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Revue des sciences de l'eau
Journal of Water Science

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Volume 32, Number 1, 2019

Received 25 October 2018, accepted 21 November 2018

URI: https://id.erudit.org/iderudit/1059877ar
DOI: https://doi.org/10.7202/1059877ar

See table of contents

Publisher(s)

Université du Québec - INRS-Eau, Terre et Environnement (INRS-ETE)

ISSN

1718-8598 (digital)

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Cite this article

Bouslimi, H., Jouili, H., Caçador, I. & Sleimi, N. (2019). Assessment of phenol compound removal from olive oil mill wastewater by using peroxidases extracted from radish and nettle leaves. *Revue des sciences de l'eau / Journal of Water Science*, *32*(1), 13–19. https://doi.org/10.7202/1059877ar

Article abstract

The aim of this work is to evaluate the degree of degradation phenolic compounds in olive mill wastewater (OMW), using crude plant peroxidases. In fact, OMW was treated with crude peroxidases extracted from radish (*Raphanus sativus* L.) and nettle (*Urtica urens* L.) leaves. A significant reduction of more than 60% of total phenols was observed in OMW incubated with peroxidase crude extract during seven days. The present chromatographic data obtained by high performance liquid chromatography (HPLC) show that enzymatic treatment may change the composition of the polyphenols contained in the OMW. Thus, we observed a significant decrease in some phenolic acid levels, such as gallic acid, *p*-coumaric acid and hydroxytyrosol, and the disappearance of vanillic acid, compared to the non-treated OMW. Finally, phytotoxicity of the treated and non-treated OMW was tested by means of young sunflower plants (*Helianthus annuus*) grown in hydroponic medium. Our results showed that sunflower plants grow normally when the nutrient medium contains treated OMW; whereas they fade rapidly in the presence of non-treated OMW. We conclude that treatment of OMW with radish and nettle crude extracts could attenuate OMW phytotoxicity considerably.

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ASSESSMENT OF PHENOL COMPOUND REMOVAL FROM OLIVE OIL MILL WASTEWATER BY USING PEROXIDASES EXTRACTED FROM RADISH AND NETTLE LEAVES

Évaluation de l'élimination des composés phénoliques des margines en utilisant des peroxydases extraites de feuilles de radis et d'ortie

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Received 25 October 2018, accepted 21 November 2018

ABSTRACT

The aim of this work is to evaluate the degree of degradation phenolic compounds in olive mill wastewater (OMW), using crude plant peroxidases. In fact, OMW was treated with crude peroxidases extracted from radish (*Raphanus sativus* L.) and nettle (*Urtica urens* L.) leaves. A significant reduction of more than 60% of total phenols was observed in OMW incubated with peroxidase crude extract during seven days. The present chromatographic data obtained by high performance liquid chromatography (HPLC) show that enzymatic treatment may change the composition of the polyphenols contained in the OMW. Thus, we observed a significant decrease in some phenolic acid levels, such as gallic acid, *p*-coumaric acid and hydroxytyrosol, and the disappearance of vanillic acid, compared to the non-treated OMW. Finally, phytotoxicity of the treated and non-treated OMW was tested by means of young sunflower plants (*Helianthus annuus*) grown in hydroponic medium. Our results showed that sunflower plants grow normally when the nutrient medium contains treated OMW; whereas they fade rapidly in the presence of non-treated OMW. We conclude that treatment of OMW with radish and nettle crude extracts could attenuate OMW phytotoxicity considerably.

Key words: olive mill wastewater, phenols, plant peroxidases, nettle, radish.

RÉSUMÉ

Le but de ce travail est d'évaluer le degré de dégradation des composés phénoliques contenus dans les margines en utilisant des peroxydases végétales brutes. En fait, la margine a été traitée avec des peroxydases brutes extraites de feuilles de radis (*Raphanus sativus* L.) et d'ortie (*Urtica urens* L.). Une réduction

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ISSN : 1718-8598

significative de plus de 60 % des phénols totaux a été observée dans la margine incubée avec un extrait brut de peroxydase pendant sept jours. D'autre part, le profil chromatographique obtenu par chromatographie liquide à haute performance (HPLC) a montré que le traitement enzymatique est capable de changer la composition des polyphénols contenus dans la margine. Ainsi, nous avons observé une diminution significative de certains niveaux d'acides phénoliques, tels que l'acide gallique, l'acide p-coumarique et l'hydroxytyrosol, et la disparition de l'acide vanillique, en comparant à la margine non traitée. Enfin, la phytotoxicité des margines traitées et non traitées a été testée à l'aide de jeunes plants de tournesol (*Helianthus annuus*) cultivés en milieu hydroponique. Nos résultats montrent que les plantes de tournesol poussent normalement lorsque le milieu nutritif contient des margines traitées, alors qu'elles se fanent rapidement en présence des margines non traitées. Ainsi, il semble que le traitement de margine avec des extraits bruts de radis et d'ortie pourrait considérablement atténuer leur phytotoxicité.

Mots-clés : margines, phénols, peroxydases végétales, ortie, radis.

1. INTRODUCTION

The increase of olive oil production activity generates large quantities of wastewater which could cause environmental damage (SALMAN *et al.*, 2014; ESFANDYARI *et al.*, 2015). In fact, olive mill wastewater (OMW) can be considered as a potential pollutant which could affect seed germination, plant growth (SAADI *et al.*, 2013; MASSOUDINEJAD *et al.*, 2014; RUSAN *et al.*, 2015), physicochemical soil properties (BUCHMANN *et al.*, 2015), microorganisms (MEKKI *et al.*, 2007), groundwater and aquatic organisms (BOUKHOUBZA *et al.*, 2008). The OMW toxicity was attributed to the presence of non-degradable compounds such as the phenolic compounds which could be present at high levels (4-15 $g \cdot L^{-1}$) (EL HAJJOUJI *et al.*, 2007). To reduce the OMW toxicity, several treatment methods have been developed: physical (PARASKEVA and DIAMADOPOULOS, 2006), chemical (HODAIFA *et al.*, 2013) and biological (DEMIAN and MAKRIS, 2013; SALMAN *et al.*, 2014; DAÂSSI *et al.*, 2016). Several studies have shown that biological treatment based on enzymatic incubation has more advantages than the physical and chemical treatments (DEMIAN and MAKRIS, 2013). Biological treatments implicate, essentially, laccases, polyphenol oxidases or peroxidases provided from fungi and microorganisms (SAAVEDRA *et al.*, 2006; ASGHER *et al.*, 2008). Recent work has demonstrated that plant peroxidases could be used to remove phenolic compounds in OMW (SERGIO *et al.*, 2010; DEMIAN and MAKRIS, 2013). The elimination of phenols using plant peroxidases was described by SERGIO *et al.* (2010). These poly-functional oxidoreductases are widely distributed in all plant tissues and are involved in several physiological processes such as cell wall lignification (MADER *et al.*, 1980), auxin catabolism (FAIVRE-RAMPANT *et al.*, 1998) and antioxidant metabolism (ASADA, 1999; GILL and TUTEJA, 2010). In this paper, we report the capacity of peroxidases provided from crude extracts of nettle and radish leaves to remove polyphenol compounds present in OMW.

2. MATERIALS AND METHODS

2.1 Olive mill wastewater (OMW)

The OMW used in the present study was obtained from a traditional press. OMW was collected directly after the extraction of olive oil in a clean container and stored at -18°C.

2.2 Enzyme assay

The crude peroxidase extracts were obtained from fresh leaves of radish (*Raphanus sativus* L.) and nettle (*Urtica urens* L.); 0.4 g of leave tissues were homogenized with a pestle and a mortar in 2 mL of phosphate buffer (50 mM KH_2PO_4/K_2HPO_4; pH 7.0) containing 5% polyvinylpyrrolidone to remove phenols. After being filtred by a paper filter, the homogenate was centrifuged at 10 000 *g* for 15 min. Resulting supernatant was used as a crude enzymatic source. GPX (guaiacol: H_2O_2 oxidoreductase, EC1.11.1.7) activity was determined by adding the enzymatic preparations to 2 mL of 9 mM guaiacol and 10 mM hydrogen peroxide in 25 mM K-phosphate buffer (pH 7.0) and following the increase in absorbance at 470 nm (FIELDING and HALL, 1978). All operations were performed at 4°C.

2.3 OMW treatment

The waste was homogenized by vigorous shaking, and then centrifuged at 6 000 *g* to remove suspended materials. The experiment is essentially based on the preparation of a buffer mixture containing 0.1 mL of the first substrate of the enzyme (electron donor): H_2O_2 (10 mM H_2O_2 in 50 mM KH_2PO_4), 0.5 mL crude enzymatic extract from each plant and 3 mL OMW. The final mixture was placed in a beaker at 25°C with stirring using an orbital stirrer.

2.4 Total phenol level assay

The determination of total polyphenols (TP) in OMW, before and after incubation with peroxidase radish and nettle leave extracts, was carried out by spectrophotometry (DEWANTO *et al.*, 2002). In an alkaline medium and in the presence of polyphenols, the Folin-Ciocalteu reagent is reduced to tungsten oxide and molybdenum giving a blue color. A 125 µL portion of the extract was mixed with 500 µL of distilled water and 125 µL of Folin-Ciocalteu. After vigorous stirring of the mixture followed by a 3 min pause, a 1 250 µL of Na_2CO_3 7% was added. Finally, the mixture obtained was adjusted with distilled water to 3 mL after 90 min pause. The measurement was done at 760 nm. Gallic acid was used as standard reference for the quantitative estimation.

2.5 Toxicity assay

OMW phytotoxicity assays were performed using *Helianthus annuus* germinated seeds. Four-day sunflower seedlings were grown on Hogland medium containing treated or untreated OMW. Phytotoxicity was evaluated by the addition of treated or untreated waste to the transplanting medium containing the 4-day sunflower seedlings.

2.6 HPLC analysis of phenolic compounds

The analysis of the main phenolic compounds present in OMW, before and after enzymatic treatment, was performed using a liquid chromatography (HPLC, Perkin Elmer 373) equipped with a quaternary gradient pump and a spectrophotometric photodiode array detector (UV6000 Lp detector). The column used for peak separation was a Phenomene Synergi HydroRP18, 4 µm, 250 × 4.6 mm, protected by a guard volume packed with the same material. Both columns were maintained at 40°C. Eluent (A) and eluent (B) were 0.05% aqueous trifluoroacetic acid (TFA) and acetonitrile (MeCN) containing 0.05% TFA, respectively. The low rate was 1 mL·min^{-1}, and the elution program used was as follows: 5 min, 5% B; 65 min, 50% B. Chromatographic profiles were monitored at 275 nm.

2.7 Statistical analysis

All the data were subjected to analysis of variance (ANOVA) and the significance of the Tukey test was determined at the 5% level. Statistical analyses were performed with Statistica 8 for Windows software.

3. RESULTS AND DISCUSSION

The expression of the peroxidase activities in OMW solution cannot refer to the quantity of total proteins due to the possible interference with phenolic compounds. Thus, we expressed the total peroxidases activity in $\Delta OD \cdot min^{-1} \cdot g^{-1}$ where OD is the optical density. This activity showed some fluctuations during the period of incubation (Figure 1). The persistence of peroxidase activity during treatment is accompanied by a continuous decrease in the concentration of total polyphenols.

The idea that plant peroxidases of class III (EC 1.11.1.7) contributing to the reduction of phenolic compounds of the OMW is recent. In fact, SERGIO *et al.* (2010) suggest that treatment with crude peroxidases extracted from artichoke leaves is efficient to reduce the total polyphenol content. The same effect was observed after varying the peroxidases sources. Thus, several plant tissues were used, such as onion (BARAKAT *et al.*, 2010) and potato peel (DEMIAN and MAKRIS, 2013). In this work, crude peroxidases extracted from radish and nettle leaves were used to remove phenolic compounds available in OMW.

Present data showed the efficiency of the two types of treatment to decrease the amount of total polyphenols in the OMW (Figure 2). Indeed, the treatment of OMW by crude peroxidases extracted from radish leaves for seven days is more efficient than the second treatment involving crude nettle peroxidases. This efficiency is much better than those revealed by other authors (BARAKAT *et al.*, 2010; DEMIAN and MAKRIS, 2013). Indeed, in these reports, only 45% of total phenols were degraded, whereas the present application reduces more efficiently the concentration of the total phenolic compounds available in OMW, with a removal exceeding 60% after a 7-day incubation.

On the other hand, after peroxidase treatment for seven days, HPLC analysis of the sample showed qualitative and quantitative changes in phenolic compounds between untreated and treated OMW by radish and nettle leaves peroxidase (POD) (Table 1). In particular, treatments reduce the levels of gallic acid, *p*-coumaric acid and hydroxytyrosol and remove the peak of vanilic acid in the case of treatment with radish peroxidase. Caffeic acid disappeared after treatment with nettle peroxidase extract and increased considerably after treatment with radish crude peroxidases, compared to untreated OMW. This revealed that POD treatment had led to significant changes in the phenolic composition of the treated waste.

Finally, the efficiency of the two employed treatments was also tested by evaluating the degree of phytotoxicity of both treated and untreated OMW. Our results showed clearly that

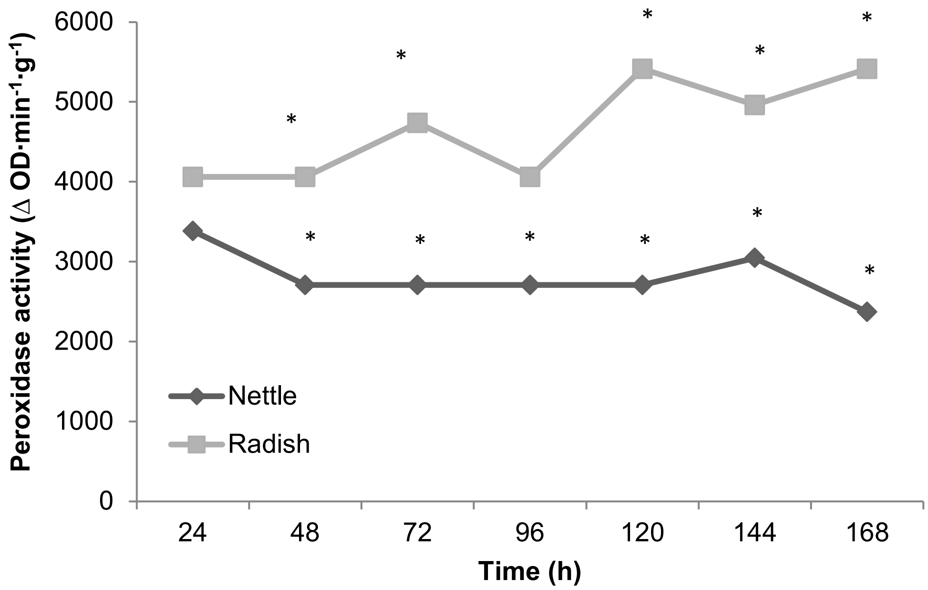

Figure 1. **Monitoring of peroxidase activity during the processing of vegetable raw extracts from the leaves of radish or nettle (*n* = 3, OD : optical density). *$p < 0.05$, values are significantly different.**
Surveillance de l'activité de la peroxydase lors du traitement par des extraits végétaux bruts des feuilles de radis ou d'ortie (n = 3, OD : densité optique). *p < 0,05, les valeurs sont significativement différentes.

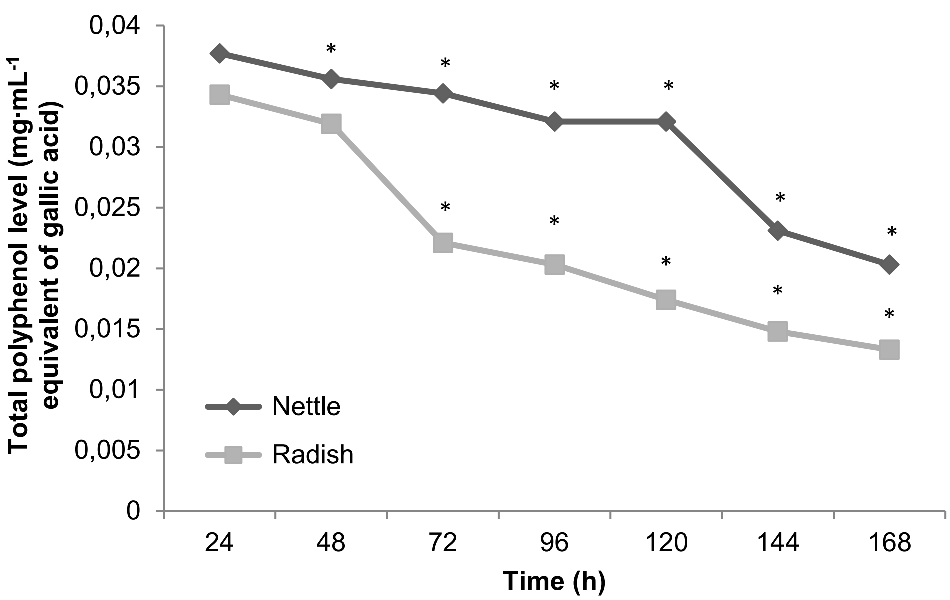

Figure 2. **Variation of total polyphenol content in the olive mill wastewater (OMW) treated with crude extracts from leaves of radish or nettle (*n* = 3). *$p < 0.05$, values are significantly different.**
Variation de la teneur en polyphénols totaux dans les margines traitées avec l'extrait brut des feuilles de radis ou d'ortie (n = 3). *p < 0,05, les valeurs sont significativement différentes.

Table 1. Percentage ([g/100 mL]*100) of phenolic compounds contained in olive mill wastewater (OMW) treated or not with crude peroxidase extracts.
Tableau 1. Pourcentage ([g/100 mL]*100) de composés phénoliques contenus dans les margines traitées ou non par des extraits peroxydasiques bruts

Phenols		Non treated OMW (%)	Treated OMW (%)	
			With crude extract of nettle leaves	With crude extract of radish leaves
Phenolic alcohols	Tyrosol	11.8	10	0
	Hydroxytyrosol	45.3	37	6
Phenolic acids	*p*-Coumaric acid	28.58	12	22.7
	Cafeic acid	1.37	0	8.11
	Gallic acid	12.62	8.66	4.66
	Vanilic acid	3.54	0	0
Flavonoïds	Coumarine	3.45	1.21	1.44

untreated OMW is extremely toxic for sunflower seedlings. These data are in accordance with other work (DERMECHE *et al*., 2013) which revealed the OMW phytotoxicity and showed that this effluent could inhibit plant growth and seed germination. According to KOMILIS *et al*. (2005), phytotoxicity is due to the presence of phenolic compounds and other constituents.

We have also based on morphological symptoms, demonstrated that peroxidasic treatment considerably attenuates the toxicity of OMW. In fact, seedlings grown on OMW treated with radish or nettle peroxidase extracts grew normally and developed to the first leaf stage.

So, we can suggest that the attenuation of OMW toxicity is related to the reduction of the level of phenolic compounds after enzymatic treatment. Thus, it appears that the recycled OMW could be used as irrigation water in agricultural areas and could be an attractive prospect for the Mediterranean countries in which water resources have been scarce in recent years. In addition, OMW could be used, after treatment, as a natural plant fertilizer, because they contain many essential nutrients for plant growth. In conclusion, the present study confirms the possibility of using radish and nettle leaves to remove phenols from OMW. Furthermore, the proposed method promises to be very cheap and interesting because olives, radish and nettle are produced in the same season and in the same geographic areas.

Nettle, which is widespread in almost all the temperate regions of the world, is a spontaneous plant and very abundant. Radish is a primary source of peroxidases; "Horse Radish Peroxidase" refers to marketed peroxidases extracted therefrom. The POD enzyme is extracted from leaves and not tubers because leaves are the non-consumable parts of the plants. We thought to enhance the part of the plant that is rejected during consumption. Also, the extraction protocol of the enzyme is simple. The treatment of OMW by the enzyme extracted from radish and nettle leaves shows a significant reduction of phenols.

4. CONCLUSION

The bioremediation of OMW by crude peroxidase extracts from nettle or radish leaves has been effective since it has achieved the following results:

i. Lowering the total polyphenols content of treated OMW.
ii. Change of chromatograms of treated OMW phenolic compounds; all the treatments reduced the contents of gallic acid, *p*-coumaric acid and hydroxytyrosol and removed the peak of vanilic acid. Caffeic acid disappeared after treatment with nettle extract.
iii. Reduced phytotoxicity of OMW.

Thus, the recycling of OMW and its use in irrigation water in agriculture is an attractive prospect for the Mediterranean countries in which water resources have been severely scarce in recent years.

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