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Phenolic compounds-enriched extract recovered from two-phase olive pomace serves as plant immunostimulants and broad-spectrum antimicrobials against phytopathogens including *Xylella fastidiosa*

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ABSTRACT

The production of extra virgin olive oil generates significant amounts of olive mill waste, whose disposal leads to severe environmental impacts, especially due to the high content of phenolic compounds. In this study, a pomace phenolic extract composed of hydroxytyrosol, tyrosol, verbascoside, and oleuropein was obtained from the liquid fraction of two-phase olive pomace and explored for its antimicrobial properties and potential as plant immunostimulants. The olive pomace extract exhibited a broad range of antimicrobial activity against important phytopathogens, including the bacteria *Xylella fastidiosa, Pseudomonas syringae*, and *Pectobacterium carotovorum*, as well as the fungi *Colletotrichum graminicola, Fusarium graminearum*, and *Botrytis cinerea*. The extract induced key features of plant innate immunity in Arabidopsis seedlings, including hydrogen peroxide production, phosphorylation of mitogen-activated protein kinase MAPK6, and upregulation of defence genes, such as *CYP81F2, FRK1*, and *WRKY53*, suggesting the activation of early signalling cascades leading to the production of indole glucosinolates and salicylic acid. The immune activation pathways induced by the phenolic extract did not always match those triggered by well-known oligogalacturonide elicitors. Notably, pretreatment of adult Arabidopsis and tomato plants with the phenolic compounds-enriched extract primed responses and enhanced their resistance against *B. cinerea* and *P. syringae*. Our findings demonstrate the potential to upcycle two-phase olive pomace into plant protectants, offering a promising alternative to reduce reliance on chemically synthesized pesticides in integrated pest management programs.

1. Introduction

A substantial global increase in the extraction and consumption of olive oil has been documented, facilitated by its favourable organoleptic characteristics and the expanding recognition of its health-promoting properties. However, olive oil production generates significant amounts of olive mill wastes (OMW) including small branches, leaves, crushed pits and olive pomace (Berbel and Posadillo 2018; Enaime et al., 2024). Compared to traditional methods of olive oil extraction, such as pressing and three-phase extraction, the two-phase extraction using horizontal centrifugation is more efficient. It produces greater quantities of oil, reduces water consumption, minimizes waste, and still preserves the oil's quality and nutritional properties. Olive pomace is the main OMW produced by the two-phase olive oil extraction system and is primarily composed of olive skin, pulp, stone fragments, vegetable water, and water-soluble materials (Foti et al. 2021). Unfortunately, the uncontrolled discard of OMW into the environment leads to severe ecological consequences due to their high content of tannins, long-chain

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fatty acids, and phenolic compounds (Pinho et al. 2017; Malapert et al. 2018; Enaime et al. 2020; Foti et al. 2021; Popolizio et al. 2022). These waste products can inhibit seed germination and early plant growth, alter soil characteristics, and create reducing conditions that affect microbial diversity in the soil (Saez et al. 1992; Ben Sassi et al. 2006; Karpouzas et al. 2010; Al-Eitan et al. 2020). According to EU Directive 2018/851, the reduction and valorisation of agro-industrial biowastes is considered a priority, reducing the polluting practice of landfilling and emphasizing the need to close material cycles through a circular economy (Kircher et al. 2023).

Another serious problem affecting agriculture, including olive growing, is the massive use of chemical compounds to combat plant diseases. Currently, farmers are largely dependent on chemical pesticides to prevent economically important food losses caused by plant pathogens (Tudi et al. 2021). Chemical pesticides can cause negative effects on human health, in particular on agricultural workers due to exposure during farm works and on consumers due to their possible presence in food (Rather et al. 2017; Curl et al. 2020). Treatment of plants with elicitors represents an excellent solution to stimulate immunity and improve crop protection. Several endogenous molecules can act as elicitors of plant immunity (De Lorenzo et al. 2018; Del Corpo et al. 2024). The most extensively studied elicitors are oligogalacturonides (OG), pectic oligomers composed of α−1,4-linked galacturonosyl residues (Galletti et al. 2008; Lionetti et al. 2012; Giovannoni et al. 2021). Among the hallmarks of plant immunity are the production of apoplastic hydrogen peroxide (H_2O_2), cytoplasmic calcium influxes, the activation of Mitogen-Activated Protein Kinases (MAPK), and the induction of immune-related genes (Zhou and Zhang 2020; DeFalco and Zipfel 2021; Ge et al. 2022). Elicitor application can confer to plants a greater ability to detect pathogens and activate defence responses faster than untreated crops (Hönig et al. 2023). This results from priming, a sensory state induced by elicitors, wherein plants develop immune memory following previous elicitation events, leading to faster and stronger activation of defence mechanisms in response to subsequent stresses (Conrath et al. 2015; Hilker and Schmülling 2019).

Generally, phenolic compounds are naturally occurring compounds found largely in fruits, vegetables, cereals that can help plants cope with stressors (Chowdhary et al. 2021). Due to their chemical structure, phenolics, such as the flavonoid quercetin and the stilbene resveratrol produced from grape, are known for their antimicrobial and antioxidant properties, useful to safeguard essential tissues against both abiotic stresses and pathogenic infections (Singh et al. 2023). These characteristics render them versatile molecules with promising human therapeutic applications (Rana et al. 2022). Salicylic acid is also a phenolic compound that act as a key signalling molecule in plant immunity, providing resistance against a wide range of pathogens (Maruri-López et al. 2019). Salicylic acid (SA) can boost the plant immune system through systemic acquired resistance (Dempsey and Klessig 2017). Exogenous application of with SA or SA like compounds was reported to induce plant resistance to several pathogens and suitable for plant protection (Bektas and Eulgem 2015; Tripathi et al. 2019). Moreover, phenolic compounds are components of lignin and suberin, polymers employed to strengthen host cells, thereby providing physical barriers to pathogens (Lee et al. 2019; Kashyap et al. 2022; Vicré and Lionetti 2023). Lignin and some related compounds can play as a signal to activate plant-specific immune response (Miedes et al. 2014; Swaminathan et al., 2021).

Although some phenolic compounds are involved in plant defence, the possibility that phenolics present in olive pomace can play a role as elicitors of defence responses has not been explored yet. Two-phase olive pomace represents a significant source of phenolic compounds, that comprises approximately 98 % of the total phenolic compounds found in olive fruits (Chanioti and Tzia 2017). Membrane processes are promising approach for purifying and concentrating bioactive phenolic compounds from OMW (Ochando-Pulido and Martinez-Ferez 2018; Sygouni et al. 2019). These environmentally sustainable processes operate without solvents, handle large sample volumes rapidly, are characterised by low processing temperatures and energy requirements, offer high separation efficiency, and achieve both high extraction yields and ease of scalability (Zagklis et al. 2022). The sequence of microfiltration, ultrafiltration, nanofiltration, and reverse osmosis techniques can effectively recover and purify high concentrations of phenolics from olive mill wastewater (Paraskeva et al. 2007; Cassano et al. 2013; Di Lecce et al. 2014).

In this work, we exploited the phenolic compounds recovered from Coratina olive pomace as plant protectors. An extract called Olive Pomace Phenolics (OPP), which includes hydroxytyrosol, tyrosol, verbascoside, and oleuropein, was obtained through tangential flow membrane filtration of the liquid fraction from two-phase olive pomace derived from processing Coratina olive fruits. OPP exhibited in vitro antimicrobial effects against important fungal and bacterial plant pathogens, which have significant economic implications for agriculture. Notably, OPP is effective against *Xylella fastidiosa*, a highly destructive pathogen that affects olive trees, as well as other species like grapevines, citrus, and various ornamental plants. OPP treatment induced significant immune responses in plants, including H_2O_2 accumulation, MAPK phosphorylation, and induction of defence gene expression. OPP operates by activating a signalling pathway that differs from the one triggered by OG oligosaccharide elicitors. OPP treatments protected Arabidopsis and tomato plants against *Botrytis cinerea* and *Pseudomonas syringae* infections.

2. Material and methods

2.1. Chemical characterization of OPP extract

Olive pomace from a two-phase olive oil extraction system fed with Coratina cultivar green olives was collected from the Agrolio SRL olive mill in Andria, Italy. Three independent pomace samples were collected during the first 15 days of November of each year across three consecutive harvest years (from 2021 to 2023). The liquid part of two-phase olive pomace was recovered following pitting and sedimentation in silos. The liquid fraction was subsequently pretreated and subjected to a tangential flow membrane filtration (TFMF) (Patent registration number: IT202100019226A1process designed to isolate an extract enriched in low molecular weight phenolic compounds (MW< 1000 Daltons) (Arnoldi et al. 2023). The concentrate obtained from reverse osmosis (referred as Olive Pomace Phenolics (OPP) was characterised for the presence of phenolic compounds using HPLC as previously reported (Sheng 2014; Curci et al. 2022). Briefly, the analysis was carried out with an HPLC 1220 Infinity LC (Agilent Technologies, Palo Alto, CA, USA) equipped with a degasser, quaternary pump solvent delivery, and thermo-stated column compartment. The elution solvents were acidified H_2O by phosphoric acid (pH 3.2), CH_3CN, and MeOH. The flow rate was 1 mL/min, with an injection volume of 20 μL. The phenolic compounds were identified by comparison of their retention times with those of the injected standard solution (hydroxytyrosol, tyrosol, verbascoside, and oleuropein) under the same operatory conditions. Total phenolic concentration was assayed by the Folin–Ciocâlteu colorimetric method (Singleton and Rossi 1965). The reaction mixture contained 50 μL of OPP solution diluted 1:1000, 475 μL of Folin–Ciocâlteu reagent, and 475 μL of sodium carbonate 1 M After 1 h of reaction in the dark at room temperature, the absorbance of reaction mixture was measured at 760 nm and used to calculate the phenolic content using gallic acid as a standard. OPP extracts were filtered using Primo Syringae filters 30 mm diameter, with polyethersulfone (PES) membrane filter 0.22 μm (ERUCLONE, EPSPE2230) before being used in all the following analyses.

2.2. Antibacterial/Bactericidal activity

X. fastidiosa subsp. *pauca* strain ST53 was taken from the microbial collection of the Institute for Sustainable Plant Protection (IPSP-CNR)

Unit of Bari (IT) and all the experiments were performed in the quarantine institute laboratories. *X. fastidiosa* was cultured on Buffered Charcoal Yeast Extract agar medium (BCYE) (Wells et al. 1981) for 7 days at 28 °C, and further sub-cultured for 10 days at 28 °C on Pierce's Disease (PD) 3 agar medium (PD3) (Davis et al. 1981). The bacterial biomass was taken with a sterile 10 μL loop and suspended in 2 mL of 1x Phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4) solution to adjust the bacterial suspension to 0.5 OD_{600} that was used as inoculum for all the experiments on the appropriate medium. To determine the minimum inhibitory concentration that reduce bacterial growth (MIC) and the minimum bactericidal concentration (MBC) of OPP against *X. fastidiosa*, the disk diffusion assay and the time-kill contact assay were used, according to Del Grosso (2022) (PhD Thesis). Briefly, for the disk diffusion assay, 0.5 OD_{600} cell suspension was spread on agar plates containing PD3 medium. Different concentrations of OPP (0.8, 1.0, 1.2, and 1.4 mg/mL) were tested using sterile filter paper disks (6 mm diameter), each inoculated with 20 μL of the respective OPP solution. These disks were placed radially on agar plates, with a central disk containing a mixture of streptomycin (100 μg/mL) and ampicillin (250 μg/mL) serving as a positive control. A total of 9 independent biological replicates (plates) were prepared for each concentration ($n = 9$). The plates were incubated at 28 °C for 7–10 days, and daily observed. The diameter of the inhibition zone was measured, and the MIC was defined as the lowest concentration that inhibited bacterial growth. For bactericidal activity, the field dose concentration of each product was tested using a modified time-kill assay. The concentrations of OPP (1.2 and 1.4 mg/mL) were included in the PD3 medium. PD3 plus kanamycin (50 μg/mL) was used as positive control. 20 μL of 0.5 OD_{600} *X. fastidiosa* bacterial suspension were pipetted on each plate. After 0, 4, 8 and 24 h the bacteria were then recovered, resuspended in 5 mL of sterile deionized water, diluted in serial decimal dilutions, and then plated on fresh BCYE medium for colony count after incubation at 28 °C for 20 days. A total of 3 biological replicates (plates) were analysed ($n = 3$). *P. syringae* pv. *tomato* DC3000 and *P. carotovorum* subsp. *carotovorum* (strain DSM 30,169, obtained from DSMZ GmbH Germany) were refreshed from frozen glycerol stock in 10 ml Luria-Bertani (LB) liquid medium containing 1 % tryptone, 0.5 % yeast extract, and 1 % NaCl. 25 μg/mL rifampicin was added in *P. syringae* liquid colture. Bacteria were grown at 28 °C in dark overnight with 180 rpm agitation in tubes lightly sloped to avoid clump formation. Bacteria were collected by centrifugation of 6000 × *g* for 10 min and suspended in 10 mL of 10 mM $MgCl_2$ or 50 mM potassium-phosphate buffer before inoculum preparation of *P. syringae* or *P. carotovorum,* respectively. To assess the antibacterial/bactericidal activity, and consequently the MIC and the MBC of OPP against *P. syringae* and *P. carotovorum*, the broth dilution assay was used, with some modifications (Amini Tapouk et al. 2020). Briefly, bacterial suspension 0.5 OD_{600} was incubated in LB liquid medium containing different doses of OPP such as 0.1, 0.2, 0.4, 0.8 or 1.6 mg/mL for *P. syringae*, and 0.4, 0.8, 1.6, 3.2 or 4.8 mg/mL for *P. carotovorum*. LB plus kanamycin (50 μg/mL) was used as positive control while only LB as negative control. After 24 h incubation at 28 °C with 180 rpm agitation, turbidity was evaluated by measuring the absorbance at 600 nm (OD_{600}) with Varian Cary 50 UV–VIS Spectrophotometer (Agilent, Santa Clara, CA, USA)and the MIC was quantified as the lowest concentration that inhibited bacterial turbidity. In parallel, 1 mL of each liquid culture of *P. syringae* or *P. carotovorum* was centrifuged for 10 min at 6000 × *g*, resuspended in 1 mL of sterile 10 mM $MgCl_2$ or 50 mM potassium-phosphate buffer, respectively, and used for serial decimal dilutions. For each dilution, 10 μL of cell suspension was spotted on LB agar medium and incubated at 28 °C overnight. The MBC was determined by counting colonies and evaluating bacteria viability. A total of 9 biological replicates were analysed for each condition ($n = 9$).

2.3. Preparation of oligogalacturonides (OGs)

Unbranched oligogalacturonides (OGs) with a degree of polymerization (DP) ranging from 10 to 15 were employed in all analyses. OG were generated as previously described (Benedetti et al. 2017). High molecular weight un-methylated polygalacturonic acid (PGA; Alfa Aesar) was dissolved in 100 mL of 50 mM sodium acetate, pH 5.0, to achieve a 2 % (w/v) concentration. The solution was digested for 180 min with 0.018 RGU of *Aspergillus niger* endo-polygalacturonase (a kind gift from Prof. Jaap Visser's laboratory at Wageningen University). The enzyme was inactivated by boiling the digest at 100 °C for 10 min. After inactivation, the sample was diluted to 0.5 % PGA with 50 mM sodium acetate. Cold ethanol was then added to the digest to a final concentration of 11 % (v/v) to precipitate the OGs. The sample was incubated overnight at 4 °C with shaking and subsequently centrifuged at 30,000 g for 30 min. The OGs, recovered in the pellet, were re-dissolved in water, dialyzed against ultrapure water using a dialysis tube with a molecular mass cutoff (MWCO) of 1000 Da (Spectra/Por®), and lyophilized. The final preparation was analyzed by HPAEC-PAD.

2.4. Antifungal activity

To evaluate the capability of OPP in inhibiting fungal growth, the agar dilution method was used (Förster et al. 2004). Briefly, molten Potato Dextrose Agarose (PDA) medium was cooled to approximately 50 °C, and different OPP amounts (1 or 2 mg/mL) were added. Only PDA medium was used as negative control and hydroxytyrosol (700 μg/mL) as positive control. 1 μL from 10^6 spores/mL solutions of *Colletotrichum graminicola, Fusarium graminearum*, and *B. cinerea* was applied to the center of PDA Petri dish (Yugueros et al. 2024). The PDA plates were incubated at 25 °C. Seven days post-inoculation, the diameter of mycelium growth in mm was measured. The percentage of mycelium growth inhibition was calculated as [(C-T)/C]·100, where C = diameter of mycelium growth in PDA, and *T*= diameter of mycelium of fungi grown in presence of HTyr or OPP (Drais et al. 2021). A total of 3 biological replicates (plates) were analysed ($n = 3$).

2.5. Plant growth conditions

Arabidopsis thaliana wild-type Columbia (Col-0) seeds were washed in 2 mL of isopropanol for 30 s followed by washing in 2 mL of ultrapure sterile water for 3 min in slow agitation. Seeds were sterilized with 2 mL of sterilization solution (20 % v/v NaClO in ultrapure sterile water) for 5 min in slow agitation, followed by 4 washing steps in 2 mL of ultrapure sterile water. Seeds were stored in dark at 4 °C for 2 days to promote germination. For seedling treatments, seeds were germinated and grown in multiwell plates (approximately 10 seeds/well) containing 1 mL per well of liquid Murashige and Skoog (MS)/2 medium Sigma (2.2 g/L MS medium basal salt with vitamins, 0.5 % sucrose, pH 5.7) (Murashige and Skoog, 1962). Multiwells were incubated in a controlled environmental chamber maintained at 22 °C with a 16 h light/8 h dark cycle and a light intensity of 120 mmol m^{-2} s^{-1}. To evaluate the dose-effect of OPP on Arabidopsis seedling growth, 7-day-old seedlings were treated with sterile OPP (10 ng/ml) and sterile water as mock. The fresh weight was measured 5 days after treatment.

For adult plant treatments, Arabidopsis wild-type Col-0 seeds were grown in solid MS/2 medium (2.2 g/L MS Medium basal salt, 1 % sucrose, 0.8 % plant agar, pH 5.7) in the same condition above described for the seedlings treatments. 7-day-old seedlings grown in solid MS/2 medium were transferred in sterile soil in growth chamber at 22 °C with a 12 h light/12 h dark cycle (PAR level of 100 μmol m-2 s-1). Tomato (Solanum lycopersicum 'Minibel') seeds were soaked on filter paper overnight. Then, the seeds were transferred on sterile soil (peat-based) and were grown in greenhouse under 16 h light/8 h dark cycle (PAR level of 75 μmol/m2/s) at 23 ± 1 °C and 35–40 % humidity.

2.6. Hydrogen peroxide quantification

Four mm diameter leaf-discs from four-week-old *A. thaliana* plants and from five-week-old *Solanum lycopersicum* 'Minibel' were used to determine hydrogen peroxide (H_2O_2) production after treatment with OG (200 μg mL^{-1}) or OPP (10 ng mL^{-1}). Sterile distilled water was used as mock. H_2O_2 production was measured by determining the luminescence produced by luminol-peroxidase reaction in a Varioskan Lux luminescence reader (Thermo Scientific, Waltham, MA, USA) (Fuertes-Rabanal et al. 2024). Six biological replicates (considering each leaf-disc collected from a single adult plant as one biological replicate) were used for each experiment. The experiment was performed three times with similar results.

2.7. Immunoblot analysis for MAPK activation

Arabidopsis seedlings (10-day-old) grown in liquid medium were treated with OG (200 μg mL^{-1}) or OPP (10 ng mL^{-1}) for 5, 10 and 20 min and frozen with liquid nitrogen. Sterile distilled water was used as mock. Protein extraction and detection of activated MAPKs were performed as previously described (Fuertes-Rabanal et al. 2024). Briefly, frozen seedlings were homogenized and protein extraction was performed with 50 μL of extraction buffer containing 25 mM Tris–HCl pH 7.8, 75 mM NaCl, 15 mM Egtazic acid (EGTA), 10 mM magnesium chloride, 15 mM sodium β-glycerophosphate pentahydrate, 15 mM bis (4-nitrophenyl) phosphate, 1 mM 1,4-dithiothreitol, 1 mM sodium fluoride, 0.5 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 % (v/v) Tween 20, and protease inhibitor cocktail (#P9599; Sigma). Total proteins were quantified by Bradford reagent (Bio-Rad, Hercules, CA, USA). Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes which were blocked with Protein-Free Blocking Buffer (TBS; Thermo Scientific) for 2 h at room temperature in agitation. The membranes were incubated overnight in agitation at 4 °C with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#4370; Cell Signalling Technology Danvers, MA, USA) diluted 1:1000 in TBS. Then, membranes were washed three times with Tris-Buffered Saline that contains 0.1 % (v/v) Tween 20 and then incubated with horseradish-peroxidase-goat anti-rabbit polyclonal secondary antibody (#10,035,943; Thermo Fisher Scientific, Waltham, MS, USA) diluted 1:250 in TBS. Membranes were developed using ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA). Additionally, the membranes were stained with Ponceau S solution to evaluate equal loading. Experiments were conducted three times with similar results.

2.8. Gene expression analysis

10-days-old Arabidopsis seedlings grown in liquid MS/2 medium were treated for 1 h with OG (200 μg mL^{-1}) or OPP (10 ng mL^{-1}). Sterile distilled water was used as mock. Seedlings were homogenized in liquid nitrogen. *B. cinerea*-infected or mock-inoculated leaves were frozen in liquid nitrogen. Tissues were homogenized using the mixer mill MM301 (RETSCH GmbH, Haan, Germany) and using stainless steel beads (5 mm in diameter) for about 1 min at 30 Hz. Total RNA was extracted with RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was extracted with Isol-RNA Lysis Reagent (5′-Prime) according to the manufacturer's instructions. RNA was treated with RQ1 DNase (Promega, Southampton, UK), and first-strand complementary DNA (cDNA) was synthesized using ImProm-II reverse transcriptase (Promega, Southampton, UK). Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analyses were performed as previously reported (Coculo et al. 2023). Quantitative Reverse Transcription PCR analysis was performed using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). One microliter of cDNA (corresponding to 50 ng of total RNA) was amplified in 20 μL of reaction mix containing 1 × Go Taq qPCR Master Mix (Promega, Southampton, UK) and 0.5 μM of each primer. The conditions for amplification were 95 °C for 2 min; 46 cycles of amplification: 95 °C for 15 s, 58 °C for 15 s, and a final extension of 72 °C for 15 s. Primer sequences were generated with Primer3 software (https://primer3.ut.ee/) (Supplemental Table S1). The expression levels were determined using a modification of the Pfaffl method (Pfaffl 2001). Briefly, the average cycle threshold (aCT) for each gene was calculated from three biological replicates and adjusted for PCR efficiency (E) using the formula: $aCT \times \log_2(1 + E)$ to obtain the corrected CT value. For each sample, the relative expression of each gene was determined by calculating the difference in CT values (ΔCT) between the target gene and the reference gene *Ubiquitin 5 (UBQ5)* (Lionetti et al. 2017; Giovannoni et al. 2021). Gene expression levels were calculated using the formula 2^–ΔCT, and the results were expressed in arbitrary units. Three biological replicates (considering each well of the plate with ten seedlings per well as one biological replicate) were performed. The experiment was repeated three times with similar results.

2.9. Arabidopsis infection assay with Botrytis cinerea

B. cinerea strain SF1 (Lionetti et al. 2007; Del Corpo et al., 2020) was grown for 20 days on malt extract agar at 20 g L^{-1} with mycological peptone at 10 g L^{-1} and micro agar at 12 g L^{-1} (MEP) at 23 °C and 70 % relative humidity in the dark before conidia collection. Conidia were harvested by washing the surface of the mycelium with sterile distilled water. Conidia suspensions were filtered in sterile conditions to remove residual mycelium and the conidia concentration was determined using a Thoma cell counting chamber. For the protection assay, 4-week-old plants were sprayed with OG (200 μg mL^{-1}) or OPP (10 ng mL^{-1}) containing 0.05 % Tween 24 MBAL (Croda, Snaith, UK) as adjuvant. Adjuvant solution in water was used as mock. After 24 h, fully developed leaves were infected with 1×10^6 conidia mL^{-1} incubated in Potato Dextrose Broth (PDB) at 24 g L^{-1}. Six droplets of spore suspension (5 μL each) were placed on the surface of each leaf. Negative control was performed using PDB. Plants were incubated at 24 °C with a 12 h/12 h day/night photoperiod. The priming effect of pretreatment was evaluated by RNA extraction from infected leaves collected at 8 h post infection (hpi) as described below. The lesion size produced by *B. cinerea* was determined by measuring necrotic tissues using ImageJ software at 48 h post infection (hpi) and was evaluated as an indicator of susceptibility to the fungus. Lesion size is expressed as a mean of a total of 50 lesions. The experiment was repeated three times with similar results.

2.10. Arabidopsis and tomato infection assay with Pseudomonas syringae

P. syringae pv. *tomato* DC3000 was refreshed from frozen glycerol stock in King's Basal medium (KB) containing 2 % (w/v) protease peptone, 0.15 % (w/v) $MgSO_4$, 0.15 % (w/v) KH_2PO_4, and 1.5 % (v/v) glycerol with 25 μg/mL rifampicin. Bacteria were grown at 28 °C in dark for three days before inoculum preparation. Bacteria were collected by washing the KB plates with 2 mL of sterile 10 mM $MgCl_2$. Four-week-old Arabidopsis and five-week old tomato plants were sprayed with 2 mL of OG (200 μg mL^{-1}) or OPP (10 ng mL^{-1}) containing 0.05 % Tween 24 MBAL (Croda, Snaith, UK) for Arabidopsis pre-treatment, and 2.5 % Tween 24 MBAL and 2.5 % UEP-100 (Croda, Snaith, UK) for tomato pretreatment as adjuvant (Rebaque et al. 2023). Adjuvant solution in water was used as mock. After 24 h, Arabidopsis plants were infected with a bacterial suspension of $OD_{600} = 0.1$ added with 0.001 % of Silwet L-77, and tomato plants were infected with a bacterial suspension of $OD_{600} = 0.5$ added with 0.002 % of Silwet L-77. Priming effect was evaluated in Arabidopsis by collecting infected leaves at 8 hpi. Two Arabidopsis and tomato leaf discs were collected at 0 and 3 days post infection (dpi). Colony forming units (CFU) per foliar area were determined after plating serial dilutions onto KB plates with 25 μg/mL rifampicin. Six biological replicates (considering each serial dilution of bacteria collected from a single adult plant as one biological replicate)

were used. The experiment was repeated three times with similar results.

2.11. Data analysis

Data were presented as mean ± standard deviation (SD) or standard error (SE) as indicated in the figure legends. Data were assessed for the normality using the Shapiro-Wilk test ($p \geq 0.05$). For the statistical analysis of the disk diffusion assay, the Kruskal-Wallis test ($p \leq 0.001$) was used. For the time kill contact assay, the ANOVA followed by Tukey's test ($p \leq 0.001$) was conducted. For the dilution assay data and Arabidopsis seedlings growth, the Student's t-test was performed ($p \leq 0.05$). The statistical analysis of elicitor activity and protection assay data were evaluated by ANOVA followed by Tukey's test ($p \leq 0.05$). Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. A phenolic compound-enriched extract from Coratina two-phase olive pomace exhibited antimicrobial activity against a broad spectrum of bacterial and fungal phytopathogens

Tangential flow membrane filtration was employed to isolate a reverse osmosis concentrate from two-phase olive pomace enriched in low molecular weight phenolic compounds (<1 Kda), referred to as Olive Pomace Phenols (OPP). To explore potential variations in the phenolic profiles of olive pomace extracts over the years, three independent pomace samples were collected in November for each of the three consecutive harvest years (2021, 2022, and 2023) and processed for OPP isolation. The total OPP phenolic concentration (~74 mg/ml) was similar among the three years (Table 1). The OPP phenolics profiles, analysed by High-Performance Liquid Chromatography (HPLC), were characterised by the presence of 4 main abundant phenolic compounds identified as hydroxytyrosol (~77 %), tyrosol (~18.5 %), verbascoside (~4 %), and oleuropein (~0.5 %). Additionally, very small peaks were observed, but their nature remains unidentified. Also in this case, no significant differences in the phenolic profiles were observed between the three years (Table 1). An OPP isolated from pomace samples collected in the 2022 was used for subsequent analyses. The OPP ability to contrast the microbial growth was tested (Fig. 1). We started to analyse the effect of OPP against the hemibiotrophic bacterium *X. fastidiosa*, a devastating pathogen of olive tree representing a serious threat to the agriculture economy and the biodiversity of Mediterranean area (Surano et al. 2024) (Fig. 1A and B). With the aim of identifying the lowest concentration of OPP that inhibits detectable growth of *X. fastidiosa* (minimum inhibitory concentration; MIC), bacterial growth was challenged with OPP at different concentrations (0.8, 1, 1.2, and 1.4 mg/mL) using a disk diffusion assay (Fig. 1A). Sterile distilled water (mock) and a mixture of streptomycin and ampicillin (Amp/Str) (100 and 250 μg/mL) were tested as positive control. OPP significantly inhibited bacterial growth at 1.2 mg/mL (MIC = 1.2 mg/mL). To explore if OPP have bacteriostatic or bactericidal effect, we quantified the lowest concentration that killed at least 99.9 % of the bacteria in the original inoculum after subculture to antibiotic-free medium (minimal bactericidal concentration; MBC). 1.2 and 1.4 mg/mL OPP were evaluated against *X. fastidiosa* by performing a time kill-contact assay at different times after incubation (Fig. 1B). At 1.2 mg/mL OPP dose, no bacterial growth was observed after 8 h incubation, identifying 1.2 mg/mL as MBC. OPP at a concentration of 1.4 mg/mL completely killed *X. fastidiosa* cells already after 4 h, indicating that rapid *X. fastidiosa* death can be achieved by increasing the OPP concentration. To investigate if OPP can effectively counteract other bacterial diseases that significantly impact agricultural crops, its antimicrobial effects were also tested on the hemibiotrophic *P. syringae* (Fig. 1C and D) and the necrotrophic *P. carotovorum* (Fig. 1E and F). Exploiting a broth dilution assay, different concentrations were used for *P. syringae* (0.1, 0.2, 0.4, 0.8 or 1.6 mg/mL) and *P. carotovorum* (0.4, 0.8, 1.6, 3.2 or 4.8 mg/mL). Kanamycin (Kan; 50 μg/mL) and LB medium were used as positive and negative controls, respectively. A partial inhibition was observed for *P. syringae* and *P.* carotovorum growth in presence of 0.4 mg/mL and 1.6 mg/mL OPP, respectively (Fig. 1C and E). The MIC and MBC values identified for *P. syringae* were 0.8 mg/mL OPP (Fig. 1C and D). For *P. carotovorum assays*, higher MIC and MBC values were observed corresponding with 3.2 mg/mL OPP (Fig. 1E and F).

Next, we extended the antimicrobial activity of OPP on different fungal phytopathogens. The antifungal activity of OPP was tested on *C. graminicola, F. graminearum*, and *B. cinerea*, three pathogens that strongly decrease crop yield and quality and cause huge losses in agricultural production (Rogério et al. 2022; Bi et al. 2023; Jiang et al. 2023). The diameter of mycelium expansion was measured in an agar dilution assay in absence and in presence of the OPP at 1 or 2 mg/mL (Fig. 2). Plates containing 700 μg/ml of hydroxytyrosol, which corresponds to the concentration of hydroxytyrosol present in plates with 1 mg/ml of OPP, were utilized as a positive control. After 7 days of incubation, OPP significantly reduced the mycelium growth of all the fungal pathogens tested in a dose dependent manner. Interestingly, OPP appeared to be more effective against *C. graminicola* with respect to *F.* graminearum and *B. cinerea* (Fig. 2A-D). In particular, the treatment with 2 mg/mL of OPP caused a greater growth inhibition (96.5 %) of *C. graminicola* compared to *F. graminearum* (78.1 %) and *B. cinerea* (61.7 %). OPP showed a greater ability to inhibit fungal growth compared to hydroxytyrosol, suggesting an antifungal activity also for the other phenolic compounds present in the extract. Overall, these results indicate that OPP has broad-spectrum antibacterial and antifungal activity.

Table 1
Total phenolic concentration and phenolic compounds of OPP extracts.

Harvest years	2021	2022	2023
Total phenolic concentration	$72.09 \pm 5.36_a$	$77.19 \pm 7.26_a$	$70.74 \pm 5.51_a$
Hydroxytyrosol	$52.95 \pm 3.62_a$	$56.32 \pm 3.38_a$	$49.63 \pm 1.37_a$
Tyrosol	$12.27 \pm 1.26_a$	$13.96 \pm 2.72_a$	$11.94 \pm 0.97_a$
Verbascoside	$2.94 \pm 1.94_a$	$3.52 \pm 2.58_a$	$2.39 \pm 1.70_a$
Oleuropein	$0.47 \pm 0.12_a$	$0.38 \pm 0.14_a$	$0.48 \pm 0.08_a$

The total phenolic concentrations were calculated using Folin–Ciocâlteu assay and single phenolic compounds were quantified by HPLC, as reported in material and method section. Data are expressed in mg/ml as mean ± SD (n = 3). Datasets are not significantly different based on ANOVA followed by Tukey's test ($p \leq 0.05$).

3.2. OPP induced H_2O_2 production, MAPK6 phosphorylation and defence gene expression in Arabidopsis plants

Next, we investigated whether the OPP extract could act as an elicitor of plant immunity. Key features of plant defence responses, including hydrogen peroxide (H_2O_2) production, activation of MAPK phosphorylation cascades, and upregulation of defence-related genes were analysed (Withers and Dong 2017; Tian et al. 2021) (Fig. 3). The production of H_2O_2 was quantified in leaf discs of Arabidopsis plants, after treatment of with 10 ng/mL OPP or water (mock), by luminol-based assays (Fig. 3A). OPP induced a transient burst of H_2O_2 that reached its maximum level 10 min after treatment. The elicitor capacity of OPP was compared with that induced by OG (200 μg/mL). Interestingly, this response was faster and more transient respect to the response induced by OG (200 μg/mL), where the peak occurred at 20 min and persisted over time (Fig. 3B). Mitogen-activated protein kinases (MAPKs) play a crucial role in coordinating defence mechanisms against pathogen invasions (Dvořák et al. 2021). We next evaluated the temporal patterns of phosphorylation of MAPKs (MAPK6/MAPK3/-MAPK4/MAPK11) in mock- and elicitor-treated Arabidopsis seedlings. OPP-treated seedlings showed the phosphorylation of MAPK6 after 5 min reaching a peak at 10 min (Fig. 3C). Interestingly, after treatment

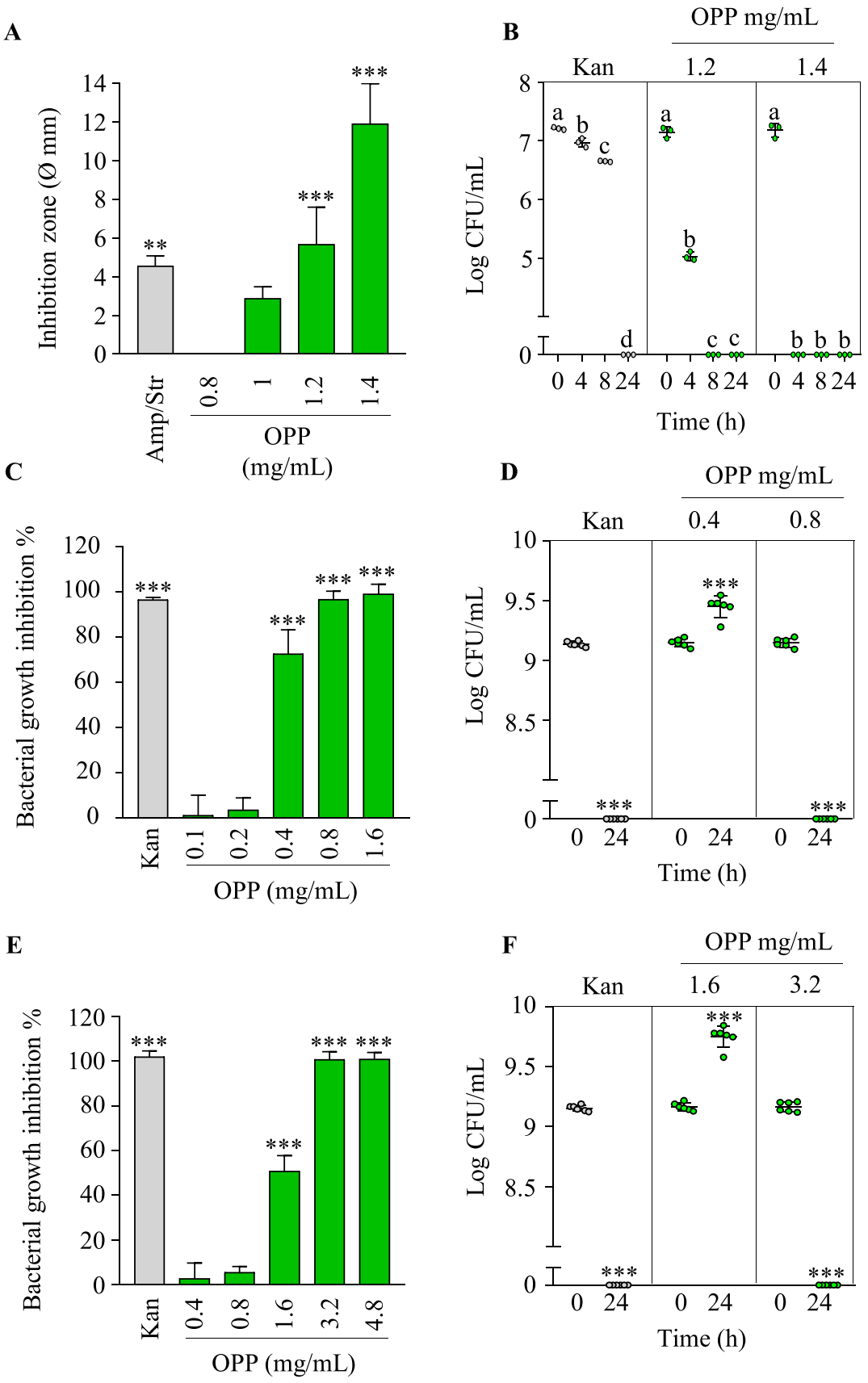

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Fig. 1. OPP showed antibacterial effects against *Xylella fastidiosa, Pseudomonas syringae* and *Pectobacterium carotovorum* (A) Inhibition of *Xylella fastidiosa* growth by OPP, quantified by disk diffusion assay. The diameter of the bacterial growth inhibition zones (Ø, mm) induced by various OPP concentrations (ranging from 0.8 mg/mL to 1.4 mg/mL) on *Xylella fastidiosa* growth was measured. A mixture of 100 μg/mL ampicillin and 250 μg/mL streptomycin (Amp/Str) was used as positive control. Data represent mean ± SD ($n = 9$). Statistical analysis was performed using the Kruskal-Wallis test comparing all concentrations against 0.8 mg/mL (**, $p \leq 0.01$; ***, $p \leq 0.001$). (B) Colony forming units (cfu) determined by time kill contact assay at times 0, 4, 8, and 24 h (h) after incubation of *Xylella fastidiosa* in PD3 medium mixed with 1.2 and 1.4 mg/mL OPP. 50 μg/mL kanamycin (Kan) was used as a positive control. Data represent the mean ± SD ($n = 3$). Different letters indicate significantly different datasets according to ANOVA followed by Tukey's test ($p \leq 0.001$) comparing the different times for each concentration. Bacterial growth inhibition of *Pseudomonas syringae* (C) or *Pectobacterium carotovorum* (E) determined by broth dilution assay at 24 h after incubation in LB medium (negative control) or LB mixed with OPP dose from 0.1 to 1.6 mg/mL for *Pseudomonas syringae* or from 0.4 to 4.8 mg/mL for *Pectobacterium carotovorum*. LB medium mixed with 50 μg/mL kanamycin (Kan) was used as positive control. The values are expressed as percentage of optical density unity 600 nm (OD_{600}) of treatments related to negative control. Data represent the mean ± SD ($n = 9$).Asterisks indicate significant differences between OPP doses respect to the lowest dose (0.1 mg/mL) according to Student's t-test (***, $p \leq 0.001$). Colony forming units (cfu) determined at 0 and 24 h (h) after incubation of *Pseudomonas syringae* (D) or *Pectobacterium carotovorum* (F) in LB medium mixed with 0.4 or 0.8 mg/mL OPP for *Pseudomonas syringae* and 1.6 or 3.2 mg/mL OPP for *Pectobacterium carotovorum*. LB medium mixed with 50 μg/mL Kan was used as positive control. Data represent the mean ± SD ($n = 6$). Asterisks indicate significant differences between 0 h and 24 h in each treatment according to Student's t-test (***, $p \leq 0.001$).

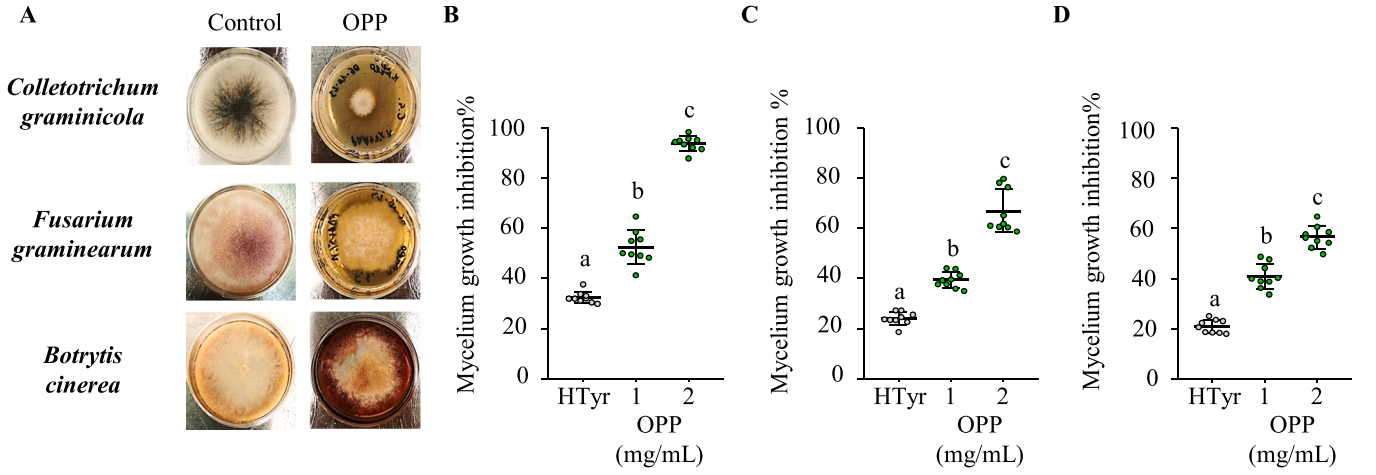

Fig. 2. OPP exhibited broad-spectrum antifungal activity. (A) Images of plates wherein fungi were incubated with 2 mg/mL OPP and with Potato Dextrose Agarose as negative control. The effect of hydroxytyrosol (Htyr) (700 μg/mL) or OPP (1 and 2 mg/mL) on the growth of *Colletotrichum graminicola* (B), *Fusarium graminearum* (C), and *Botrytis cinerea* (D) was measured. Results are expressed as percentage (%) of mycelium growth inhibition (percentage of treatment respect to negative control) measured after 7 days incubation. Data represent the mean ± SD ($n = 9$). Different letters indicate significantly different datasets according to ANOVA followed by Tukey's test ($p \leq 0.05$).

with OG, a different phosphorylation pattern was revealed after 10 min of treatment, involving, in addition to MAPK6, also MAPK3. MAPK4/11 phosphorylation was not observed in both OPP- or OG-treated seedlings. To explore on the ability of OPP activity to induce defence gene expression, the expression of three important genes for Arabidopsis immunity, *CYP81F2, WRKY53*, and *FRK1*, was assessed after 1 h of seedling treatment with OPP (10 ng/mL), OG (200 μg/mL) or mock (Fig. 3D-F). *CYP81F2* (*CYTOCHROME P450, FAMILY 81*) encodes a cytochrome P450 monooxygenase that participates in the biosynthesis of indole glucosinolates (Pfalz et al. 2009); *WRKY53* (*WRKY DNA-BINDING PROTEIN 33*) encodes a WRKY transcription factor associated with the salicylic acid signalling pathway, that can play a role in basal resistance against *P. syringae* (Murray et al. 2007); *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*) encodes a leucine-rich repeat receptor kinase involved in the initiation of early defence signalling cascades (Asai et al. 2002). The OPP or OG treatments stimulated the expression of all genes selected in comparison with mock-treated seedlings. However, an intriguing difference arises when comparing the gene induction patterns of OPP with OG. OG were more effective at inducing *CYP81F2*, whereas OPP primarily enhanced *WRKY53* expression. Both OPP and OGs showed similar levels of *FRK1* expression. The growth of Arabidopsis seedlings treated with 10 ng/mL OPP was not significantly different from control (Figure S1). Overall, our results indicate that exogenous application of OPP to Arabidopsis can trigger multiple early immune hallmarks without affecting plant growth and that some may differ from those triggered by OG.

3.3. Pretreatment of Arabidopsis with OPP reduced disease symptoms caused by Botrytis cinerea and Pseudomonas syringae

The evidence that OPP can trigger defence responses prompted us to evaluate the possible protective effect of pretreatment of Arabidopsis with OPP against the necrotrophic fungus *B. cinerea*. Four-week-old adult plants were foliar-sprayed with OPP (10 ng/mL), OG (200 μg/mL), or mock and 24 h after the pretreatment, leaves were inoculated with *B. cinerea* spores (Fig. 4A). The primed expression of the defence-related genes *CYP81F2* and *PAD3* was evaluated at 8 h post infection (hpi) (Fig. 4B and C). *PAD3* (*PHYTOALEXIN DEFICIENT 3*) encodes a key biosynthetic enzyme involved in the synthesis of the antimicrobial compound camalexin and is essential for elicitor-induced resistance to *B. cinerea* (Schuhegger et al. 2006; Ferrari et al. 2007). OPP- and OG-pretreated plants showed a significant accumulation of *CYP81F2* and *PAD3* transcripts. While *CYP81F2* induction levels were comparable between the two elicitors, OG elicited a significantly higher induction of *PAD3* expression compared to OPP. After 48 hpi, OPP and OG pretreatments significantly improved resistance of Arabidopsis to *B. cinerea* (Fig. 4D and E). Plants pretreated with OPP showed a greater reduction of lesion area (about 64 %) than OG pretreated ones (about 48 %).

The OPP induction of *WRKY53* expression previously observed in seedlings suggested a potential priming and protective effect of the extract against *P. syringae* (Hu et al. 2012). To confirm this hypothesis, adult Arabidopsis plants were pretreated with OPP (10 ng/mL), OG (200 mg/mL) and after 24 h, they were sprayed with a suspension of

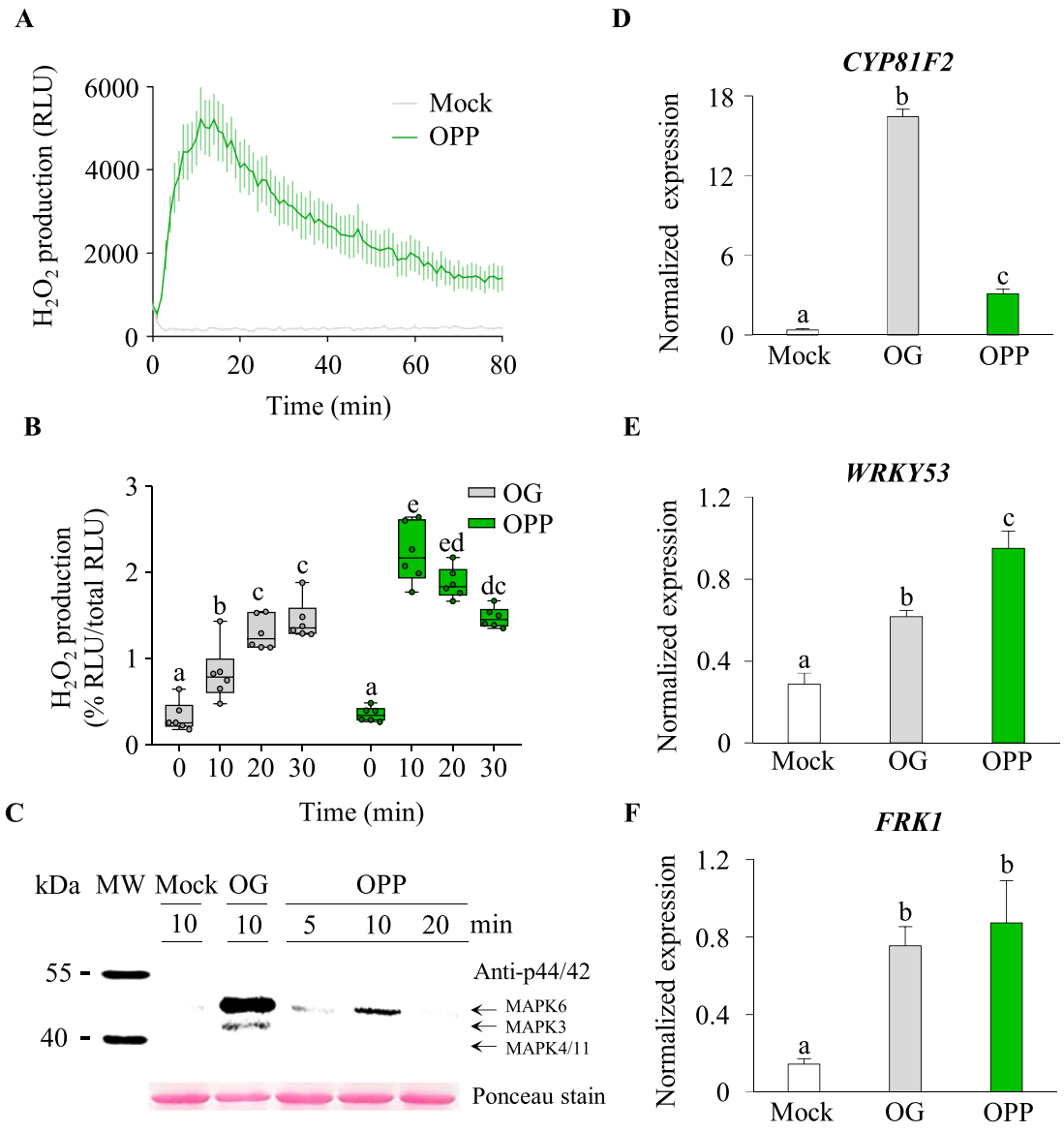

Fig. 3. OPP activated immunity hallmarks in Arabidopsis tissues. (A) Kinetics of H_2O_2 production measured by Luminol reaction after treatment with sterile distilled water (mock) or OPP (10 ng/mL) in four-week-old Arabidopsis leaf-discs and reported as Relative Luminescence Units (RLU). Data represent mean ± SE ($n = 6$). The experiments were repeated three times with similar results. (B) Ratio of RLU values after of OG (200 μg/mL) or OPP (10 ng/mL) at 10, 20, and 30 min respect to the total RLU. Data are presented as box plots, with the centre line showing the median, the box limits showing the 25th and 75th percentiles, and the whiskers showing the full range of data. The experiments were repeated three times with similar results. (C) MAPK activation in Arabidopsis seedlings in response to mock, OG (200 ug/ml), or OPP (10 ng/mL) treatment. MAPK phosphorylation was determined by western blot using the phospho-p44/42 MAPK antibody at different time points (5, 10, and 20 min). Equal loading was confirmed by Ponceau staining. Experiments were conducted three times with similar results. MW = Molecular Weight marker. Expression of (D) *CYP81F2*, (E)*WRKY53*, and (F) *FRK1* after mock, OG (200ug/ml), or OPP (10 ng/mL) treatment of Arabidopsis seedlings analysed by quantitative RT-PCR. The expression levels were normalized to *UBQ5* expression. Data represent the mean ± SE ($n = 3$). Different letters indicate significantly different datasets according to ANOVA followed by Tukey's test ($p \leq 0.05$).

P. syringae pv. tomato DC3000 (Fig. 5A). To explore on a possible OPP-induced priming effect, the expression of *WRKY53* and *CYP81F2* genes was monitored at 8 hpi and compared with mock pre-treatment (Fig. 5B and C). Pretreatment of Arabidopsis with OPP and OG significantly induced, to a similar extent, the expression of *WRKY53* and *CYP81F2*. The ability of OPP to protect Arabidopsis against *P. syringae* was investigated at 3 days post bacterial inoculation. A bacterial quantification was performed and expressed as CFU per leaf area (cm^2) (Fig. 5D). Bacterial growth was significantly reduced of about of 13 % in the OPP- and OG-pretreated plants compared with mock-pretreated plants. Overall, the results indicate that preventive pretreatments with OPP enhanced Arabidopsis immune performance and provided protection against *B. cinerea* and *P. syringae*.

3.4. OPP triggered H_2O_2 production and protected tomato against *Pseudomonas syringae*

The capacity of OPP to trigger immunity was tested also on economically important crop as tomato. Leaf discs collected from five-weeks old tomato plants were treated with OPP (10 ng/mL), or mock and H_2O_2 production was quantified by luminol-based assays (Fig. 6A and B). OPP induced a transient burst of H_2O_2 that reached its maximum level 20 min after treatment (Fig. 6A). As previously observed in Arabidopsis, the response of OPP is faster and more transient compared to that induced by OG (200 μg/mL) (Fig. 6B). These results prompted us to investigate on a possible tomato protection against pathogens. Five-week-old tomato plants were pretreated with OPP (10 ng/nL), OG

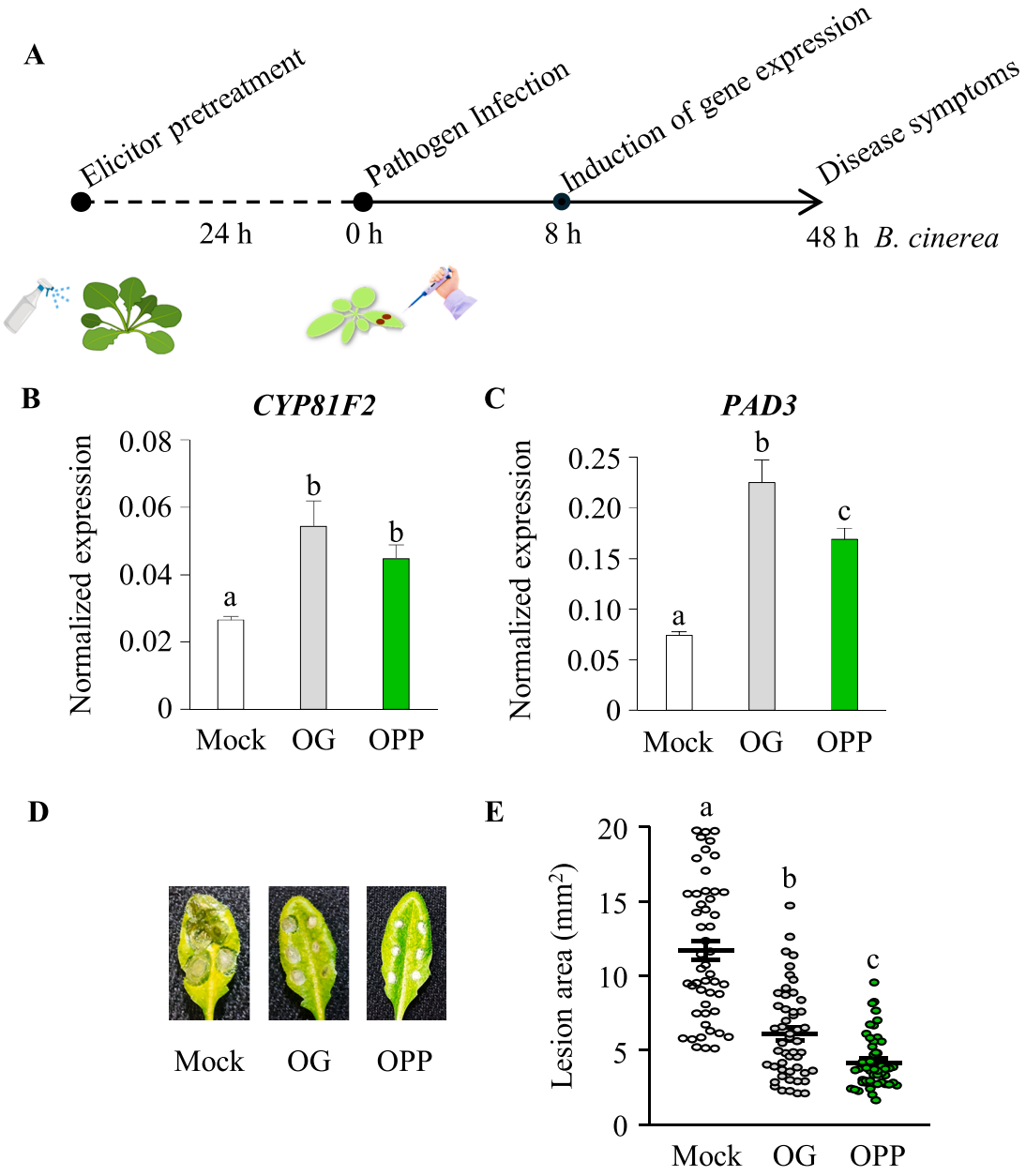

Fig. 4. Pretreatment of Arabidopsis with OPP enhanced immune response and protection against *B. cinerea*. (A) Four-week-old Arabidopsis Col-0 leaves were pretreated with OG (200 ug/ml), OPP (10 ng/mL) and mock and 24 h later were inoculated with *Botrytis cinerea*. (B) Quantitative RT-PCR analysis in *Botrytis cinerea*-infected leaves collected at 8 h post infection (hpi). mRNA expression levels are normalized to *UBQ5* expression. Data represent the mean ± SE ($n = 3$). Different letters indicate significantly different datasets according to ANOVA followed by Tukey's test ($p \leq 0.05$) (C-D-E) Lesion areas of *Botrytis cinerea* -infected leaves were measured at 48 h after the inoculation. Values are means ± SE of at least 50 lesions. Different letters indicate significantly different datasets according to ANOVA followed by Tukey's test ($p \leq 0.05$).

(200 μg/mL), or mock 24 h prior spray-inoculation with *P. syringae* (Fig. 6C). Notably, a 10 % reduction of bacterial accumulation was observed in OPP- and OG-pretreated tomato at 3 dpi compared to mock pretreated tomato. These results indicated that OPP can promptly stimulate the tomato immune system and its protection against *P. syringae*.

4. Discussion

In this work, we attempted to valorise two-phase olive pomace obtained from Coratina green fruit processing as source of phenolics with immunostimulant properties. A green-patented process based on a tangential flow membrane filtration of the liquid pomace allows us to extract a mixture of phenolic compounds named OPP with a total phenolic content of about 74 mg/mL. Interestingly, an higher yield of phenolics was extracted compared to other reports (Leouifoudi et al. 2015; Sklavos et al. 2015; Yangui and Abderrabba 2018; Frascari et al. 2019). This can be favoured by the extraction method used like also from the nature of the pomace used. The process involves fermentation and enzymatic digestion pretreatments, leading to a OPP with low sugar content and high phenolic concentration. Moreover, olive pomace used derived from a two-phase extraction, that does not require the addition of process water, reducing water consumption and wastewater production (Dahdouh et al. 2023). Furthermore, the cultivar and the degree of ripeness of the olives from which the pomace derives could also have contributed to a high yield of phenolic compounds (Aggoun et al. 2016). Coratina is one of the olive tree cultivars with the highest phenol content (Dauber et al. 2022; Yılmaz-Düzyaman et al. 2023). The biosynthesis of

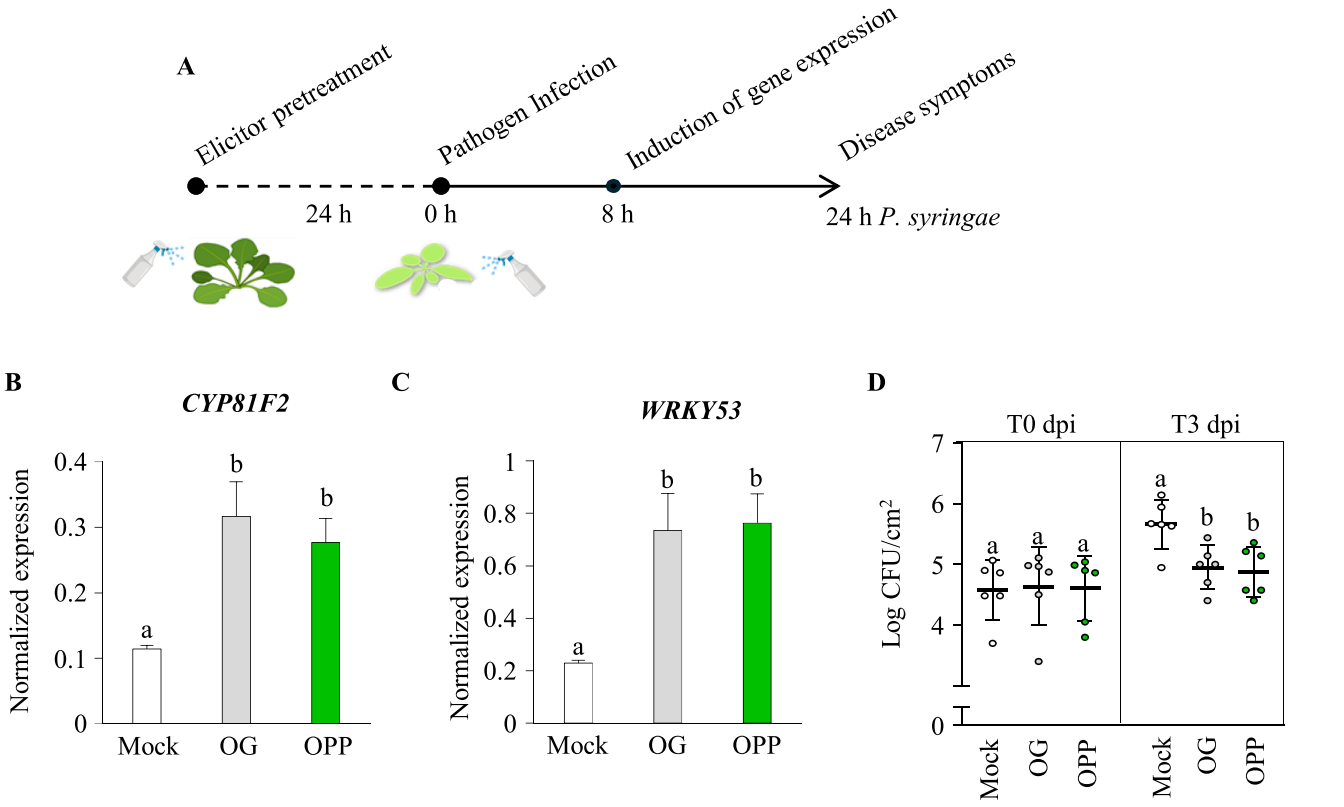

Fig. 5. Pretreatment of Arabidopsis with OPP enhanced immune response and protection against *Pseudomonas syringae*. (A) Leaves of 4-weeks-old Arabidopsis Col-0 plants were pre-treated with OG (200 μg/mL), OPP (10 ng/mL) and mock 1 day before bacterial inoculation. (B-C) Quantitative RT-PCR analysis in *Pseudomonas syringae* -infected leaves collected at 8 hpi. mRNA expression levels are normalized to *UBQ5* expression. Data represent the mean ± SE ($n = 3$). (D) Colony forming units (cfu) of *Pseudomonas syringae* per leaf area (cm^2) were determined at 3 h and 3 days post infection (T0 dpi and T3 dpi). Data represent mean ± SD ($n = 6$). Different letters indicate significantly different datasets according to ANOVA followed by Tukey's test ($p \leq 0.05$).

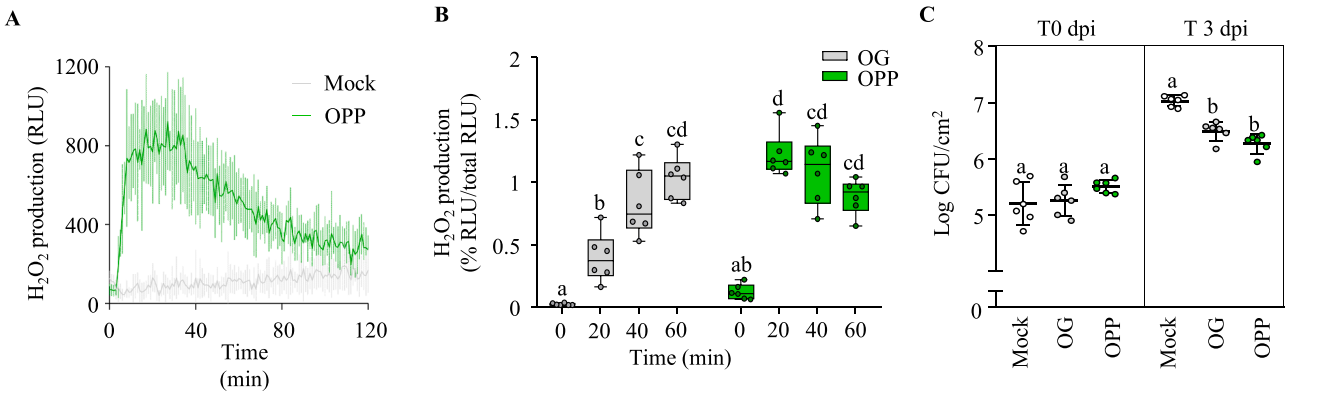

Fig. 6. OPP activated H_2O_2 production and induced *Pseudomonas syringae* resistance in tomato. (A) H_2O_2 production by luminol reaction after treatment with OG (200 μg/mL) or OPP (10 ng/mL) and mock, in five-week-old tomato leaf-discs. Data represent mean ± SE ($n = 6$). The experiments were repeated three times with similar results. (B) Ratio of RLU values after of OG (200 μg/mL) or OPP (10 ng/mL) at 20, 40, and 60 min respect to the total RLU. The experiments were repeated three times with similar results. (C) Leaves of five-week-old tomato Minibel plants were pre-treated with OG (200 μg/mL), OPP (10 ng/mL) and mock 1 day before *Pseudomonas syringae* inoculation. Bacterial CFU per leaf area (cm^2) were determined at 3 h and 3 days post infection (T0 dpi and T3 dpi). Data represent mean ± SD ($n = 6$). Different letters indicate significantly different datasets according to ANOVA followed by Tukey's test ($p \leq 0.05$).

phenolic compounds in olive tree is influenced by several factors including pedoclimatic conditions, cultivation practices, and ripening stages of fruits (Stanković et al. 2017; Sáinz et al. 2019; Jukić Špika et al. 2022). The fruits of the Coratina olive accumulate a greater quantity of phenolic compounds in the early stages of ripening, decreasing as ripening progresses (Dag et al. 2014; Kong et al. 2019). We aimed to obtain extracts enriched in low molecular weight compounds (<1 kDa), as these molecules may penetrate microbial and plant cell membranes to enhance plant protection better than larger molecules. The phenolic compounds characterization revealed that OPP is a mixture mainly enriched in hydroxytyrosol and tyrosol, with minor amounts of verbascoside, and traces of oleuropein. It should be considered that OPP does not encompass all phenolic compounds in Coratina cultivar pomace. Some higher molecular weight phenolics, such as tannins, lignans, and polyphenols like quercetin and luteolin, may also be present but are not targeted in our extraction process.

Previous evidence indicates olive fruits phenolic compounds as antimicrobial agents against specific bacteria and fungi (Drais et al. 2021; Pannucci et al. 2021). This work reveals new opportunities for the exploitation of phenolic compounds present in olive pomace as antimicrobial agents against key phytopathogenic bacteria and fungi. OPP showed a broad spectrum of inhibitory activity against the growth of a wide range of pathogens. Importantly, OPP was able to contrast the growth of *X. fastidiosa,* the bacterium responsible for the devastating plant disease Olive Quick Decline Syndrome (Surano et al. 2024). The same value of MIC and MBC (1.2 mg/mL) indicate that OPP have a bactericidal action against this microbe. This is the first time that a treatment with an hydroxytyrosol-enriched mixture extracted from pomace has proven effective against *X. fastidiosa* subsp. *pauca*, the strain that causes the great devastation in the Mediterranean countries (Surano et al. 2024). This discovery opens significant possibilities for treating *X. fastidiosa*-infected olive trees with OPP through foliar and/or endo-therapy. OPP was also effective against the bacterial phytopathogen *P. syringae* (MIC/MBC=0.8 mg/mL). This bacterium causes disease symptoms ranging from leaf spots to stem cankers, that can lead to significant crop losses in hundreds of plant species (Santamaría-Hernando et al. 2022; El-Fatah et al. 2023). Our evidence indicates for the first time a strong susceptibility of *P. syringae* to pomace phenolic compounds. *P. carotovorum* is also sensitive to OPP. *P. carotovorum* causes the destructive soft rot disease to many economically important vegetables (Abd-El-Khair et al. 2021). *P. carotovorum* was less sensitive to OPP than the other two pathogenic bacteria previously tested (MIC/MBC=3.2 mg/mL). Future studies will be necessary to understand the ability of this bacterium to better tolerate phenolic compounds. The bactericidal activity of OPP could be attributed to their potential effects on virulence determinants, such as motility, biofilm formation, and extracellular enzyme activities (Joshi et al. 2015) or to their ability to chelate transition metals like iron and copper, thereby reducing their bioavailability and limiting bacterial growth and virulence (Shina et al. 2012).

The antifungal activities of OPP were also investigated in vitro against three economically important pathogens, *C. graminicola, F. graminearum*, and *B. cinerea*. In particular, *Colletotrichum* causes anthracnose disease, one of the most important diseases in maize production (Rogério et al. 2022). *F. graminearum* causes fusarium head blight disease in cereal crops and downgrades grain quality because of its mycotoxin production (Jiang et al. 2023; Loron et al. 2023). *B. cinerea* causes gray mold disease in a wide range of important crops (Bi et al. 2023). OPP exhibited significant antifungal activity against all fungal pathogens. At comparable concentrations, OPP appeared to more effectively inhibit fungal growth than hydroxytyrosol alone. This suggests that the other phenolic components of the OPP mixture may act synergistically with hydroxytyrosol. The low susceptibility of *B. cinerea* to OPP could be attributed to the ability of *B. cinerea* to synthesize laccase enzymes, known to deactivate phenolic compounds by oxidation (Claus, 2017).

Our results highlight for the first time the role of phenolic compounds present in olive pomace, and therefore in green olive drupes, as inducers of plant immunity. OPP pretreatment induced several Arabidopsis defence responses including H_2O_2 production, MAPK phosphorylation, and defence gene expression reported to mediate increased indole glucosinolate biosynthesis, initiation of early defence signalling cascades and the salicylic acid signalling pathway. Interestingly, some differences in the ability to elicit defenceresponses was observed by OPP treatment respect to OG. A faster and more transient H_2O_2 oxidative burst was induced by OPP respect to OG. OPP treatment induced phosphorylation of MAPK6 while OG treatment activated the phosphorylation of both MAPK6 and MAPK3. Furthermore, a different pattern of induction of *WRKY53, CYP81F2*, and *FRK1* expression was triggered by OPP respect to OG. These results indicate that OPP and OG may activate distinct signal transduction pathways and defence responses. The two elicitors could be combined to obtain immunity based on the activation of multiple pathways, thus providing broader effectiveness against pathogens. We plan to focus some of our upcoming experiments on testing this hypothesis. OG and arabino-oligosaccharides have been identified in olive pomace (Greco et al., 2024). Therefore, it is essential to consider the co-presence of at least two types of elicitors in this by-product.

We proved that OPP pretreatment primed the expression of defence genes in adult Arabidopsis and reduced *B. cinerea* or *P. syringae* disease incidences. The ability of OPPs to combat phytopathogens may result from the synergy between their immunostimulant effects and their antifungal activity. OPP pretreatments enhanced the ability of Arabidopsis to express *CYP81F2* and *PAD3* and to resist to *B. cinerea.* This could lead to an higher production of the antimicrobial compounds indole glucosinolate and the phytoalexin camalexin (Schuhegger et al. 2006; Pfalz et al. 2009). OPP pretreatments also enhanced the ability of Arabidopsis to express *CYP81F2* and *WRKY53* to resist to *P. syringae.* The induction of the latter could imply the ability of OPP to induce the salicylic acid dependent pathway (Hu et al. 2012; Bresson et al. 2022). OPP concentrations that we found to elicit plant immunity did not affect Arabidopsis growth. Importantly, similar results were observed in tomato, one of the most important commercial crops worldwide. OPP pretreatment of tomato plants induced H_2O_2 accumulation and improved resistance to *P. syringae.*

Further research is necessary to improve the understanding of the OPP composition and its mechanism of action in plant immunity and pest control. Trace of other low molecular weight phenolic compounds (e.g., caffeic acid, gallic acid, ferulic acid, coumaric acid, and quercetin) may be present in the OPP (Sciubba et al. 2020). Additionally, TFMF may extract small amounts of other non-phenolic molecules. Future analyses, including LC-MS and NMR, will enhance our understanding of other potential compounds present in OPP.The use of OPP in agriculture could face limitations regarding their stability and bioavailability in different environmental conditions. Investigating OPP stability and bioavailability is crucial, along with optimizing extraction methods and dosages for effective use as biostimulants or biopesticides. Incorporating suitable excipients and standardizing application procedures will be important for field implementation. Additionally, the need for regulatory approval and the challenges associated with standardizing application methods may hinder widespread adoption. Conducting a life cycle assessment and a technology evaluation assessment will help to evaluate the environmental and economic impacts. Addressing these issues through further research will be essential for maximizing the potential of these compounds as biostimulants or biopesticides. OPP immunostimulants and antimicrobial properties could enhance olive tree resistance to *X. fastidiosa*. Utilizing OPP can promote a circular economy by upcycling olive mill wastes in green molecules for olive growth and health.

In conclusion, the results reported in this work can pave the way for upcycling two-phase olive pomace into a valuable mine of natural non-synthetic biopesticides and immunostimulants phenolic compounds to be used in eco-friendly plant disease control strategies against a broad spectrum of phytopathogens. The use of OPP represents a promising opportunity for integrated pest management programs to reduce the use of chemical pesticides and mitigating environmental pollution related to olive pomace disposal in the soil.

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CRediT authorship contribution statement

Marco Greco: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **María Fuertes-Rabanal:** Visualization, Investigation. **Carlos Frey:** Visualization, Investigation. **Carmine Del Grosso:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Daniele Coculo:** Visualization, Writing – review & editing. **Pasquale Moretti:** Supervision, Methodology, Investigation. **Pasquale Saldarelli:** Writing – review & editing, Supervision. **Savino Agresti:** Resources, Conceptualization. **Rosanna Caliandro:** Visualization, Investigation. **Hugo Mélida:** Writing – review & editing, Supervision, Funding acquisition. **Vincenzo Lionetti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100655.

Data availability

The data that has been used is confidential.

References

Abd-El-Khair, H., Abdel-Gaied, T.G., Mikhail, M.S., Abdel-Alim, A.I., El-Nasr, H.I.S, 2021. Biological control of Pectobacterium carotovorum subsp. carotovorum, the causal agent of bacterial soft rot in vegetables, in vitro and in vivo tests. Bull. Natl. Res. Cent. 45 (1), 37. https://doi.org/10.1186/s42269-021-00491-4.

Aggoun, M., Arhab, R., Cornu, A., Portelli, J., Barkat, M., Graulet, B., 2016. Olive mill wastewater microconstituents composition according to olive variety and extraction process. Food Chem. 209, 72–80. https://doi.org/10.1016/j.foodchem.2016.04.034.

Al-Eitan, L., Alkhatib, R., Mahawreh, B., Tarkhan, A., Malkawi, H., Rusan, M., 2020. The effects of olive mill wastewater on soil microbial populations. Jordan J. Biol. Sci. https://doi.org/10.54319/jjbs/140321.

Amini Tapouk, F., Nabizadeh, R., Mirzaei, N., Hosseini Jazani, N., Yousefi, M., Valizade Hasanloei, M.A, 2020. Comparative efficacy of hospital disinfectants against nosocomial infection pathogens. Antimicrob. Resist. Infect. Control 9 (1), 115. https://doi.org/10.1186/s13756-020-00781-y.

Arnoldi, A., Clodoveo, M.L., Corbo, F.F.R., Franchini, C., Lammi, C., Lentini, G., Lorenzo, V., Massari, C.D., Milani, G., Moretti, P., et al., 2023. Processo produttivo di complessi polifenolici da acque di vegetazione olearie con processo fermentativo e relativi complessi polifenolici prodotti. Patent IT202100019226A1.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., Sheen, J., 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415 (6875), 977–983. https://doi.org/10.1038/415977a.

Bektas, Y., Eulgem, T., 2015. Synthetic plant defense elicitors. Front. Plant Sci. 5. https://doi.org/10.3389/fpls.2014.00804.

Ben Sassi, A., Boularbah, A., Jaouad, A., Walker, G., Boussaid, A, 2006. A comparison of olive oil mill wastewaters (OMW) from three different processes in Morocco. Process Biochem. 41 (1), 74–78. https://doi.org/10.1016/j.procbio.2005.03.074.

Benedetti, M., Mattei, B., Pontiggia, D., Salvi, G., Savatin, D.V., Ferrari, S., 2017. Methods of isolation and characterization of oligogalacturonide elicitors. In: Plant Pattern Recognition Receptors: Methods and Protocols, L Shan and P He, eds, Methods in Molecular Biology. Springer, New York, NY, pp. 25–38. https://doi.org/10.1007/978-1-4939-6859-6_3.

Berbel, J., Posadillo, A., 2018. Review and analysis of alternatives for the valorisation of agro-industrial olive oil by-products. Sustainability 10 (1), 237. https://doi.org/10.3390/su10010237.

Bi, K., Liang, Y., Mengiste, T., Sharon, A, 2023. Killing softly: a roadmap of Botrytis cinerea pathogenicity. Trends Plant Sci. 28 (2), 211–222. https://doi.org/10.1016/j.tplants.2022.08.024.

Bresson, J., Doll, J., Vasseur, F., Stahl, M., von, Roepenack-Lahaye E, Kilian, J., Stadelhofer, B., Kremer, J.M., Kolb, D., Wenkel, S., et al., 2022. The genetic interaction of REVOLUTA and WRKY53 links plant development, senescence, and immune responses. PLoS One 17 (3), e0254741. https://doi.org/10.1371/journal.pone.0254741.

Cassano, A., Conidi, C., Giorno, L., Drioli, E, 2013. Fractionation of olive mill wastewaters by membrane separation techniques. J. Hazard. Mater. 248–249, 185–193. https://doi.org/10.1016/j.jhazmat.2013.01.006.

Chanioti, S., Tzia, C., 2017. Optimization of ultrasound-assisted extraction of oil from olive pomace using response surface technology: oil recovery, unsaponifiable matter, total phenol content and antioxidant activity. LWT - Food Sci. Technol. 79, 178–189. https://doi.org/10.1016/j.lwt.2017.01.029.

Chowdhary, V., Alooparampil, S., Pandya, R.V., Tank, J.G., 2021. Physiological function of phenolic compounds in plant defense system. Phenolic Compounds - Chemistry, Synthesis, Diversity, Non-Conventional Industrial, Pharmaceutical and Therapeutic Applications. IntechOpen. https://doi.org/10.5772/intechopen.101131.

Claus, H., 2017. Laccases of botrytis cinerea. Biology of Microorganisms on Grapes, in Must and in Wine, pp. 339–356. https://doi.org/10.1007/978-3-319-60021-5_14.

Coculo, D., Del Corpo, D., Martínez, M.O., Vera, P., Piro, G., De Caroli, M., Lionetti, V., 2023. Arabidopsis subtilases promote defense-related pectin methylesterase activity and robust immune responses to botrytis infection. Plant Physiol. Biochem. 201, 107865. https://doi.org/10.1016/j.plaphy.2023.107865.

Conrath, U., Beckers, G., Langenbach, C., Jaskiewicz, M., 2015. Priming for enhanced defense. Annu. Rev. Phytopathol. 53. https://doi.org/10.1146/annurev-phyto-080614-120132.

Curci, F., Corbo, F., Clodoveo, M.L., Salvagno, L., Rosato, A., Corazza, I., Budriesi, R., Micucci, M., LB, Mattioli, 2022. Polyphenols from olive-mill wastewater and biological activity: focus on irritable bowel syndrome. Nutrients 14 (6), 1264. https://doi.org/10.3390/nu14061264.

Curl, C.L., Spivak, M., Phinney, R., Montrose, L, 2020. Synthetic pesticides and health in vulnerable populations: agricultural workers. Curr. Environ. Health Rep. 7 (1), 13–29. https://doi.org/10.1007/s40572-020-00266-5.

Dag, A., Harlev, G., Lavee, S., Zipori, I., Kerem, Z., 2014. Optimizing olive harvest time under hot climatic conditions of Jordan Valley, Israel. Eur. J. Lipid Sci. Technol. 116. https://doi.org/10.1002/ejlt.201300211.

Dahdouh, A., Khay, I., Le Brech, Y., El Maakoul, A., Bakhouya, M, 2023. Olive oil industry: a review of waste stream composition, environmental impacts, and energy valorization paths. Environ. Sci. Pollut. Res. 30, 1–25. https://doi.org/10.1007/s11356-023-25867-z.

Dauber, C., Carreras, T., Fernández Fernández, A., Irigaray, B., Albores, S., Gámbaro, A., Ibáñez, E., Vieitez, I, 2022. Response surface methodology for the optimization of biophenols recovery from "alperujo" using supercritical fluid extraction. comparison between Arbequina and Coratina cultivars. J. Supercrit. Fluids 180, 105460. https://doi.org/10.1016/j.supflu.2021.105460.

Davis, M.J., French, W.J., Schaad, N.W., 1981. Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. Curr. Microbiol. 6 (5), 309–314. https://doi.org/10.1007/BF01566883.

De Lorenzo, G., Ferrari, S., Cervone, F., Okun, E., 2018. Extracellular DAMPs in plants and mammals: immunity, tissue damage and repair. Trends Immunol. 39 (11), 937–950. https://doi.org/10.1016/j.it.2018.09.006.

DeFalco, T.A., Zipfel, C., 2021. Molecular mechanisms of early plant pattern-triggered immune signaling. Mol. Cell 81 (17), 3449–3467. https://doi.org/10.1016/j.molcel.2021.07.029.

Del Corpo, D., Coculo, D., Greco, M., De Lorenzo, G., Lionetti, V., 2024. Pull the fuzes: processing protein precursors to generate apoplastic danger signals for triggering plant immunity. Plant Commun 5 (8), 100931. https://doi.org/10.1016/j.xplc.2024.100931.

Del Corpo, D., Fullone, M.R., Miele, R., Lafond, M., Pontiggia, D., Grisel, S., Kieffer-Jaquinod, S., Giardina, T., Bellincampi, D., Lionetti, V., 2020. AtPME17 is a functional Arabidopsis thaliana pectin methylesterase regulated by its PRO region that triggers PME activity in the resistance to Botrytis cinerea. Mol. Plant Pathol. 21, 1620–1633. https://doi.org/10.1111/mpp.13002.

Del Grosso, C. 2022. Selection, evaluation and characterization of bioactive compounds for their activity against Xylella fastidiosa and other phytopathogenic bacteria. PhD thesis. Università del Molise.

Dempsey, D.A., Klessig, D.F., 2017. How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans? BMC Biol. 15 (1), 23. https://doi.org/10.1186/s12915-017-0364-8.

Di Lecce, G., Cassano, A., Bendini, A., Conidi, C., Giorno, L., TG, Toschi, 2014. Characterization of olive mill wastewater fractions treatment by integrated

membrane process. J. Sci. Food Agric. 94 (14), 2935–2942. https://doi.org/10.1002/jsfa.6637.
Drais, M.I., Pannucci, E., Caracciolo, R., Bernini, R., Romani, A., Santi, L., Varvaro, L., 2021. Antifungal activity of hydroxytyrosol enriched extracts from olive mill waste against Verticillium dahliae, the cause of Verticillium wilt of olive. Phytopathol. Mediterr. 60 (1), 139–148. https://doi.org/10.36253/phyto-12019.
Dvořák, P., Krasylenko, Y., Zeiner, A., Šamaj, J., Takáč, T., 2021. Signaling toward reactive oxygen species-scavenging enzymes in plants. Front. Plant Sci. 11. https://doi.org/10.3389/fpls.2020.618835.
El-Fatah, B.E.S.A., Imran, M., Abo-Elyousr, K.A.M., AF, Mahmoud, 2023. Isolation of Pseudomonas syringae pv. tomato strains causing bacterial speck disease of tomato and marker-based monitoring for their virulence. Mol. Biol. Rep. 50 (6), 4917–4930. https://doi.org/10.1007/s11033-023-08302-x.
Enaime, G., Baçaoui, A., Yaacoubi, A., Belaqziz, M., Wichern, M., Lübken, M., 2020. Phytotoxicity assessment of olive mill wastewater treated by different technologies: effect on seed germination of maize and tomato. Environ. Sci. Pollut. Res. 27 (8), 8034–8045. https://doi.org/10.1007/s11356-019-06672-z.
Enaime, G., Dababat, S., Wichern, M., Lübken, M, 2024. Olive mill wastes: from wastes to resources. Environ. Sci. Pollut. Res. 31 (14), 20853–20880. https://doi.org/10.1007/s11356-024-32468-x.
Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F.M., Dewdney, J, 2007. Resistance to botrytis cinerea induced in arabidopsis by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. Plant Physiol. 144 (1), 367–379. https://doi.org/10.1104/pp.107.095596.
Förster, H., Kanetis, L., Adaskaveg, J, 2004. Spiral gradient dilution, a rapid method for determining growth responses and 50% effective concentration values in fungus-fungicide interactions. Phytopathology 94, 163–170. https://doi.org/10.1094/PHYTO.2004.94.2.163.
Foti, P., Romeo, F.V., Russo, N., Pino, A., Vaccalluzzo, A., Caggia, C., CL, Randazzo, 2021. Olive mill wastewater as renewable raw materials to generate high added-value ingredients for agro-food industries. Appl. Sci. 11 (16), 7511. https://doi.org/10.3390/app11167511.
Frascari, D., Rubertelli, G., Arous, F., Ragini, A., Bresciani, L., Arzu, A., Pinelli, D., 2019. Valorisation of olive mill wastewater by phenolic compounds adsorption: development and application of a procedure for adsorbent selection. Chem. Eng. J. 360, 124–138. https://doi.org/10.1016/j.cej.2018.11.188.
Fuertes-Rabanal M., Largo-Gosens A., Fischer A., Munzert K.S., Carrasco-López C., Sánchez-Vallet A., Engelsdorf T., and Mélida H. Linear β-1,2-glucans trigger immune hallmarks and disease resistance in plants. 2024:2024.05.30.596602. 10.1101/2024.05.30.596602.
Galletti, R., Denoux, C., Gambetta, S., Dewdney, J., Ausubel, F.M., De Lorenzo, G., Ferrari, S., 2008. The AtrbohD-mediated oxidative burst elicited by oligogalacturonides in Arabidopsis is dispensable for the activation of defense responses effective against Botrytis cinerea. Plant Physiol. 148 (3), 1695–1706. https://doi.org/10.1104/pp.108.127845.
Ge, D., Yeo, I.-C., Shan, L, 2022. Knowing me, knowing you: self and non-self recognition in plant immunity. Essays Biochem. 66 (5), 447–458. https://doi.org/10.1042/EBC20210095.
Giovannoni, M., Marti, L., Ferrari, S., Tanaka-Takada, N., Maeshima, M., Ott, T., De Lorenzo, G., Mattei, B., 2021. The plasma membrane–associated Ca2+ -binding protein, PCaP1, is required for oligogalacturonide and flagellin-induced priming and immunity. Plant Cell Environ. 44 (9), 3078–3093. https://doi.org/10.1111/pce.14118.
Greco, M., Kouzounis, D., Fuertes-Rabanal, M., Gentile, M., Agresti, S., Schols, H.A., Mélida, H., Lionetti, V., 2024. Upcycling olive pomace into pectic elicitors for plant immunity and disease protection. Plant Physiol. Biochem. 217, 109213. https://doi.org/10.1016/j.plaphy.2024.109213.
Hilker, M., Schmülling, T., 2019. Stress priming, memory, and signalling in plants. Plant Cell Environ. 42 (3), 753–761. https://doi.org/10.1111/pce.13526.
Hönig, M., Roeber, V.M., Schmülling, T., Cortleven, A., 2023. Chemical priming of plant defense responses to pathogen attacks. Front. Plant Sci. 14. https://doi.org/10.3389/fpls.2023.1146577.
Hu, Y., Dong, Q., Yu, D, 2012. Arabidopsis WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen Pseudomonas syringae syringae. Plant Sci. 185–186, 288–297. https://doi.org/10.1016/j.plantsci.2011.12.003.
Jiang, H., Zhong, S., Schwarz, P., Chen, B., Rao, J., 2023. Antifungal activity, mycotoxin inhibitory efficacy, and mode of action of hop essential oil nanoemulsion against Fusarium graminearum. Food Chem. 400, 134016. https://doi.org/10.1016/j.foodchem.2022.134016.
Joshi, J.R., Burdman, S., Lipsky, A., Yedidia, I, 2015. Effects of plant antimicrobial phenolic compounds on virulence of the genus *Pectobacterium*. Res. Microbiol. 166 (6), 535–545. https://doi.org/10.1016/j.resmic.2015.04.004.
Jukić Špika, M., Liber, Z., Montemurro, C., Miazzi, M.M., Ljubenkov, I., Soldo, B., Žanetić, M., Vitanović, E., Politeo, O., Škevin, D, 2022. Quantitatively unraveling hierarchy of factors impacting virgin olive oil phenolic profile and oxidative stability. Antioxidants 11 (3), 594. https://doi.org/10.3390/antiox11030594.
Karpouzas, D.G., Ntougias, S., Iskidou, E., Rousidou, C., Papadopoulou, K.K., Zervakis, G. I., Ehaliotis, C, 2010. Olive mill wastewater affects the structure of soil bacterial communities. Appl. Soil. Ecol. 45 (2), 101–111. https://doi.org/10.1016/j.apsoil.2010.03.002.
Kashyap, A., Jiménez-Jiménez, Á.L., Zhang, W., Capellades, M., Srinivasan, S., Laromaine, A., Serra, O., Figueras, M., Rencoret, J., Gutiérrez, A., et al., 2022. Induced ligno-suberin vascular coating and tyramine-derived hydroxycinnamic acid amides restrict Ralstonia solanacearum colonization in resistant tomato. New Phytol. 234 (4), 1411–1429. https://doi.org/10.1111/nph.17982.
Kircher, M., Aranda, E., Athanasios, P., Radojcic-Rednovnikov, I., Romantschuk, M., Ryberg, M., Schock, G., Shilev, S., Stanescu, M.D., Stankeviciute, J., et al., 2023. Treatment and valorization of bio-waste in the EU. EFB Bioeconomy J. 3, 100051. https://doi.org/10.1016/j.bioeco.2023.100051.
Kong, W., Han, R., Liu, N., Bai, W., Ma, J., Bai, X., Liang, J., Wang, J., Zhang, J., 2019. Dynamic assessment of the fruit quality of olives cultivated in Longnan (China) during ripening. Sci. Hortic. 253, 8–16. https://doi.org/10.1016/j.scienta.2019.04.037.
Lee, M., Jeon, H.S., Kim, S.H., Chung, J.H., Roppolo, D., Lee, H., Cho, H.J., Tobimatsu, Y., Ralph, J., Park, O.K., 2019. Lignin-based barrier restricts pathogens to the infection site and confers resistance in plants. EMBO J. 38 (23), e101948. https://doi.org/10.15252/embj.2019101948.
Leouifoudi, I., Harnafi, H., Zyad, A, 2015. Olive mill waste extracts: polyphenols content, antioxidant, and antimicrobial activities. Adv. Pharmacol. Sci. 2015, 714138. https://doi.org/10.1155/2015/714138.
Lionetti, V., Cervone, F., Bellincampi, D., 2012. Methyl esterification of pectin plays a role during plant-pathogen interactions and affects plant resistance to diseases. J. Plant Physiol. 169 (16), 1623–1630. https://doi.org/10.1016/j.jplph.2012.05.006.
Lionetti, V., Fabri, E., De Caroli, M., Hansen, A.R., Willats, W.G.T., Piro, G., Bellincampi, D., 2017. Three pectin methylesterase inhibitors protect cell wall integrity for arabidopsis immunity to botrytis. Plant Physiol. 173 (3), 1844–1863. https://doi.org/10.1104/pp.16.01185.
Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., Favaron, F., Cervone, F., Bellincampi, D., 2007. Overexpression of pectin methylesterase inhibitors in arabidopsis restricts fungal infection by botrytis Cinerea. Plant Physiol. 143 (4), 1871–1880. https://doi.org/10.1104/pp.106.090803.
Loron, A., Wang, Y., Atanasova, V., Richard-Forget, F., Gardrat, C., Coma, V., 2023. Chitosan for eco-friendly control of mycotoxinogenic Fusarium graminearum. Food Hydrocoll. 134, 108067. https://doi.org/10.1016/j.foodhyd.2022.108067.
Malapert, A., Reboul, E., Loonis, M., Dangles, O., Tomao, V., 2018. Direct and rapid profiling of biophenols in olive Pomace by UHPLC-DAD-MS. Food Anal. Methods 11 (4), 1001–1010. https://doi.org/10.1007/s12161-017-1064-2.
Maruri-López, I., Aviles-Baltazar, N.Y., Buchala, A., Serrano, M., 2019. Intra and extracellular journey of the phytohormone salicylic acid. Front. Plant Sci. 10, 423. https://doi.org/10.3389/fpls.2019.00423.
Miedes, E., Vanholme, R., Boerjan, W., Molina, A., 2014. The role of the secondary cell wall in plant resistance to pathogens. Front. Plant Sci. 5, 358. https://doi.org/10.3389/fpls.2014.00358.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.
Murray, S.L., Ingle, R.A., Petersen, L.N., KJ, Denby, 2007. Basal resistance against pseudomonas syringae in arabidopsis involves WRKY53 and a protein with homology to a nematode resistance protein. Mol. Plant-Microbe Interactions 20 (11), 1431–1438. https://doi.org/10.1094/MPMI-20-11-1431.
Ochando-Pulido, J.M., Martinez-Ferez, A., 2018. Operation setup of a nanofiltration membrane unit for purification of two-phase olives and olive oil washing wastewaters. Sci. Total Environ. 612, 758–766. https://doi.org/10.1016/j.scitotenv.2017.08.287.
Pannucci, E., Caracciolo, R., Romani, A., Cacciola, F., Dugo, P., Bernini, R., Varvaro, L., Santi, L., 2021. An hydroxytyrosol enriched extract from olive mill wastewaters exerts antioxidant activity and antimicrobial activity on Pseudomonas syringae savastanoi pv. savastanoi and Agrobacterium tumefaciens. Nat. Prod. Res. 35 (16), 2677–2684. https://doi.org/10.1080/14786419.2019.1662006.
Paraskeva, C.A., Papadakis, V.G., Kanellopoulou, D.G., Koutsoukos, P.G., KC, Angelopoulos, 2007. Membrane filtration of olive mill wastewater and exploitation of its fractions. Water Environ. Res. 79 (4), 421–429. https://doi.org/10.2175/106143006X115345.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids. Res. 29 (9), e45. https://doi.org/10.1093/nar/29.9.e45.
Pfalz, M., Vogel, H., Kroymann, J, 2009. The gene controlling the indole glucosinolate modifier1 quantitative trait locus alters indole glucosinolate structures and aphid resistance in arabidopsis. Plant Cell 21 (3), 985–999. https://doi.org/10.1105/tpc.108.063115.
Pinho, I.A., Lopes, D.V., Martins, R.C., MJ, Quina, 2017. Phytotoxicity assessment of olive mill solid wastes and the influence of phenolic compounds. Chemosphere 185, 258–267. https://doi.org/10.1016/j.chemosphere.2017.07.002.
Popolizio, S., Fracchiolla, M., Leoni, B., Cazzato, E., Camposeo, S., 2022. Phytotoxic effects of retentates extracted from olive mill wastewater suggest a path for bioherbicide development. Agronomy 12 (6), 1378. https://doi.org/10.3390/agronomy12061378.
Rana, A., Samtiya, M., Dhewa, T., Mishra, V., RE, Aluko, 2022. Health benefits of polyphenols: a concise review. J. Food Biochem. 46 (10), e14264. https://doi.org/10.1111/jfbc.14264.
Rather, I.A., Koh, W.Y., Paek, W.K., Lim, J., 2017. The sources of chemical contaminants in food and their health implications. Front. Pharmacol. 8. https://doi.org/10.3389/fphar.2017.00830.
Rebaque, D., López, G., Sanz, Y., Vilaplana, F., Brunner, F., Mélida, H., Molina, A., 2023. Subcritical water extraction of Equisetum arvense biomass withdraws cell wall fractions that trigger plant immune responses and disease resistance. Plant Mol. Biol. 113 (6), 401–414. https://doi.org/10.1007/s11103-023-01345-5.
Rogério, F., Baroncelli, R., Cuevas-Fernández, F.B., Becerra, S., Crouch, J., Bettiol, W., Azcárate-Peril, M.A., Malapi-Wight, M., Ortega, V., Betran, J., et al., 2022. Population genomics provide insights into the global genetic structure of

colletotrichum graminicola, the causal agent of maize anthracnose. mBio 14 (1). https://doi.org/10.1128/mbio.02878-22 e02878–e02822.
Saez, L., Perez, J., Martinez, J., 1992. Low molecular weight phenolics attenuation during simulated treatment of wastewaters from olive oil mills in evaporation ponds. Water Res. 26 (9), 1261–1266. https://doi.org/10.1016/0043-1354(92)90187-9.
Sáinz, J.A., Garrido, I., Hernández, M., Montaño, A., Llerena, J.L., Espinosa, F., 2019. Influence of cultivar, irrigation, ripening stage, and annual variability on the oxidant/antioxidant systems of olives as determined by MDS-PTA. PLoS One 14 (4), e0215540. https://doi.org/10.1371/journal.pone.0215540.
Santamaría-Hernando, S., Á, López-Maroto, Galvez-Roldán, C., Munar-Palmer, M., Monteagudo-Cascales, E., Rodríguez-Herva, J.-J., Krell, T., López-Solanilla, E, 2022. Pseudomonas syringae pv. tomato infection of tomato plants is mediated by GABA and L-Pro chemoperception. Mol. Plant Pathol. 23 (10), 1433–1445. https://doi.org/10.1111/mpp.13238.
Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B.L., Olsen, C.E., Svatoš, A., Halkier, B.A., Glawischnig, E, 2006. CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. Plant Physiol. 141 (4), 1248–1254. https://doi.org/10.1104/pp.106.082024.
Sciubba, F., Chronopoulou, L., Pizzichini, D., Lionetti, V., Fontana, C., Aromolo, R., Socciarelli, S., Gambelli, L., Bartolacci, B., Finotti, E, 2020. Olive mill wastes: a source of bioactive molecules for plant growth and protection against pathogens. Biology (Basel) 9 (12), 450. https://doi.org/10.3390/biology9120450.
Sheng, L., 2014. Determination of Biophenols in Olive Oil. https://doi.org/10.14293/P2199-8442.1.SOP-CHEM.PJCXGW.v1.
Shina, S., Shuaibu, M., Bello, A.B., Tureta, S.G., Isah, A., Izuagie, T., Nasiru, S., MB, Kamaru, 2012. Phytochemistry and antimicrobial activities of Cassia occidentalis used for herbal remedies. J. Che. Eng. 1, 38–41.
Singh, J., Rasane, P., Kaur, R., Kaur, H., Garg, R., Kaur, S., Ercisli, S., Choudhary, R., Skrovankova, S., Mlcek, J., 2023. Valorization of grape (Vitis vinifera) leaves for bioactive compounds: novel green extraction technologies and food-pharma applications. Front. Chem. 11. https://doi.org/10.3389/fchem.2023.1290619.
Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16 (3), 144–158. https://doi.org/10.5344/ajev.1965.16.3.144.
Sklavos, S., Gatidou, G., Stasinakis, A.S., Haralambopoulos, D., 2015. Use of solar distillation for olive mill wastewater drying and recovery of polyphenolic compounds. J. Environ. Manage. 162, 46–52. https://doi.org/10.1016/j.jenvman.2015.07.034.
Stanković, M., Ćurčić, S., Zlatić, N., Bojović, B., 2017. Ecological variability of the phenolic compounds of Olea europaea L. leaves from natural habitats and cultivated conditions. Biotechnol. Biotechnol. Equip. 31 (3), 499–504. https://doi.org/10.1080/13102818.2016.1275804.
Surano, A., del Grosso, C., Musio, B., Todisco, S., Giampetruzzi, A., Altamura, G., Saponari, M., Gallo, V., Mastrorilli, P., Boscia, D., et al., 2024. Exploring the xylem-sap to unravel biological features of Xylella fastidiosa subspecies pauca ST53 in immune, resistant and susceptible crop species through metabolomics and in vitro studies. Front. Plant Sci. 14. https://doi.org/10.3389/fpls.2023.1343876.
Swaminathan, S., Reem, N.T., Lionetti, V., OA, Zabotina, 2021. Coexpression of fungal cell wall-modifying enzymes reveals their additive impact on arabidopsis resistance to the fungal pathogen. Botrytis Cinerea. Biol. 10 (10), 1070. https://doi.org/10.3390/biology10101070.
Sygouni, V., Pantziaros, A.G., Iakovides, I.C., Sfetsa, E., Bogdou, P.I., Christoforou, E.A., Paraskeva, C.A., 2019. Treatment of two-phase olive mill wastewater and recovery of phenolic compounds using membrane technology. Membranes (Basel) 9 (2), 27. https://doi.org/10.3390/membranes9020027.
Tian, H., Wu, Z., Chen, S., Ao, K., Huang, W., Yaghmaiean, H., Sun, T., Xu, F., Zhang, Y., Wang, S., et al., 2021. Activation of TIR signalling boosts pattern-triggered immunity. Nature 598 (7881), 500–503. https://doi.org/10.1038/s41586-021-03987-1.
Tripathi, D., Raikhy, G., Kumar, D., 2019. Chemical elicitors of systemic acquired resistance—salicylic acid and its functional analogs. Curr. Plant Biol. 17, 48–59. https://doi.org/10.1016/j.cpb.2019.03.002.
Tudi, M., Daniel Ruan, H., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., DT, Phung, 2021. Agriculture development, pesticide application and its impact on the environment. Int. J. Environ. Res. Public Health 18 (3), 1112. https://doi.org/10.3390/ijerph18031112.
Vicré, M., Lionetti, V., 2023. Editorial: plant cell wall in pathogenesis, parasitism and symbiosis, Volume II. Front. Plant Sci. 14, 1230438. https://doi.org/10.3389/fpls.2023.1230438.
Wells, J.M., Raju, B.C., Nyland, G., SK, Lowe, 1981. Medium for isolation and growth of bacteria associated with plum leaf scald and phony peach diseases. Appl. Environ. Microbiol. 42 (2), 357–363. https://doi.org/10.1128/aem.42.2.357-363.1981.
Withers, J., Dong, X, 2017. Post-translational regulation of plant immunity. Curr. Opin. Plant Biol. 38, 124–132. https://doi.org/10.1016/j.pbi.2017.05.004.
Yangui, A., Abderrabba, M., 2018. Towards a high yield recovery of polyphenols from olive mill wastewater on activated carbon coated with milk proteins: experimental design and antioxidant activity. Food Chem. 262, 102–109. https://doi.org/10.1016/j.foodchem.2018.04.091.
Yılmaz-Düzyaman, H., Medina-Alonso, M.G., Sanz, C., Pérez, A.G., de la Rosa, R., León, L, 2023. Influence of genotype and environment on fruit phenolic composition of olive. Horticulturae 9 (10), 1087. https://doi.org/10.3390/horticulturae9101087.
Yugueros, S.I., Peláez, J., Stajich, J.E., Fuertes-Rabanal, M., Sánchez-Vallet, A., Largo-Gosens, A., Mélida, H., 2024. Study of fungal cell wall evolution through its monosaccharide composition: an insight into fungal species interacting with plants. Cell Surf. 11, 100127. https://doi.org/10.1016/j.tcsw.2024.100127.
Zagklis, D.P., Papageorgiou, C.S., Paraskeva, C.A., 2022. Valorization of phenolic extracts from *Olea europaea* L. by membrane operations. In: Iulianelli, A., Cassano, A., Conidi, C., Petrotos, K. (Eds.), Membrane Engineering in the Circular Economy. Elsevier, pp. 495–524. https://doi.org/10.1016/B978-0-323-85253-1.00003-4.
Zhou, J.-M., Zhang, Y., 2020. Plant immunity: danger perception and signaling. Cell 181 (5), 978–989. https://doi.org/10.1016/j.cell.2020.04.028.