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Upcycling olive pomace into pectic elicitors for plant immunity and disease protection

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A R T I C L E I N F O

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A B S T R A C T

Olive oil production generates substantial quantities of pomace, which are often disposed of in soil, leading to adverse effects on agriculture and the environment. Furthermore, climate change exacerbates plant diseases and promotes the use of toxic phytochemicals in agriculture. However, olive mill wastes can have high potential as reusable and valuable bioresources. Using diluted ethanol, an environmentally friendly solvent, we extracted a fraction containing short and long oligogalacturonides, short arabino-oligosaccharides and polysaccharides. The obtained extract elicited key features of plant innate immunity in Arabidopsis seedlings, including the phosphorylation of mitogen-activated protein kinases MPK3 and MPK6 and the upregulation of defence genes such as *CYP81F2*, *WRKY33*, *WRKY53*, and *FRK1*. Notably, pretreatment of adult Arabidopsis and tomato plants with the olive pomace extract primed defence responses and enhanced their resistance to the phytopathogens *Botrytis cinerea* and *Pseudomonas syringae*. Our results highlight the opportunity to upcycle the two-phase olive pomace collected at the late stage of olive oil campaign, in low-cost and sustainable glycan elicitors, contributing to reducing the use of chemically synthesized pesticides.

1. Introduction

Due to the growing market demand for agricultural products, traditional cultivation systems are turning into intensive plantations, that are heavily dependent on chemical fertilizers and pesticides, unsustainable for the environment (Pathak, 2018; Mairech et al., 2020). Agrochemical application causes severe environmental impacts, producing negative effects on non-target organisms, human health, and plant disease diffusion due to the onset of pathogen resistance (Miller et al., 2022; Soriano et al., 2022). Agricultural and environmental policies worldwide, including the Farm-to-Fork strategy within the European Green Deal, aim to replace chemical plant protection products with natural/organic preparations (Jin and Fang, 2018; Drobek et al., 2019; Farm to Fork, 2020; Panyasing et al., 2022).

A natural alternative to chemical pesticides which has gained momentum in recent times is the pretreatment of plant tissues with natural plant extracts which, acting as elicitors, activate the immune system protecting plant from pathogen attacks (Yang et al., 2022). Special emphasis is placed on plant oligosaccharides released during disease processes from the plant cell wall (CW) (Swaminathan et al., 2022; Vicré and Lionetti, 2023). In this work, we focused on CW oligosaccharides extracted from the pomace resulting from the pressing of the olive fruit. The CW of the pulp of this drupe is mainly composed of pectic polysaccharides, primarily homogalacturonan (HG; a linear pectin polymer composed of galacturonic acid (GalA) with a higher degree of methyl esterification) and arabinans (30–45%), with low amounts of rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Jiménez et al., 1994; Mafra et al., 2001). RG-I is a heteropolysaccharide composed of a repeating disaccharide unit, (1,2) α-L-rhamnosyl-(1,4) α-GalA. It features side chains of arabinan, galactan, arabinogalactan, and xylan. RG-II is a highly complex polysaccharide, composed of a short stretch of HG substituted with four different side chains. It contains a total of 12 different monosaccharides linked in over 21 distinct

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Abbreviations

AIS	Alcohol-insoluble solids
Ara	Arabinose
ARA-I	α-L-arabinofuranosidase/β-D-xylosidases
BXL4	β-D-XYLOSIDASE 4
cDNA	Complementary cDNA
Cfu	Colony forming units
Col-0	Columbia
CW	Cell wall
CYP81F2	*CYTOCHROME P450, FAMILY 81*
DA	Degree of acetyl-esterification
DAMP	Damage-Associated Molecular Patterns
DM	Degree of methyl-esterification
DP	Degree of polymerization
Dpi	Days post infection
DTI	Damage-Triggered Immunity
FRK1	*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GC	Gas chromatography
Glc	Glucose
GlcA	Glucuronic acid
HG	Homogalacturonan
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
Hpi	Hours post infection
HPLC-RI	High performance liquid chromatography with Refractive Index detector
Man	Mannose
MAPK	Mitogen-Activated Protein Kinases
MS	Murashige and Skoog
MWCO	Molecular weight cut-off
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
OG	Oligogalacturonides
OPE	Olive pomace extract
PAD3	*PHYTOALEXIN DEFICIENT 3*
PDB	Potato Dextrose Broth
PRR	Pattern-Recognition Receptors
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
Rha	Rhamnose
ROS	Reactive Oxygen Species
UBQ5	*UBIQUITIN5*
WRKY	*WRKY DNA-BINDING PROTEIN*
Xyl	Xylose

glycosidic linkages (Mohnen, 2008; Ralet et al., 2016; Amos et al., 2022). Hemicellulosic polysaccharides are also abundant (20–30%) in the olive pulp and may include xyloglucan, glucuronoxylan, and arabinoxylan. Mannans may also be present in trace amounts. Cellulose constitutes approximately 15–25% of the CW of the pulp. The olive fruit contains lignin primarily in the stone, which can account for a significant amount while the pulp holds much smaller amounts (1–5%). Lignin content can increase significantly in olive pomace due to stone milling (reaching about 20–35% of total CW polymers). Differences in CW composition can be observed between cultivars and ripening stages (Huisman, 1996; Jiménez et al., 2001). Pectin-related sugars (Galacturonic Acid; GalA, Rhamnose; Rha, and Arabinose; Ara) are abundant in olives early during ripening, whereas hemicellulose monosaccharides (Xylose; Xyl, Mannose; Man, Galactose; Gal, and Glucose; Glc) seems to increase later during ripening. Proteins and water are also present as CW components.

The biogenesis of CW oligosaccharides occurs when pathogens, to penetrate and spread into host tissues, degrade CW using an arsenal of CW-degrading enzymes (Bellincampi et al., 2014; Swaminathan et al., 2022). Specific CW fragments can be classified as Damage-Associated Molecular Patterns (DAMP), because, upon recognition by specific Pattern-Recognition Receptors (PRR), they can activate the Damage-Triggered Immunity (DTI) (De Lorenzo et al., 2018; Hou et al., 2019). DTI includes production of apoplastic Reactive Oxygen Species (ROS), cytoplasmic calcium influxes, activation of Mitogen-Activated Protein Kinases (MAPK) and induction of immune-related genes (Zhou and Zhang, 2020; DeFalco and Zipfel, 2021; Ge et al., 2022). Treatment of plants with DAMP represents an excellent solution to stimulate immunity and improve crop protection. Using natural alternatives to chemical pesticides fosters a more sustainable approach to agriculture. DAMP application confers to plants a greater ability to detect pathogens and activate defence responses faster than untreated crops (Hönig et al., 2023). This is a consequence of priming, a sensory state induced by the elicitors, remembering past elicitation events and responding to the next stresses with faster and stronger activation of defence (Conrath et al., 2015; Hilker and Schmülling, 2019). The most studied oligosaccharide DAMPs are oligogalacturonides (OG), which are pectic oligomers of α-1, 4-linked galacturonosyl residues which can be saturated or unsaturated and C-6 methyl esterified or O-2/O-3 acetyl-substituted (Lionetti et al., 2012). The activities of plant and microbial pectin methyl esterases and acetyl esterases, regulated by their inhibitors and activators, modulate the degree of pectin methyl- or acetyl esterification (DM and DA, respectively) (Lionetti, 2015; Lionetti et al., 2015a, 2015b; Swaminathan et al., 2021; Tundo et al., 2016). These biochemical CW traits can depend on several physiological factors such as the plant source, fruit ripening as well as biotic and abiotic stresses (Lionetti et al., 2015a; Del Corpo et al., 2020; Diarte et al., 2021; Mehari et al., 2019). DM and DA can impact on the release of pectic oligosaccharides as well as on their biological activity (Pogorelko et al., 2013; Coculo and Lionetti, 2022; Del Corpo et al., 2024). OG elicit a wide range of defense responses, including accumulation of phytoalexins, glucanases, and chitinases, deposition of callose, and production of ROS and nitric oxide (Ferrari et al., 2013). OG treatment primes and improves protection in different plants, such as *Arabidopsis thaliana*, grapevine (*Vitis vinifera*), and tomato (*Solanum lycopersicum*) against infections by the necrotrophic fungus *Botrytis cinerea* (Aziz et al., 2007; Ferrari et al., 2007; Gamir et al., 2021). In addition, the repertoire of CW-derived DAMP have recently expanded. The arabinosidase activities of the apoplastic β-D-XYLOSIDASE 4 (BXL4), a homolog of the barley bifunctional α-L-arabinofuranosidase/β-D-xylosidases (ARA-I) in Arabidopsis, was proposed to release arabinans from side-chains from RG-I and to contribute to immunity against *B. cinerea* and *Pseudomonas syringae* (Guzha et al., 2022; Bauer et al., 2023). Highly purified xyloglucan oligomers can activate immune responses, including MAPKs activation and callose deposition in grapevine and *Arabidopsis*, resulting in plant resistance against necrotrophic *B. cinerea* and biotrophic *Hyaloperonospora arabidopsidis* (Claverie et al., 2018). Tomato and pepper plants pretreated with arabinoxylan oligosaccharides are more resistant to *P. syringae* pv. t*omato* and to *Sclerotinia sclerotiorum* pathogens respectively (Mélida et al., 2020). CW-derived mixed-linked β-1,3/1,4-glucan oligosaccharides trigger immune responses and disease resistance in Arabidopsis, tomato, barley, and pepper to different bacterial and fungal pathogens (Barghahn et al., 2021; Rebaque et al., 2021).

The possibility of developing crop protection technologies based on the use of oligosaccharides as DAMP is hampered by the high costs for their large-scale production, mainly linked to the use of commercial

enzymes and substrates, and by the sustainability of the production processes (Giovannoni et al., 2020). The use of DAMP extracted from waste plant biomass could be a more sustainable and economic solution. DAMP can be extracted from plant CW using chemical and physical agents, such as Chelating agents (e.g. trans-1, 2-cyclohexanediaminetetraacetic acid) basic (e.g. KOH, NaOH, Na_2CO_3) or acidic solutions (e.g. HCl, trifluoroacetic acid), reducing agents (e.g. NaBH4) and high temperature (Francocci et al., 2013; Lionetti et al., 2015a, 2017; Lionetti, 2015; Bacete et al., 2017; Benedetti et al., 2017; Rebaque et al., 2023). These chemical substances, listed as corrosive, irritant, and toxic, could remain in traces in DAMP extracts, with the strong risk of being released in cultivated fields during DAMP treatments, causing strong environmental pollution. Aqueous ethanol solutions were used for the extractions of poly- and oligosaccharides from plant material for basic research objectives (Coculo et al., 2023; Guo et al., 2017). Pectins have a strong affinity for water molecules when in an aqueous solution. Organic solvents can be employed to disrupt these interactions between pectin and water, facilitating the pectin precipitation from the solution. Ethanol, in particular, has been commonly utilized for purifying pectins from aqueous mixtures (Yapo et al., 2007; Roman-Benn et al., 2023) and was previously used to precipitate bioactive OG from pectin digested with pectinases (Spiro et al., 1993; Pontiggia et al., 2015). Water and ethanol are considered as green solvent in the industrial applications because it is environmentally friendly, not hazardous and also can be produced from green source (Capello et al., 2007; Byrne et al., 2016; Yilmaz and Soylak, 2020).

One significant issue facing the olive mill industry is the substantial volume of agro-industrial waste generated during production processes (Fleyfel et al., 2022). Of particular concern is olive pomace, a semi-liquid waste frequently disposed in evaporation ponds near olive cultivation areas, posing significant risks to aquatic and terrestrial ecosystems (Batuecas et al., 2019; Foti et al., 2021). Milling waste is recognized as a valuable reservoir of biocompounds, but efforts to develop extraction procedures aimed at their valorisation remain scarce (Müller and Laibach, 2022). The two-phase system in olive oil extraction is a significant advancement for sustainability. Using a horizontal decanter, it relies on the natural water content of olives, cutting down on external water usage and wastewater production (Di Giacomo and Romano, 2022). In this study, we attempted to recover oligosaccharide-based elicitors from the liquid fraction of two-phase olive pomace, a semi-solid byproduct rich in carbohydrates, mainly derived from the CW of olive pulp, as well as lipids, proteins, and polyphenols (Cardoso et al., 2007; Sygouni et al., 2019; Khdair and Abu-Rumman, 2020; Alkhalidi et al., 2023). In this work, our hypothesis is based on the assumption that CW oligosaccharides could potentially be released in olive pomace through the extensive degradation of the CW of olive pulp by CW-degrading enzymes produced by plant tissues during olive ripening, as well as by the olive milling process aimed at oil extraction, or by microbial communities resident in the pomace (Cano et al., 2020; Sciubba et al., 2020). Ethanol fractionation was selected for its ability to isolate active components without the use of harsh chemical solvents, contributing to an environmentally friendly and green extraction process. We have identified the pomace collected in the advanced stages of the olive oil campaign, obtained from the processing of ripe olives, as the best candidate for this valorisation. We obtained an olive pomace extract (OPE) containing OG with an average degree of polymerization (DP) mainly ranging from 1 to 30 and short arabino-oligosaccharides (DP 2–8). Our next aim was to test whether OPE could prime plant defence mechanisms and resistance to phytopathogens. Application of OPE on Arabidopsis and tomato plants triggered several immune responses and enhanced protection against fungal and bacterial pathogens without affecting plant growth.

2. Materials and methods

2.1. Sample collection

Olive pomace was collected from the Agrolio olive mill at Andria, Bari, Italy, at three different periods (November, December, and January) during the olive oil production season of three consecutive harvesting years (from 2021 to 2023). The samples were collected from an olive oil two-phase extraction system fed with olives of olive cultivar Coratina.

2.2. Plant growth conditions

A. 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% 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 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, 0.5% sucrose, pH 5.7) (Murashige and Skoog, 1962). To evaluate the dose-effect of extracts on Arabidopsis seedling growth, 7-day-old Arabidopsis seedlings were grown in MS/2 liquid medium containing sterile water or extracts at different concentrations. Extracts were sterilized by vacuum filtering through an MCE membrane filter (0.22 μm pore size). 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). Both plates 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-1.7-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-2s-1). Tomato (*Solanum lycopersicum*, Minibel) seeds were germinated on wet paper overnight, were transferred on soil, and were grown in greenhouse under 16 h light/8 h dark cycle (PAR level of 75 $\mu mol\ m^{-2}s^{-1}$) at 23 °C and 35–40% humidity.

2.3. Fungi and bacteria cultivation

B. cinerea strain SF1 (Lionetti et al., 2007) 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 $g\ L^{-1}$ at 23 °C and 70% relative humidity in the dark before conidium collection. *P. syringae* pv. t*omato* DC3000 was refreshed from frozen glycerol stock on King Agar B (KB) medium containing 2% peptone protease, 0.15% K_2HPO_4, 1.5% glycerol, 1.5% agarose, 25 μg ml^{-1} rifampicin, and 5 mM of $MgSO_4$, and was grown at 28 °C in dark for three days before inoculum preparation.

2.4. Extraction of alcohol insoluble solids and OPE from olive pomaces

The olive pomace samples, deprived of stones, were lyophilized using a Savant™ Universal SpeedVac™ Vacuum System and the dried matter was ground using a mixer mill (Retsch Mixer Mill MM301) and stainless-steel beads (5 mm in diameter) for 1 min at 30 Hz. The obtained powders (300 mg) were treated for the extraction of alcohol-insoluble solids (AIS) as previously described (Lionetti et al., 2017). For OPE extraction, pomace fractions (50 ml) were centrifuged at 10, 000×*g* for 10 min and the supernatant containing the pomace liquid fraction were collected. The isolation of oligosaccharides with a DP > 6 was attempted exploiting a size-selective precipitation by adjusting the pomace liquid fraction to contain 50 mM NaOAc, pH 5 and 11% EtOH (v/v), adding cold 100% EtOH solvent while stirring on ice (Spiro et al., 1993). The samples were incubated overnight at 4 °C with constant orbital shaking (50 rpm) and then centrifuged at 30,000×*g* for 30 min at

4 °C. The resulting supernatant was discarded and the pellet (alcohol insoluble solids; AIS) was collected for subsequent steps. AIS was re-dissolved at 1 g/ml in water, dialyzed against ultrapure water in a dialysis tube with a molecular weight cut-off (MWCO) of 1000 Da (Spectra/Por®, 6 Dialysis Membrane, part number: 132,636), and dried. The resulting precipitate was named OPE. The OPE yields were quantified as the ratio between OPE dry weight (mg) and the volume of pomace liquid fraction (L) used for the extraction. The yield of OPE respect to the Olive weight were quantified as the ratio between OPE dry weight (mg) and the weight of the olive (Kg).

2.5. OPE biochemical characterization

Sugar composition analysis of OPE was performed by methanolysis in combination with TFA hydrolysis (Broxterman and Schols, 2018). OPE was dissolved at 2.5 mg/ml in 50 mM sodium acetate (NaOAc) buffer, pH 5.0. Next, aliquots were hydrolyzed and analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a ICS7000 HPLC system (Dionex, Sunnyvale, CA) with and ICS7000 ED PAD detector (Dionex) and CarboPac™ PA1 IC column (length, 250 mm, internal diameter 2 mm) and a CarboPac™ PA guard column (length, 50 mm, internal diameter 2 mm) using the same conditions described elsewhere (Kouzounis et al., 2022). For quantification, a standard solution containing fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man), galacturonic acid (GalA), and glucuronic acid (GlcA) was prepared, treated similarly to samples, and diluted in the range of 1–50 μg/ml. The sugar composition of AIS extracted from olive pomace was determined as previously described (Lionetti et al., 2017). The molecular weight distribution of glycans present in OPE (2.5 mg/ml) was determined by high performance size-exclusion chromatography with refractive index detection (HPSEC-RI), as previously described (Kouzounis et al., 2022). Pectin standards of known molecular weight were used for calibration (Deckers et al., 1986).

2.6. Oligosaccharide profiling

The oligosaccharide profile of OPE was investigated by HPAEC-PAD using a ICS7000 HPLC system (Dionex, Sunnyvale, CA) with ICS7000 ED PAD detector (Dionex) and equipped with a CarboPac™ PA1 IC column and a CarboPac™ PA guard column. The column temperature was set at 20 °C and the injection volume was 10 μL. Two mobile phases were used: 0.1 M sodium hydroxide (NaOH) (A) and 1 M sodium acetate in 0.1 M NaOH (B). The flow rate was 0.3 ml/min. The separation was performed by using the following elution profile: 0–5 min 100% A (isocratic), 5–30 min from 0 % to 20 % B (linear gradient), 30–65 min from 20 % to 65 % B, 65–70 min at 100 % B (isocratic) and 70–80 min 100 % A (isocratic). Analytical standards were used for the identification of Ara and arabinan oligosaccharides (DP 2–8; Megazyme, Bray, Ireland). GalA and OG were identified based on the elution pattern of a mixture of unbranched OG with a degree of polymerization (DP) 1 to 15, produced by the enzymatic treatment of lemon pectin with polygalacturonase from *Aspergillus niger*, as previously described (Zwolschen et al., 2024). For OPE digestion with pectinase (PG), OPE was treated with 0.25 μl of PG from *A. niger* (Product number P0690, N°CAS 9032-75-1, EC 232–8856) at 37 °C O/N before HPAEC-PAD analysis (Lionetti, 2015).

2.7. Determination of the degree of methyl esterification

The degree of methyl esterification (DM) was determined by measuring the amount of methyl groups that are released after saponification by alkali (Huisman et al., 2004). 5 mg sample was weighed in sealed headspace vials, followed by the addition of 1 ml 0.1 M NaOH. The vials were placed in ice for 1 h and afterwards, they were placed at room temperature overnight. The release of methanol was determined by headspace gas chromatography (GC) using a Thermo Scientific Trace GC DSQII equipped with DB-WAX column. Non-saponified samples were also analyzed to account for the presence of free methanol. The DM values were expressed as mol of methanol per 100 mol GalA. The determination of methyl esters was also confirmed by colorimetric assay as previously described (Lionetti et al., 2017).

2.8. Determination of the degree of acetyl esterification

The degree of acetyl esterification (DA) was determined by measuring the amount of acetyl groups that are released after saponification by alkali (Voragen et al., 1986). 20 mg sample was weighed in a reaction vial, followed by the addition of 1 ml of 0.4 M NaOH solution in 50% (v/v) isopropanol in water. The vials were placed in ice for 1 h and afterwards, they were placed at room temperature for 2 h. The samples were centrifuged (20,000×*g*, 5 min) and the supernatant was transferred to HPLC vial. The release of acetic acid was determined by high performance liquid chromatography with Refractive Index detector (HPLC-RI) using an Ultimate 3000 HPLC System (Dionex) equipped with an Aminex HPX-87 H column (Bio-Rad, Richmond, VA, USA) and a guard column. The HPLC system was coupled to a Shodex RI-101 refractive index detector (Showa Denko KK, Kawasaki, Japan). The samples (injection volume 10 μL) were run isocratically using 5 mM H2SO4 as eluent at 0.6 ml/min flow rate, with column temperature at 40 °C. Non-saponified samples were also analyzed to account for the presence of free acetic acid. The DA values were expressed as mol of methanol and mol of acetic acid per 100 mol GalA.

2.9. Arabidopsis infection assay with Botrytis cinerea

Conidia of *B. cinerea* were harvested by washing the surface of the mycelium with sterile distilled water. Conidia suspensions were filtered in sterile condition 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) or OPE (200 μg/ml). Distilled 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.

2.10. Arabidopsis and tomato infection assay with Pseudomonas syringae

P. syringae pv. *Tomato* DC3000 bacteria were collected by washing the plate with 2 ml of sterile 10 mM $MgCl_2$ and bacteria concentration was determined by means of a spectrophotometer. Four-week-old Arabidopsis and five-week-old tomato plants were sprayed with 2 ml of OG (200 μg ml^{-1}) or OPE (200 μg ml^{-1}) containing 0.05% Tween 24 MBAL (Croda, Snaith, UK) for Arabidopsis pretreatment, and 2.5% Tween 24 MBAL and 2.5% UEP-100 (Croda, Snaith, UK) for tomato pretreatment as adjuvants (Rebaque et al., 2021). Adjuvant solutions were used as mocks. After 24 h, Arabidopsis plants were infected with a bacterial concentration of $OD_{600} = 0.1$ added with 0.001% of Silwet L-77, and tomato plants were infected with a bacterial concentration 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. Arabidopsis and tomato leaf discs were collected from four different plants at 0 (3 h) and 3 days post infection (dpi) and colony forming units (cfu) per foliar area were determined as previously described (Mélida et al., 2020).

2.11. Immunoblot analysis for MAPK activation

Arabidopsis seedlings (10-day-old) were treated with OG (100 μg/ml) or OPE (100 μg/ml) for 5, 10 and 20 min and frozen with liquid nitrogen. Distilled water was used as mock. Protein extraction and detection of activated MAPKs were performed using the Phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, https://www.cellsignal.com/) as previously described (Fuertes-Rabanal et al., 2024).

2.12. Gene expression analysis

10-days-old Arabidopsis seedlings grown in liquid MS/2 medium were treated for 1 h with OG (100 μg/mL) or OPE (100 μg/ml). Distilled water was used as mock. Seedlings were frozen in liquid nitrogen and homogenized using the mixer mill MM301 (RETSCH) and stainless-steel beads (5 mm in diameter) for about 1 min at 30 Hz. Total RNA was extracted with RNA isolation NucleoZol reagent (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA was treated with RQ1 Rnase-FreeDnase (Promega, Madison, WI, USA), and first-strand complementary DNA (cDNA) was synthesized using ImProm-II™ (Promega, Madison, WI, USA). Analysis was performed using a CFX96 Real-Time System (Bio-Rad). One microliter of cDNA (corresponding to 50 ng of total RNA) was amplified in 10 μL of reaction mix containing 1X Go Taq qPCR Master Mix (Promega Madison, WI, USA) and 0.5 μM of each primer described in Supplemental Table S1. The expression levels of each gene, related to the *UBIQUITIN5* (*UBQ5*) gene, were determined using a modification of the Pfaffl method (Pfaffl, 2001). Primer sequences were generated with Primer 3 software (https://primer3.ut.ee/).

2.13. Data analysis

Data were presented as mean ± standard deviation (SD) or standard error (SE) as indicated in the figure legends. The significant differences were evaluated by Student's t-test or ANOVA analysis followed by Tukey's test ($p < 0.05$), as indicated in the figure legends.

3. Results

3.1. CW composition of two-phase olive pomace varies during the olive oil extraction season

Olives with different degrees of ripeness, turning from green to black, become a source for the industrial oil production process (Fig. 1A). This could lead to a different composition of CW within the pomace, that could potentially influence the release of oligosaccharide elicitors. With the aim to identify a stage of the mill quarterly activity that can produce pomace enriched in pectin-derived elicitors, olive pomaces at three times during the two-phase oil extraction season (November, December, and January) were collected (Fig. 1A). AIS containing CW glycans were extracted from de-pitted two-phase olive pomace. A significant increase (about 15%) of in AIS yield from liquid pomace was detected in intermediate and advanced stages of the olive oil season compared to November (Table 1). The isolated AIS fractions were characterized for their monosaccharide composition (Fig. 1B). All AIS analyzed were characterized by the presence of high levels of Xyl, GalA, Ara, Glc, and Gal with smaller quantities of Rha, Man, and traces of Fuc and GlcA. However, significant differences were observed between the samples collected at different timeframes along the olive oil campaign. CW material obtained from pomace collected in January was enriched in GalA, Rha, and Ara, most likely deriving from HG, RGI and

Table 1
AIS and OPE yields at different times during olive oil extraction season.

Collection period	AIS/pomace (%w/w)	OPE/olives (mg/kg)	OPE/pomace (mg/kg)	OPE/pomace liquid fraction (mg/L)
November	$52.1 \pm 1.8_a$	$25.1 \pm 1.65_a$	$34 \pm 2.2_a$	$80 \pm 5_a$
December	$60.3 \pm 0.6_b$	$47.8 \pm 1.14_b$	$64.6 \pm 1.5_b$	$152 \pm 4_b$
January	$60.2 \pm 0.4_b$	$97.9 \pm 3.52_c$	$132.2 \pm 4.8_c$	$311 \pm 11_c$

AIS yield = mg AIS/mg of pomace dry weight; OPE in liquid pomace = mg OPE/L of liquid fraction of pomace; OPE in pomace = mg OPE/kg of wet pomace; raw pomace mg OPE dry weight/kg raw pomace. The different letters per column indicate significantly different datasets according to ANOVA followed by Tukey's test ($p < 0.05$).

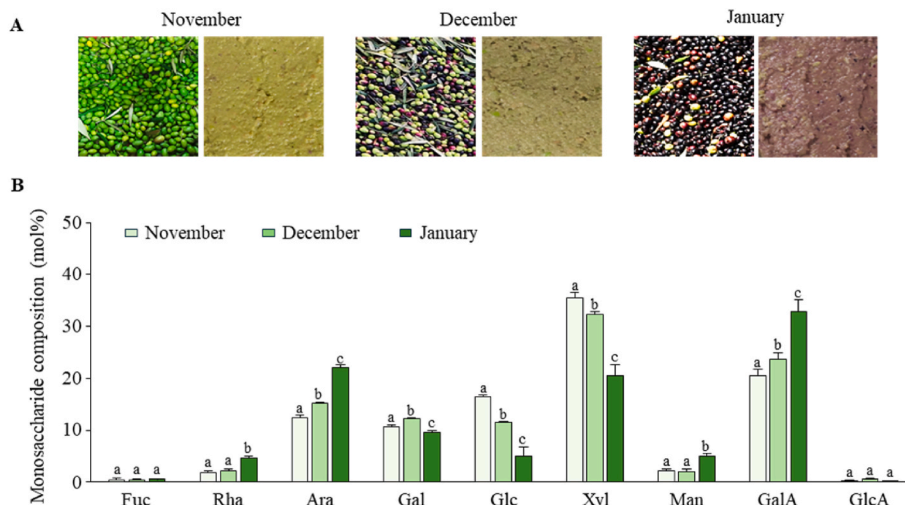

Fig. 1. Characterization of two-phase olive pomace collected at the indicated periods during the season of olive oil production. (**A**) Olives of Coratina cultivar at different degrees of ripening used as feed in olive oil extraction and resulting two-phase olive pomaces. (**B**) Monosaccharide composition of two-phase olive pomaces. The molar percentages of Fucose (Fuc), Rhamnose (Rha), Arabinose (Ara), Galactose (Gal), Glucose (Glu), Xylose (Xyl), Mannose (Man), Galacturonic Acid (Gal A), and Glucuronic Acid (Glc A) were quantified. Results represent the mean ± SD (n = 3). The different letters on the bars indicate significantly different datasets according to ANOVA followed by Tukey's test ($p < 0.05$).

RGII. Worthy of note is the 60% increase in GalA in the pomace obtained from ripe olives processing compared to pomace from green olives. A progressive reduction in hemicellulose building blocks, such as Xyl and Glc, was also observed, except mannose which instead increases in January. These data highlight a progressive change in olive pomace CW composition during the olive oil extraction season mainly characterized by a shift from hemicellulose to pectin glycans.

3.2. Liquid fraction of olive pomace contains pectic oligosaccharides

Ethanol fractionation was attempted to precipitate soluble pectic oligosaccharides from the liquid part of olive pomace. From all the samples, a white powder was obtained after centrifugation, solvent removal and drying. This precipitate resulted in the highest yield in the January samples, which were 2 and 4 times significantly higher than those of December and November, respectively (Table 1). Since our focus is on isolating pectic elicitors, we focused our subsequent experiments on pomace harvested in January, given the highest amount of GalA and OPE yields (Fig. 1B; Table 1).

The OPE fraction was evaluated for the presence and composition of carbohydrates by carrying out a monosaccharide composition analysis. Our results indicated that GalA is the main carbohydrate constituent (45.2%), followed by Ara (13.6%), Rha (13.4%), and Man (11.4%). Gal (6.8%), Glc (4.8%), Xyl (2.8%), and GlcA (0.6%) are minor monosaccharide constituents (Table 2). The high GalA content indicates that pectins are predominant in OPE. Furthermore, the presence of Ara, Rha, and Gal pointed to the presence of RGs-related carbohydrates. The high GalA/Rha ratios suggest a greater presence of HG respect to RGs. The presence of Glc, Xyl, and Man suggested the co-extraction of hemicelluloses, possibly xyloglucan, mannan or galactomannan, that are known to be present in olive pomace (Lama-Muñoz et al., 2012). Further analysis of HG substituents indicated that OPE presented DA and DM values of 24.4% and 17.3%, respectively (Table 2).

The carbohydrate profile in the OPE was further investigated by analyzing the molecular distribution. HPSEC-RI analysis indicated the presence of oligomeric material below 10 kDa oligomers suggesting the presence of oligosaccharides (Fig. S1). Polymers were also detected, ranging from 10 kDa to 100 kDa. The oligosaccharides profile in OPE was further investigated by HPAEC-PAD (Fig. 2). OPE presented several oligomeric peaks eluting between 15 and 65 min. In specific, a series of peaks eluting between 20 and 65 min shared the retention times with reference saturated OG. Oligosaccharides with a DP ranging from 1 to about 30 were recorded. The disappearance of chromatographic peaks upon treatment of OPE with *A. niger* endo-polygalacturonase confirmed the identification of these oligomers as OG (Fig. S2). Furthermore, peaks matching the elution pattern of arabinan oligosaccharides DP 2–8 were also observed (Fig. 2). These results revealed that OPE contains short and long OG and short arabino-oligosaccharides.

3.3. Arabidopsis treatment with OPE induces hallmarks of pathogen-triggered immunity

The next step was to investigate the immunostimulatory potential of OPE by analyzing hallmarks of Arabidopsis immunity. First, we conducted a dose-effect analysis of OPE treatment on Arabidopsis seedlings growth to identify a concentration suitable for immunostimulation that would not negatively impact plant yield (Davidsson et al., 2017; He et al., 2022). Arabidopsis seedlings were cultivated in liquid medium supplemented with OPE at different concentrations (100, 200 and 400 μg ml^{-1}) and the seedlings weight was quantified after 5 days of treatment (Fig. 3). Seedlings treated with OPE at 100 and 200 μg/ml showed a growth not significantly different from mock-treated seedlings. The addition of OPE at 400 μg/ml produced about 25% of seedling growth inhibition. Next, the OPE ability to mount Arabidopsis immune reactions was explored (Fig. 4). We monitored the phosphorylation of downstream mitogen-activated protein kinases MPK3, MPK4, MPK6 and MPK11, upon treatment of Arabidopsis seedlings with mock (water) and OPE (100 μg/ml) (Fig. 4A). OG (100 μg/ml) were used as positive control (Galletti et al., 2008). After 5 and 10 min of treatment with OPE, phosphorylation of MPK3 and MPK6 was activated, and the pattern was comparable to the OG treatment at 10 min.

Next, the expression of *CYP81F2*, *FRK1*, *WRKY53*, and *WRKY33* genes, important for Arabidopsis immunity against pathogens, was compared between elicitor-treated and mock-treated Arabidopsis seedlings. *CYP81F2* (*CYTOCHROME P450, FAMILY 81*) encodes a cytochrome P450 monooxygenase involved in the biosynthesis of indole glucosinolates (Pfalz et al., 2009); *WRKY33* (*WRKY DNA-BINDING PROTEIN 33*) mediated indole glucosinolate metabolic pathway, essential for defence toward the necrotrophic fungi *B. cinerea* and *Alternaria brassicicola* (Birkenbihl et al., 2012; Zheng et al., 2006); *WRKY53* is involved in basal resistance against *P. syringae* (Hu et al., 2012; Murray et al., 2007); *FRK1* (*FLG22-INDUCED RECEPTOR-LIKE KINASE 1*) encodes a leucine-rich repeat receptor kinase involved in defence signal pathway (Asai et al., 2002). Interestingly, greater induction of all selected genes was observed after OPE treatment compared to mock treatment (Fig. 4B). Notably, the *CYP81F2* gene exhibited the greatest induction, with its expression increasing by approximately 20-fold. All these results indicate that OPE have elicitor activity and OPE treatment can be exploited to trigger plant immunity.

3.4. OPE application primed plants and reduced disease symptoms caused by B. cinerea and P. syringae

The effect of OPE pretreatment on Arabidopsis protection against the necrotrophic fungus *B. cinerea* was investigated. Four-week-old adult plants were foliar-sprayed with mock and OPE (200 μg/ml) and leaves were inoculated with *B. cinerea* spores 24 h after the pretreatment (Fig. 5A). OG (200 μg/ml) were used as positive control OG (Giovannoni et al., 2021). The expression of the defense-related genes *CYP81F2* and *PAD3* was evaluated at 8 hpi. *PAD3* (*PHYTOALEXIN DEFICIENT 3*) encodes a key biosynthetic enzyme involved in the production of the antimicrobial compound camalexin and is required for elicitor-induced protection against *B. cinerea* (Ferrari et al., 2007; Chávez-Martínez et al., 2020). In plant challenged with Botrytis, a higher induction of *CYP81F2* and *PAD3* transcripts were revealed in OPE-pretreated plants respect the mock-treated plants (Fig. 5B and C). After 48 hpi, plants pretreated with OPE showed a significant reduced susceptibility (both about 40%) compared to mock-treated plants (Fig. 5D and E).

The induction of *WRKY53* by OPE observed in seedlings suggests a possible protection of Arabidopsis also against bacterium *Pseudomonas syringae* (Hu et al., 2012). We subsequently evaluated the ability of OPE to function as elicitors against the bacterium. Adult Arabidopsis plants were pretreated with OPE and OG (200 μg/ml) 24 h before challenging them with the *P. syringae* (Fig. 6A). At 8 hpi, plants pretreated with OPE

Table 2
Monosaccharide composition of OPE.

Monosaccharide composition (mol %)										
Fuc	Rha	Ara	Gal	Glc	Xyl	Man	GalA	GlcA	DA[a]	DM[a]
ND	13.4 ± 0.8	13.6 ± 1.0	6.8 ± 0.4	4.8 ± 0.3	2.8 ± 0.1	11.4 ± 0.6	45.2 ± 1.7	0.6 ± 0.5	24.4 ± 1.6	17.3 ± 0.1

[a] Expressed as mol per 100 mol GalA. DA = Degree of acetyl esterification; DM = Degree of methyl esterification.

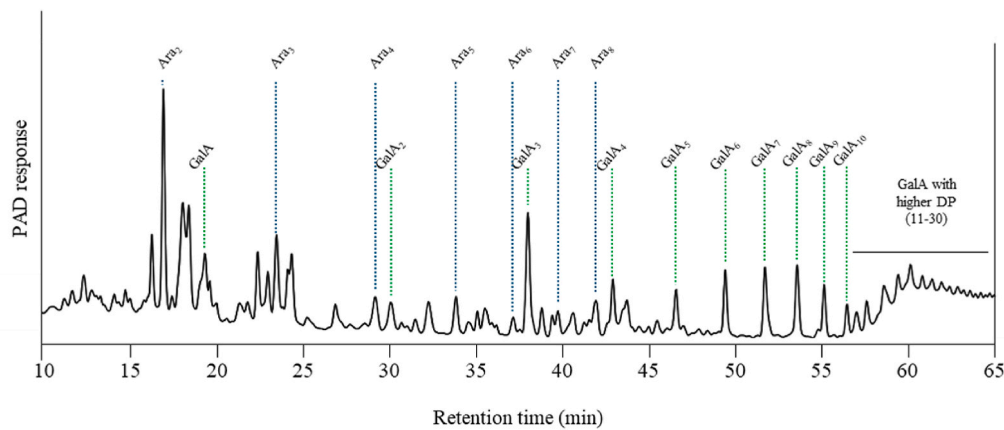

Fig. 2. Oligosaccharide profile in olive pomace extract (OPE). High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) profiles of the oligosaccharides extracted by ethanol fractionation from the liquid part of olive pomace collected late during olive mill campaign. The oligosaccharide peaks of OPE matching Oligogalacturonides reference mixture or arabino-oligosaccharides standards are indicated with $GalA_n$ and Ara_n, respectively. *N* indicates the degree of polymerization (DP).

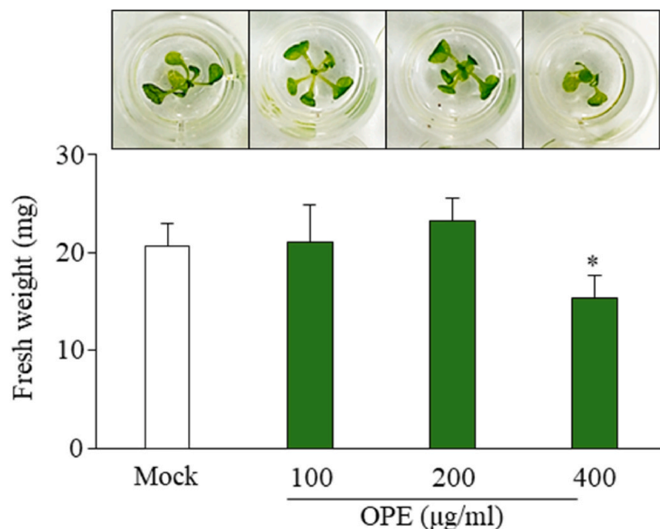

Fig. 3. Dose–effect of OPE on Arabidopsis seedling growth. Effects of OPE (100, 200, and 400 µg/ml) on the growth of 10-days-old *A. thaliana* Col-0 seedlings in liquid medium. Distilled water (mock) was used as negative control. The fresh weight was measured 5 days after treatment. Data represent the mean ± SD (n = 3). Asterisk indicate statistically significant differences between OPE and mock treatment according to Student's t-test (*p < 0.05). The experiments were repeated three times with similar results.

showed a higher induction of *CYP81F2* and *WRKY53* expression compared to those pretreated with mock during *P. syringae* infection (Fig. 6B and C). Bacterial growth was significantly lower at 3 dpi in OPE- or OG-pretreated Arabidopsis plants (both about 20%) compared with mock-pretreated plants (Fig. 6D). We extended our analysis to tomato, a crop commercially important for the fresh fruit market and the processed food industries. Five-weeks old tomato plants were pretreated with OPE and OG (200 µg/ml) at 24 h before *P. syringae* inoculation. A significant lower *P. syringae* colonies were recovered at 3 dpi in OPE- or OG-pretreated plants (both about 10%) compared with mock-pretreated plants (Fig. 6E). All these results indicate that a preventive application of plants with OPE can improve immune performance and protection against different pathogens.

4. Discussion

Olive pomace could be a natural source of oligosaccharides, which could potentially be released by CW degradation during olive ripening and/or the milling process for oil extraction (Cano et al., 2020; Sciubba et al., 2020). In this study, we aimed to identify and extract oligosaccharides from the liquid portion of two-phase olive pomace for use as immunostimulants in plant treatments for agriculture. Olives at various stages of ripeness, transitioning from green to black, are utilized as a source for the industrial oil production process. Variations in CW composition were observed among different ripening stages (Jiménez et al., 1994, 2001; Huisman, 1996; Mafra et al., 2001). This variability could lead to differences in CW composition in the pomace and in the enrichment of CW-derived elicitors. We observed a notable increase in AIS yield from olive pomace from Coratina olives during the intermediate and advanced stages of the olive oil season compared to the starting stage of campaign. Moreover, a progressive change in CW composition was observed in olive pomace biomass. A consistent increase in the content of GalA, Rha, and Ara compensated for a continual reduction in Xyl and Glc. Worthy of note is the 60% increase in GalA in the pomace obtained from ripe olives processing compared to that from green olives. These data indicate that pectins become prevalent than hemicelluloses as the olive oil extraction season progressed. These results could suggest a shift in the biosynthesis and/or degradation of the two types of polysaccharides in olive pulp, perhaps functional to seed dispersal. It cannot be excluded that the differences arise in the pomace where the activity of different microbial communities with specific degradation activities towards polysaccharides could prevail at a particular stage (Dashti et al., 2015). Interestingly, several oligosaccharide elicitors are released and produced from pectin degradation (Lionetti and Metraux, 2014; Swaminathan et al., 2022; Rebaque et al., 2023).

We then focused on extracting pectin oligosaccharides from the olive pomace. Currently, oligosaccharides are industrially obtained from biomasses rich in CWs through hydrothermal pretreatment, acid, or enzymatic hydrolysis, which entail high production costs and require specialized laboratories (Ávila et al., 2021; Yue et al., 2022). In this work, diluted ethanol was exploited as green solvent to precipitate pectic fragments from the liquid fraction of two-phase olive pomace. We successfully obtained an extract called OPE, with the highest yield (about 130 mg/kg of total pomace) from the pomace collected in later stages of olive oil extraction season. We focused our subsequent experiments on OPE extracted from pomace collected in January. The molecular distribution indicated that OPE contain oligo- and polysaccharides. HG-derived carbohydrates comprised a significant proportion of the total carbohydrates in this OPE, exhibiting a DM and DA values of about 17% and 24%, respectively. The presence of methyl- and acetyl esters and the degree of polymerization can influence the

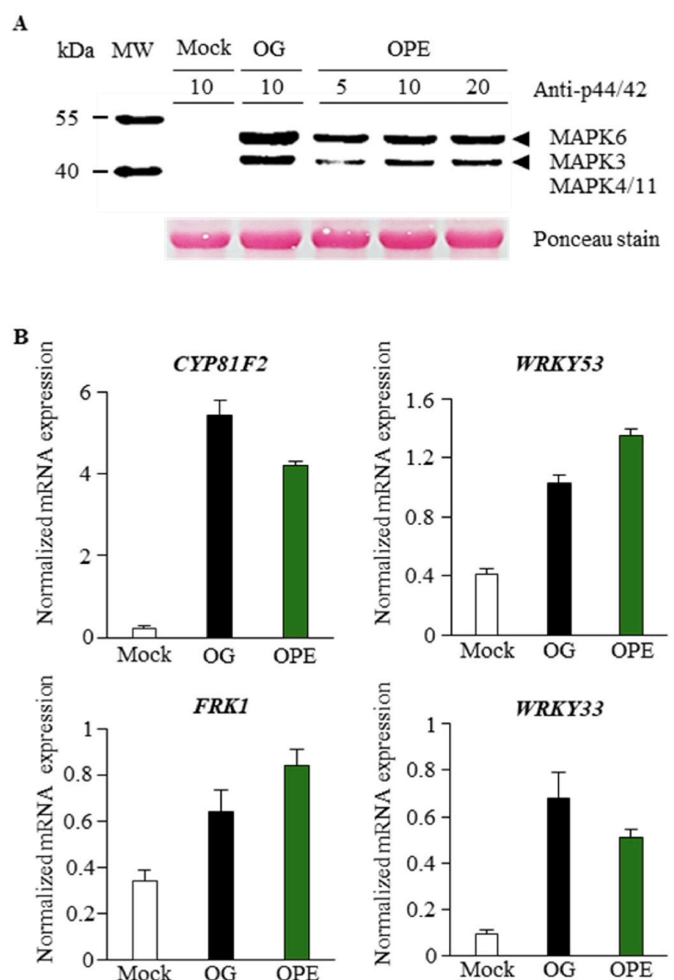

Fig. 4. Pattern-triggered immunity hallmarks activation by OPE in Arabidopsis. (**A**) MAPK activation in Arabidopsis seedlings in response to distilled water (mock), OG (100 μg/ml) or OPE (100 μg/ml) treatment. MAPK phosphorylation was determined by Western blot using the phospho-p44/42 MAPK antibody at different time after 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. (**B**) Expression of *CYP81F2*, *WRKY53*, *FRK1*, and *WRKY33* after OPE treatment. The expression of selected defence genes was analyzed by quantitative RT-PCR at 1 h after OPE treatment of 10-days-old Arabidopsis Col-0 seedlings. The expression levels were normalized to *UBQ5* expression. Data represent the mean ± SD (n = 3). The experiments were repeated three times with similar results. OG standard (DP 10–15) was used as positive control.

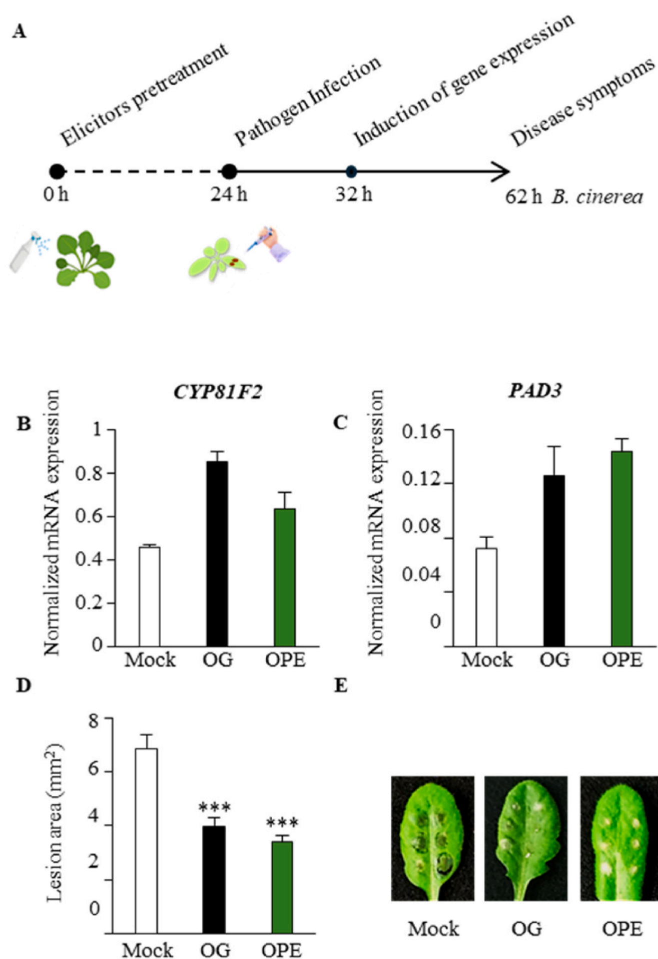

Fig. 5. Pretreatment of Arabidopsis with OPE enhances immune response and protection against *B. cinerea*. (**A**) 4-weeks-old Arabidopsis Col-0 leaves were pretreated with mock, OG (200 μg/ml) or OPE (200 μg/ml) and 24 h later were inoculated with *B. cinerea*. (**B and C**) Quantitative RT-PCR analysis in *B. cinerea*-infected leaves collected at 8 h post infection (hpi). mRNA expression levels are normalized to *UBQ5* expression. Data represent the mean ± SD (n = 3). The experiments were repeated three times with similar results. (**D and E**) Lesion areas of *B. cinerea*-infected leaves were measured at 48 h after the inoculation. Values are means ± SE of at least 30 lesions. The experiments were repeated three times with similar results. OG (DP 10–15) were used as positive control. Asterisks indicate statistically significant differences between elicitor-treated and mock treated plants according to Student's *t*-test (***$p < 0.001$).

ability of OG to elicit defence responses (Osorio et al., 2008; Lionetti et al., 2012; Voxeur et al., 2019; Xiao et al., 2024; Lionetti, 2015). OGs with a degree of polymerization (DP) 10–15 have been shown to elicit canonical PTI signaling and confer plant protection against a range of pathogens ((De Lorenzo et al., 2011; Xiao et al., 2024). More recently, shorter OGs have also been shown to trigger immune responses and defence (Davidsson et al., 2017). A low level of methyl- and acetyl esterification can improve the ability of OGs to elicit specific defence responses (Osorio et al., 2008, 2011; Randoux et al., 2010; Selim et al., 2017). The presence of relative amounts of Ara, Gal, and Rha also suggests the potential accumulation of RG and/or polysaccharides containing side chains of arabinan, galactan, and arabinogalactan (Cardoso et al., 2007; Coimbra et al., 2010; Millan-Linares et al., 2021; Vierhuis, 2002). The presence of Glc, Xyl, and Man suggested also the co-extraction of hemicellulose carbohydrates, possibly xyloglucan, mannan or galactomannan, that are known to be present in olive pomace (Lama-Muñoz et al., 2012). A further analysis of carbohydrate profile indicated that OPE contains a heterogeneous mixture of oligosaccharides. Our ability to identify oligosaccharides in liquid olive pomace can be attributed to utilizing two-phase olive pomace as a substrate, where the liquid fraction originates solely from the water content inherent in the olives. Previous studies encountered challenges in detecting oligosaccharide fragments in olive pomace, presumably due to their reliance on wastewater generated during the three-phase oil extraction process. This process involves the addition of water, which likely diluted the constituents, hampering their detection. Notably, saturated OG with a DP mainly ranging from 1 to 30 and high molecular weight pectins were detected. The presence of saturated OG rather than unsaturated ones may indicate a prevalence of polygalacturonase activity compared to pectin lyase during the ripening of olives and, consequently, in olive pomace. Arabinan oligosaccharides with a low degree of polymerization were also detected, suggesting the involvement of arabinosidase activities. A potential approach to enhance the yield of oligosaccharides from OPE could involve enzymatic digestion using CW-degrading enzymes (Ferrari et al., 2008). Additionally, innovative techniques such as subcritical water extraction hold great promise

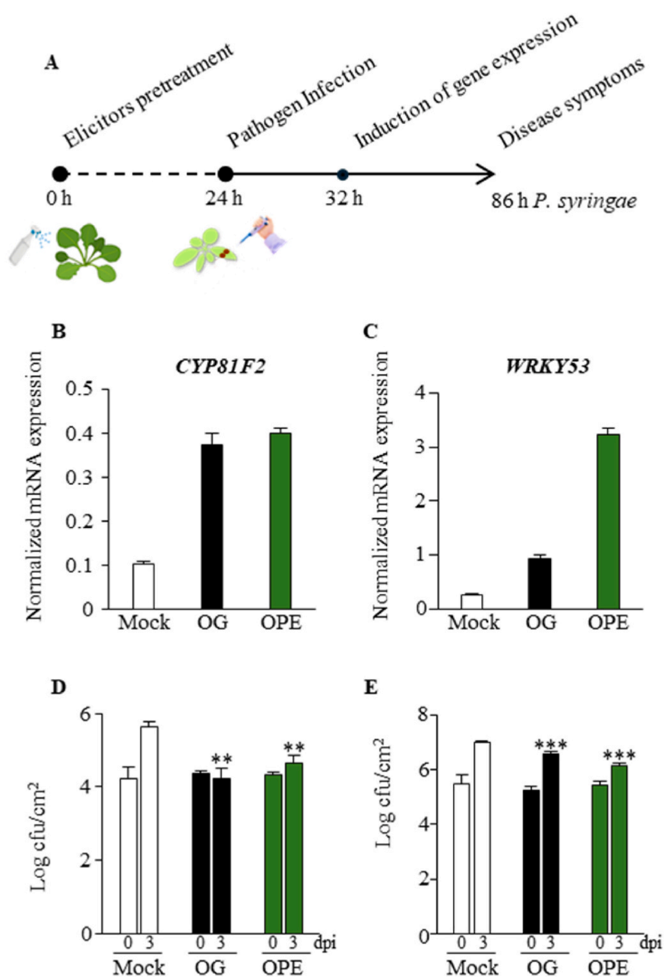

Fig. 6. Pretreatment of Arabidopsis with OPE enhances immune response and protection against *P. syringae* pv. *tomato* DC3000. (**A**) Leaves of 4-weeks-old Arabidopsis Col-0 plants were pretreated with adjuvant solutions (mock), OG (200 μg/ml) or OPE (200 μg/ml) 1 day before bacterial inoculation. (**B** and **C**) Quantitative RT-PCR analysis in *P. syringae*-infected leaves collected at 8 hpi. mRNA expression levels are normalized to *UBQ5* expression. Data represent the mean ± SD (n = 3) from one experiment out of three that gave similar results. Colony forming units (cfu) of *P. syringae* pv. *tomato* DC3000 per leaf area (cm^2) were determined in (**D**) Arabidopsis and (**E**) tomato at 3 h and 3 days post infection (0 and 3 dpi). Data represent mean ± SD (n = 6). The experiments were repeated three times with similar results. The OGs with degree of polymerization from 10 to 15 were included as positive control. Asterisks indicate statistically significant differences between elicitor-treated and mock treated plants according to Student's *t*-test (**$p < 0.05$, ***$p < 0.001$).

for improving oligosaccharide production (Basak and Annapure, 2022).

MPK3 and MPK6 phosphorylation is one of the earliest responses to elicitors (Pitzschke and Hirt, 2009). OPE treatment induced MAPK3 and MAPK6 phosphorylation and the induction of the expression of defense-related genes as *CYP81F2*, *WRKY53*, *FRK1*, and *WRKY33* in Arabidopsis. The similar patterns of phosphorylation and the gene expression observed after OG and OPE treatments supports our biochemical evidence that OPE contain OGs. The OPE induction of *CYP81F2* and *WRKY33* suggest that this extract can stimulate the production of antimicrobial compounds such as indole glucosinolates (Birkenbihl et al., 2012). The induction of *WRKY53* by OPE suggests a possible Arabidopsis protection to *P. syringae* (Hu et al., 2012). Overall, all these results indicate that OPE can promptly stimulate the Arabidopsis immune system. In Arabidopsis plants pre-treated with OPE and OGs, the expression of *PAD3* and *CYP81F2* was induced by Botrytis to a greater extent than in control plants, indicating a priming effect of the elicitors. A similar effect was also observed for *CYP81F2 and WRKY53* expression induced by Pseudomonas. These data revealed that OPE application on Arabidopsis induced priming of defence gene expression during fungal and bacterial infection. These defence responses triggered by OPE can contribute to the protection observed in Arabidopsis and tomato pretreated with OPE against *B. cinerea* and the *P. syringae* infection. These findings could have an impact on olive tree disease management. In the future, it would be intriguing to assess the elicitation activity of OPE on olive trees against *Xylella fastidiosa*, a bacterium responsible for a severe epidemic in the Mediterranean region, devastating olive trees and for which effective control measures have not been found yet.

Training plants memory for stress represents a useful approach to help safeguard crop reproduction and design climate-friendly crops for the future. In accordance with the recommendations of the EU, chemical plant protection agents are intended to be slowly replaced by natural preparations (Drobek et al., 2019). The opportunity to integrate disease management by pretreating plant tissues with single or combinations of isolated natural green elicitors is innovative and sustainable. Ever-increasing interest exists in the use of oligosaccharides as biological control agents in agriculture. IBISCO is a complex of chito-oligosaccharides and OG already on the market, able to induce resistance against several pathogens in different crop plants (Van Aubel et al., 2014; Clinckemaillie et al., 2017; Calderone et al., 2022). European Food Safety Authority (2014) and the European Commission consider IBISCO as a low-risk active substance (Commission Implementing Regulation, 2015). In conclusion, we highlight the potential for recovering oligosaccharide elicitors from pomace at specific times during the olive oil production season, using green solvents and low-cost methods, without the need for expensive commercial enzymes and substrates. This represents a cleaner circular economy strategy that can open new waste management markets for olive mill plants. OPE can complement chemical products in an integrated pest management program for protecting plants from pests, favoring a sustainable future crop production, and mitigating the negative environmental impact associated with current practices.

Author contributions

Marco Greco: Investigation, Methodology, Formal analysis, Writing - Original Draft, Visualization. **Hugo Melida**: Writing - Review & Editing, Supervision, Funding acquisition. **Henk A. Schols**: Writing - Review & Editing, Supervision. **Dimitrios Kouzounis**: Investigation, Methodology, Formal analysis, Writing - Review & Editing, Visualization. **María Fuertes-Rabanal**: Investigation, Visualization. **Mariagrazia Gentile**: Investigation, Visualization. **Savino Agresti**: Conceptualization, Resources. **Vincenzo Lionetti**: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - Original Draft, Writing - Review & Editing.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.109213.

Data availability

The data that has been used is confidential.

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