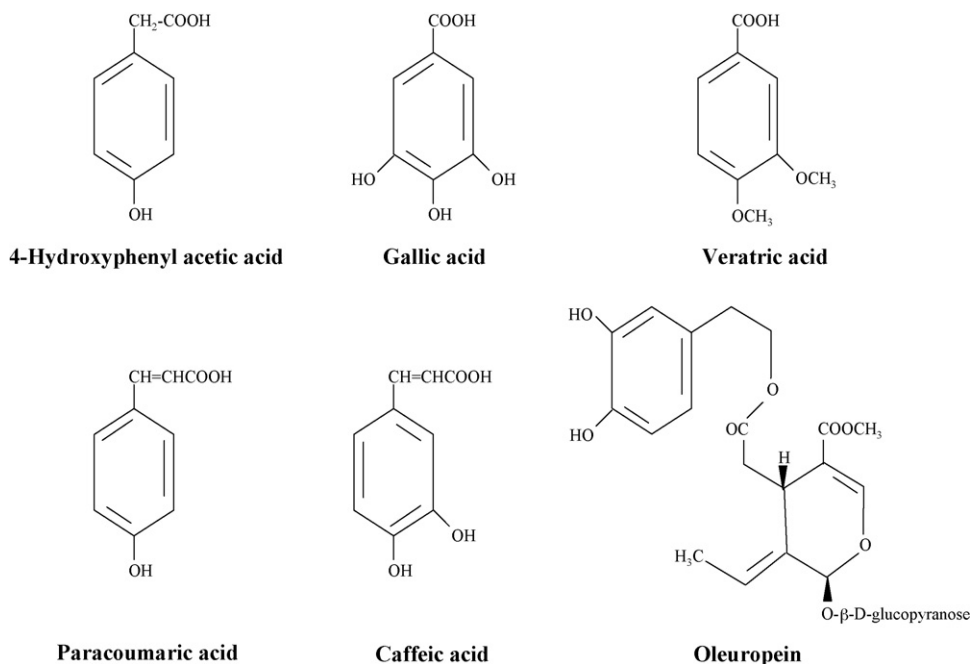
Fig. 1. Chemical structures of the phenols studied.

were allowed to germinate between two layers of moist cotton. After 3 days, the primary roots at about 2–3 cm in length were suspended in Hoagland's solution and their tips cut off in order to let the secondary roots grow. Four days were necessary to obtain secondary roots of suitable length (1–2 cm) for the test. Exposure time was 30 h for the negative control and 6 h for the treated groups followed by a 24 h recovery period. Five different concentrations of OMWW were tested (0.1, 1, 5, 10 and 20% v/v). In addition, the six major phenols identified in the OMWW (oleuropein, gallic acid, 4-hydroxyphenyl acetic acid, caffeic acid, paracoumaric acid, and veratric acid) were tested. For each phenol, the final concentration used corresponds to its respective concentration in the 10% OMWW (Table 2). For each experiment five seeds were used per treatment. Maleic hydrazide (40 μM) was used as a positive control. Aerated Hoagland's solution was used as a negative control. After treatment, root tips were fixed in Carnoy's solution (glacial acetic acid/ethanol 1:3) at 4 °C overnight and transferred into 70% ethanol for storage. They were then hydrolyzed in 1 N HCl at

60 °C for 5–7 min. Five slides were prepared for each of the five seeds. After staining the root tips with 1% aceto-orcein, the interphase cells as defined by Ma et al. [24] were scored for micronucleus frequencies at 1000× magnification. Five thousand cells per tip were counted.

In order to avoid underestimation of the micronucleus frequency due to impaired cell proliferation rate, the micronucleus test was performed only on root tips with a mitotic index superior to 2% [26].

### 2.5. Statistical analysis

A statistical analysis was performed on the collected data. The mean value of the negative and positive controls and the OMWW groups was obtained from descriptive analysis, and One ANOVA Way test was conducted to obtain $F$ values and MS errors. Tukey's test [27] was used to determine the level of significance against the negative and the positive control values in each experimental series.

## 3. Results

### 3.1. Characterization of phenols by HPLC

The use of HPLC to study the phenolic compounds in raw OMWW revealed the presence of 36 compounds (data not shown). Six of the major phenolic compounds present in the OMWW were identified in comparison with commercial standards (Table 2). Two of these, 4-hydroxyphenyl acetic acid (HA) and veratric acid (VA)

Table 2
Phenol concentration analysis in raw and 10% dilution of OMWW

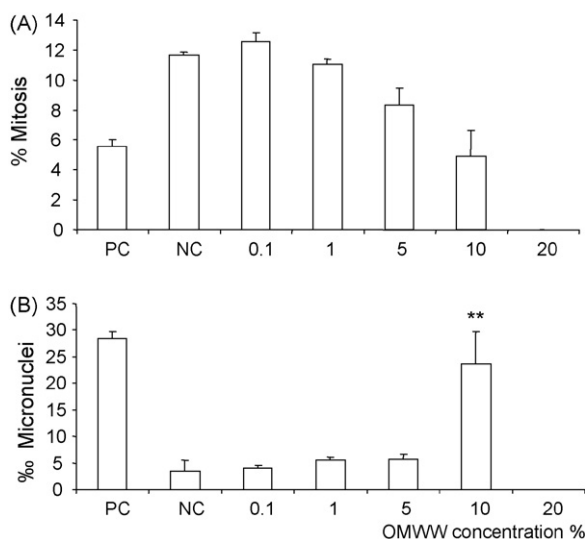| Phenols | Raw OMWW (μM) | 10% OMWW (μM) |
|---|---|---|
| 4-Hydroxyphenyl acetic acid (HA) | 3086.4 | 308.6 |
| Veratric acid (VA) | 1762.4 | 176.2 |
| Paracoumaric acid (PA) | 572.2 | 57.2 |
| Gallic acid (GA) | 361.1 | 36.1 |
| Oleuropein (Ol) | 56.7 | 5.7 |
| Caffeic acid (CA) | 31.9 | 3.2 |

Fig. 2. Mitotic index (A) and micronucleus frequency (B) values in *Vicia faba* roots exposed to different OMWW concentrations (0.1, 1, 5, 10 and 20%). PC: positive control; NC: negative control. [**]$p < 0.001$ against the NC.

were present at concentrations of, respectively, 3086.4 and 1762.4 µM. Paracoumaric acid (PA) and gallic acid (GA) were present at 572.2 and 361.1 µM, respectively, and oleuropein (Ol) and caffeic acid (CA) were present at lower concentrations of, respectively, 56.7 and 31.9 µM.

### 3.2. Micronucleus test of OMWW

The results of the *Vicia faba* root-micronucleus test of five concentrations of OMWW samples collected in an olive oil-producing factory are presented in Fig. 2. For concentrations of OMWW superior to 10%, a blackening of the root tips appeared and a loss of mitosis was observed (Fig. 2A). Under these conditions the quantification of micronuclei was impossible. For lower concentrations of OMWW from 0.1 to 5%, no significant increase in micronucleus frequency was recorded, compared with the negative control (Fig. 2B). A significant increase in micronucleus frequency was observed in *Vicia faba* roots exposed to 10% OMWW. A digital picture of the micronuclei induced by OMWW is presented in Fig. 3. Under these conditions, the number of micronuclei was not significantly different from the positive control and underlines the genotoxic potential of this effluent. In order to investigate the molecules involved in this genotoxicity, the six major phenols present in this effluent were studied at concentrations corresponding to that giving the maximum genotoxic effect of the OMWW (10%).
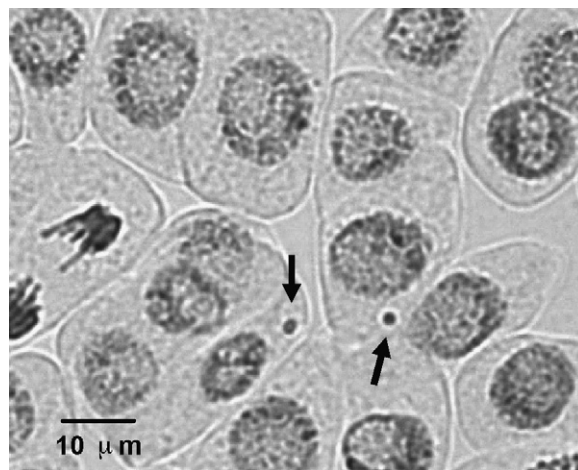


Fig. 3. Micronuclei induced by OMWW in *Vicia faba* root cells. Micronuclei are marked with an arrow.

### 3.3. Micronucleus test of phenols

The data in Fig. 4 show the effect of the six phenols tested at concentrations corresponding to those of 10% OMWW (Table 2). For all the phenols tested separately, the mitotic index ranged between 2 and 17%. Apart from GA and Ol, the four other phenols did not induce significant genotoxicity. GA induced a strong increase in micronucleus frequency, 7.5-fold above the negative control and not significantly different from the positive control (27‰ ± 7.7). Ol significantly increased the micronucleus frequency to a level that was twice as
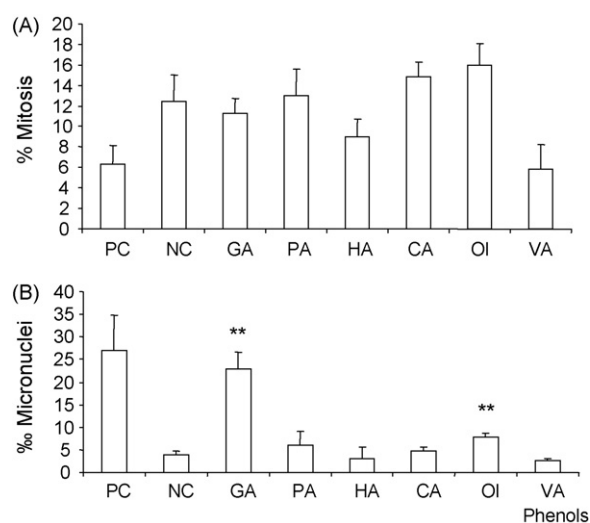


Fig. 4. Mitotic index (A) and micronucleus frequency (B) values in *Vicia faba* roots exposed to different phenol concentrations. PC: positive control; NC: negative control; HA: 4-hydroxyphenyl acetic acid; VA: veratric acid; PA: paracoumaric acid; GA: gallic acid; Ol: oleuropein; CA: caffeic acid. [**]$p < 0.001$ against the NC.

high as the negative control, but 3.5 times below the positive control.

## 4. Discussion

In this study the *Vicia faba* micronucleus test, which detects chromosomal breakage and aneuploidy, was employed in order to evaluate the genotoxic potential of OMWW. It is now well established that OMWW is toxic for bacteria, aquatic organisms and plants [28]. The results of Fig. 2 illustrate this toxicity: at a concentration of 20% of OMWW in Hoagland's solution, a blackening of the root tips and a loss of mitosis was observed. Microscopic observation revealed necrotic cells and disorders in root-tip tissue (data not shown). Further physicochemical characteristics of OMWW are in line with these toxic effects. OMWW contains nitrogenous compounds, polyphenols, volatile acids and polyalcohols [29]. The concentration of phenols, which reached up to 900 mg/L, could also have contributed to the high toxicity of the OMWW. At concentrations below 20% the toxicity disappeared and the number of mitoses became normal. Under these conditions, the 10% OMWW concentration induced a significantly higher *Vicia faba* micronucleus frequency than was observed in the negative control. These data illustrated the genotoxic potential of the OMWW. At lower concentrations the genotoxicity was no longer detected. The *Vicia faba* micronucleus test is a very sensitive and useful method that allows detection of both clastogenic and aneugenic effects [30]. Micronuclei are the result of chromosome breaks or mitotic anomalies that require a passage through mitosis to be recognisable. The molecular mechanism of DNA breakdown is not yet clearly understood. Although it does not constitute the only genotoxic pathway, oxidative stress was described to play a major role in DNA-damage induction [31]. Because phenolic compounds are important organic components of OMWW and because they promote oxidative stress [32], the present study was focused on these molecules.

In the literature there are no studies describing the genotoxic effects of phenols in plants. However, many studies on different cultured cell lines [11,33] or bacteria, e.g. the *Salmonella* mutagenicity assay (Ames test) demonstrated the genotoxic potential of some phenols. In the present work, two phenols, gallic acid (GA) and oleuropein (Ol) induced a significant increase in micronucleus frequency, whereas 4-hydroxyphenyl acetic acid (HA), veratric acid (VA), paracoumaric acid (PA) and caffeic acid (CA) did not show a genotoxic effect. It has been demonstrated that phenolic compounds can spontaneously oxidise at pH above neutrality,

giving rise to the superoxide anion [34] and semiquinone intermediates [32]. The superoxide anion can initiate a free-radical chain reaction with simultaneous production of other reactive oxygen species, such as hydrogen peroxide and the hydroxyl radical. The hydroxyl radical is the ultimate reactive oxygen species that interacts with DNA and promotes genetic damage.

In previous studies, Tayama and Nakagawa [35] demonstrated that GA autoxidation was accompanied by the production of active oxygen species. In yeast, the genotoxicity of GA increases when autoxidation is accelerated [36]. Duarte et al. [37] showed that several phenolic compounds present in roasted coffee (caffeic acid, cathecol, pyrrogallol and chlorogenic acid) in the presence of $Fe^{3+}$/EDTA show the ability to promote the degradation of deoxyribose, especially at pH values above neutral. This activity was mediated by hydrogen peroxide since it was inhibited by catalase. In the same way, Jacobi et al. [38] reported that propyl gallate damages DNA synergistically with Cu(II) and they postulated that the effect was due to reactive oxygen species produced as a result of a redox reaction between propyl gallate and copper. In our experimental conditions, the pH value was slightly acid, around 6.5 in all the experiments and Hoagland's solution contained Fe-tartrate and copper sulphate. The presence of Fe (44.5 $\mu$M) and Cu (0.15 $\mu$M) could contribute to the formation of the hydroxyl radical generated from $H_2O_2$ by the Fenton reaction [39]. Do Céu Silva et al. [32] reported that cathecol and pyrogallol show a clear clastogenic effect in a pH-dependent way. At pH 6, only pyrogallol at high concentration significantly increased the level of chromosomal aberrations in V79 cells. These results could partly explain the different effects of the phenols in the *Vicia faba* micronucleus assay. The genotoxicity of phenolic compounds could depend on (i) their concentration (which is different in all the experiments), (ii) the pH of the culture medium and (iii) the presence of metal ions (particularly Fe and Cu). The concentration of caffeic acids in our experiments was particularly low in comparison with doses used in the literature.

Some studies noted the antioxidant potential of phenols from olives [40,41]. On the basis of *in vitro* antioxidant activity, Roche et al. [42] demonstrated that oleuropein (Ol) emerges as a strong antioxidant in olives – more than hydroxytyrosol and caffeic acid – in agreement with the ranking provided by the DPPH scavenging test in methanol. Babish and Visioli [13] studied the cytotoxicity of Ol on human gingival fibroblast in culture. They found that the toxicity of Ol was higher than that of caffeic acid and coumaric acid. The authors suggested that the toxicity of these molecules should also

be associated with the formation of $H_2O_2$ in the culture media. An adverse effect of Ol was also reported by Nousis et al. [33]: in a search for compounds able to protect nuclear DNA in cells exposed to oxidative stress, these authors used the COMET assay in Jurkat cells to demonstrate the capacity of olive-oil extracts and OMWW to prevent – at low concentrations – the $H_2O_2$-induced formation of single-strand breaks in the DNA. They also showed that at higher concentrations, these products were also able to induce DNA damage by themselves. In addition, they found that in the same experimental system, Ol exerted a genotoxic effect while hydroxytyrosol and CA were protective. These contradictory results illustrate the ambivalence of these phenols, which show different effects dependent on the different experimental conditions.

Finally, the *Vicia faba* micronucleus test seems to confirm the genotoxicity *in vivo* of the two natural products GA and Ol. The results suggest that in spite of the similarity of structure between the different molecules studied, the effects are different. Do Céu Silva et al. [32] suggested that the mechanisms of genotoxicity are dependent on the structural features of the molecules, since phenol itself is not genotoxic while the compounds with two OH groups showed lower genotoxic activity than pyrogallol with three OH groups. With three OH groups, GA is very similar to pyrogallol and exhibits in our experimental conditions an important genotoxic effect, equivalent to that of the positive control (maleic hydrazide). Further work is now necessary to determine the *in vivo* mechanism of genotoxicity of these two phenols in *Vicia faba* root tips.

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