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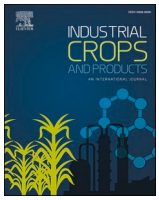

Carob and pomegranate extracts enhance plant defence mechanisms against olive anthracnose through antioxidant activity and phenolic compounds production

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ABSTRACT

Olive anthracnose (OA), caused by *Colletotrichum* species, is one of the most economically damaging disease in olive sector. This study was focused to identify sustainable control alternatives by evaluating the effect of pomegranate and carob extracts against OA and elucidating their mode of action. *In vitro* assays on mycelial growth, conidial production, and germination, and appressoria formation of *C. godetiae* and *C. nymphaeae* were performed. Neither extract significantly inhibited mycelial growth, while both reduced reproductive structures formation of the pathogens. Bioassays on detached and attached olive fruits in plants of cv. Arbequina, treated with extracts and/or inoculated with *C. godetiae* were conducted to evaluate the effect of the extracts on disease progression. Despite of the limited effect of the extracts on reducing disease progression in detached fruit, curative applications were more effective than preventive ones. Carob extract was more effective than pomegranate extract in reducing the disease incidence progression in attached fruit *in planta*. The resistance-inducing effect of plant extracts was evaluated by quantifying of H_2O_2 and phenolic compounds production in olive leaves at 0, 3, 7 and 24 h after inoculation with *C. godetiae*. Both extracts increased these parameters in the inoculated plants, with carob extract triggering an earlier activation and promoting a greater diversity of phenolic compounds accumulation. These findings reveals that these two extracts act as resistance inducers through different effective defence pathways, with carob extract standing out as a promising bioprotector against OA. These results open new possibilities for environmentally friendly management of OA using plant extracts.

1. Introduction

Olive anthracnose (*Olea europaea* L. subsp. *europaea* var. *europaea*; OA) leads to severe yield losses, fruit depreciation and oil quality impact worldwide (Cacciola et al., 2012; Moral et al., 2021; Romero et al., 2022; Talhinhas et al., 2018). The disease causes fruit rot mainly, which is characterized by dark, sunken lesions on olives, often accompanied by a characteristic pinkish-orange spore mass (Cacciola et al., 2012; Moral et al., 2021).

The disease is caused by a wide diversity of *Colletotrichum* species, that belongs to the species complexes *Colletotrichum acutatum*, *C. gloeosporioides* and *C. boninense* (Moral et al., 2021; Talhinhas et al., 2018). Although there is a global distribution of *Colletotrichum* species, normally in each geographic region, there is a dominant and some secondary species (Moral et al., 2021). In this regard, the most frequent species in the Iberian Peninsula are *C. godetiae* (in Spain) and *C. nymphaeae* (in Portugal), both belonging to the *C. acutatum* species complex (Moral et al., 2021; Silva et al., 2023; Talhinhas et al., 2018). Likewise, species such as *C. gloeosporioides* and *C. fructicola* have been reported in the Iberian Peninsula, although with a lower frequency (Moral et al., 2021).

So far, the traditional strategies adopted to control OA are mainly based on the spray of copper-based fungicides in the tree canopy (Materatski et al., 2019; Moral et al., 2018; Silva et al., 2023). However, the restrictions on the use of copper in olive groves planned for the coming years by the European Union commission, together with the high environmental impact of its accumulation in ecosystems, make it necessary to search for sustainable control alternatives. In this context,

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the efficacy of bioprotection by using plant extracts to manage plant diseases has been reported, largely due to their high content of phenolic compounds, which contributes to their protective effect (Borges et al., 2018; Santra and Banerjee, 2020; Stenberg et al., 2021). In this regard, some studies have addressed the potential use of the bioprotection with plant extracts against OA (Pangallo et al., 2017a; 2022; Varveri et al., 2024). Varveri et al. (2024) determined that three natural commercial products significantly reduced *C. acutatum* conidia production and disease progression in detached olive fruits of cv. Kalamon, although their efficacy in reducing OA in plants was not verified. Similarly, Pangallo et al. (2017a); (2022) demonstrated the efficacy of pomegranate (*Punica granatum*) peel extract obtained with ethanol:water solutions against *C. acutatum sensu stricto* on olive plants of cvs. Arbosana and Ottobratica, in both laboratory-controlled conditions and in the field. However, its efficacy against *C. godetiae* and *C. nymphaeae* and its mode of action against AO, is so far unknown. Beyond olive anthracnose, pomegranate extract has also shown *in vitro* antifungal activity against other plant pathogens such as *Aspergillus flavus*, *Botrytis cinerea*, *Fusarium oxysporum* and *Penicillium digitatum*, and has effective in reducing disease symptoms in both detached fruits and plants (Abo-Elyousr et al., 2022; Glazer et al., 2012; Ismail et al., 2021; Nicosia et al., 2016; Romeo et al., 2015; Tayel et al., 2009). Likewise, acetic or methanolic carob (*Ceratonia siliqua*) leaf extracts have shown *in vitro* efficacy against citrus sour rot (*Geotrichum candidum*) or potato soft rot (*Pectobacterium atrosepticum*) (Meziani et al., 2015; Talibi et al., 2012). Based on the efficacy shown by these extracts in the bioprotection against various diseases, in the previous study developed by Antón-Domínguez et al. (2024b), pomegranate (*P. granatum*) peel and carob (*C. siliqua*) leaf extracts were elaborated and characterized against Verticillium wilt of olive, another major disease of this crop caused by the soilborne fungi *Verticillium dahliae*. Both extracts, especially those obtained with methanol as solvent, were able to inhibit the germination of *V. dahliae* conidia and reduce disease progression (Antón-Domínguez et al., 2024b). More recently, these extracts have also been shown to act as resistance inducers in olive plants, modulating the overexpression of salicylic acid and abscisic acid pathways related-genes, as well the expression of enzymes such as polyphenoloxidase (Antón-Domínguez et al., 2025).

The effectiveness of natural plant extracts in disease management is closely linked to their rich composition of bioactive compounds such as phenols, flavonoids and terpenoids, that trigger the activation of the plant's defence system (Anjali et al., 2023; Báidez et al., 2006; Borges et al., 2018; Llorens et al., 2017; Nag et al., 2024). Furthermore, these bioactive compounds have the ability to trigger stress-related biochemical pathways, including the production of reactive oxygen species (ROS) (Baptista et al., 2007; Llorens et al., 2017; López-Moral et al., 2022b; Reglinski et al., 2023; Sahu et al., 2022).

Based on the studies described above, the protective effect of carob leaf and pomegranate peel extracts against Verticillium wilt of olive and other plant diseases, appears to be attributed to their ability to trigger plant resistance mechanisms, particularly through the activation of plant hormone-related genes. According to this background, we hypothesized that both carob and pomegranate extracts may also be effective against other major olive diseases such as OA. Therefore, the purpose of this study was to search for effective and sustainable alternatives to chemical control of OA by evaluating the potential of pomegranate peel and carob leaf extracts, determining their mechanisms of action. This study aligns with the European Green Deal and the Farm to fork Strategy towards to envision a Mediterranean olive oil sector that actively promotes resilience by improving olive health with the application of highly effective and long-lasting environmental-friendly compounds. To this end, the following specific objectives were carried out: 1) to evaluate the effect of pomegranate and carob extracts on mycelial growth, conidial production and germination, and appressoria formation of *C. godetiae* and *C. nymphaeae in vitro*; 2) to evaluate their effect on disease progression and incidence by means of assays on both detached (humidity chambers) and attached (plants) olive fruit of cv. Arbequina inoculated with *C. godetiae*; and 3) to determine their role as resistance inducers in olive plants by enhancing antioxidant activity and stimulating phenolic compound production in leaves. A laboratory copper (Cu) sulphate was included for comparative purposes, as it is the most used measure against OA nowadays. Based on previous studies, we hypothesize that both carob and pomegranate extracts should be also effective preventing *Colletotrichum* infections in olive fruit. Thus, this study was conceived to provide a comprehensive evaluation on the effectiveness and the mode of action of these plant extracts as bioprotectants against OA. Demonstrating this hypothesis, they could be potentially selected as environmentally-friendly alternatives to chemicals in olive-crop protection.

2. Materials and methods

2.1. Plant extracts and dose tested

Pomegranate (*P. granatum*) peels and carob (*C. siliqua*) leaves extracts were obtained with methanol following the protocol described by Antón-Domínguez et al. (2024b). To this end, 120 g of ground dried plant material was added to a 2 L Erlenmeyer flask filled with 1 L of methanol $\geq$ 99.5 % (Panreac Química SLU, Barcelona, Spain) as solvent and shaken at 25ºC for 48 h in the dark. The mixture was filtered through Whatman No. 4 paper (grammage 92 g m^{-2}), and the solvent was evaporated at 40ºC using a Rotavapor R-100 (BUCHI Ibérica, Barcelona, Spain). The extracts were lyophilized and stored at 5°C in the dark until use. The doses used for pomegranate and carob extracts in the *in vitro* assays were adjusted at 30; 300; and 3000 mg of extract per L of sterile distilled water (SDW). A laboratory copper sulphate (Merk Lab.) was included for comparative purposes, with the doses tested being 125 (1/16 of the high dose); 500 (1/4 of the high dose); and 2000 mg L^{-1} (high dose) (López-Moral et al., 2021; Antón-Domínguez et al., 2024a).

For both experiments, on detached olive fruits in humidity chambers and on attached olive fruits in plants of cv. Arbequina, only the maximum doses of 3000 or 2000 mg L^{-1} were used for plant extracts or Cu sulphate applications, respectively.

2.2. Fungal isolates

The isolates *Colletotrichum godetiae* Col-511 and *C. nymphaeae* Col-466 were used in this study. They were previously characterized by Moral et al. (2021) and are maintained in the fungal collection of the Department of Agronomy of the University of Cordoba (DAUCO, Spain). To prepare the inoculum, they were firstly grown on potato dextrose agar (PDA; Difco® Laboratories, MD, United States) acidified with lactic acid (APDA; 0.1 % (vol/vol); pH = 4.0–4.5) and incubated in the dark for 14 days at 24 $\pm$ 2ºC. Then, the growing colonies were transferred to PDA, incubated as described above and used as inoculum source for the experiments conducted in this study.

2.3. In vitro effect of plant extracts

Two *in vitro* assays were conducted to evaluate the effect of plant extracts against *C. godetiae* and *C. nymphaeae*. The first assay (2.3.1) assessed the impact of plant extracts or Cu sulphate on mycelial growth, and how the amended culture media (PDA) with the extracts or Cu sulphate influenced in the conidial production on the fungal colonies and in their viability (conidia germination and appressoria formation). The second assay (2.3.2) tested the effect of the extracts on conidia germination and appressoria formation by mixing conidial suspensions of the pathogen with extracts or Cu sulphate solutions.

2.3.1. Effect of plant extracts on mycelial growth of Colletotrichum spp. on amended culture media, and their influence on conidial production and viability

Appropriate weights of pomegranate or carob extracts, or Cu

sulphate, were added to sterilized PDA medium to adjust the final concentration of each tested dose. Amended PDA Petri dishes were inoculated in the center by placing a mycelial plug of the pathogen taken from the edge of 14-day-old colonies grown as described above. Non-amended PDA Petri dishes plated with each pathogen were included as controls (dose = 0 mg L^{-1}).

A factorial design was used with 18 treatments [two pathogens (*C. godetiae* and *C. nymphaeae*), three products (two plant extracts and Cu sulphate), and three doses] and a positive control for each pathogen (dose = 0 mg L^{-1}) as independent variables, and four replicate Petri dishes per treatment combination or control ($20 \times 4 = 80$ Petri dishes in total). The experiment was repeated twice. After 14 days of incubation under the conditions described above, the largest and smallest colony diameters were measured with a digital scale.

Subsequently, to determine the effect of the products and doses on conidial production, one mycelium plug taken from the colony edge of each treatment combination was transferred to 1.5 mL Eppendorf® tubes containing 1 mL of SDW. One Eppendorf® tube was used per replicate Petri dish from each experimental repetition, four tubes in total per treatment combination, a total of 80 tubes per experiment. The Eppendorf® tubes were vortexed for 1 min and the conidial concentration was determined using a hemocytometer. Finally, the viability of conidia was evaluated by inoculating a microscope coverslip center (20×20 mm) with 10 µl of a conidial suspension previously adjusted to 4×10^5 conidia mL^{-1} of SDW. The coverslips were placed in Petri dishes with water agar as humidify chambers and incubated at 24 ± 2ºC in the dark at 100 % relative humidity (RH) for 16 h (Silva et al., 2023). After the incubation period, a five µl drop of 0.01 % acid fuchsin in lactoglycerol was added to each coverslip to stop germination and the coverslips were placed on slides according to López-Moral et al. (2022a). 100 conidia were randomly observed per slide, and ungerminated and germinated conidia, and those with or without appressoria were counted using a Nikon Eclipse 80i microscope (Nikon Corp., Tokyo, Japan).

The effect of the plant extracts or Cu sulphate in inhibiting *Colletotrichum* species mycelial growth (MGI; %), conidial production (CPI; %), and their viability [conidia germination (CGI; %) as well as appressoria formation (AFI; %)] was assessed by calculating the percentage of inhibition in relation to control (i.e., nonamended PDA) for each parameter using the following equation:

$$Inhibition\ (\%) = [1 - (treatment/control)] \times 100$$

where "treatment" is the mycelial growth, conidial production, or germination, or appressoria formation of *Colletotrichum* species on PDA amended with each product (extract or Cu sulphate), and "control" is the same parameters evaluated in *Colletotrichum* species on nonamended PDA.

2.3.2. Effect of plant extracts on conidia germination and appressoria formation of Colletotrichum spp.

The effect of pomegranate or carob extracts, or Cu sulphate, on the conidia germination and appressoria formation of *C. godetiae* and *C. nymphaeae* was conducted following the protocol described by López-Moral et al. (2022a). A five µl drop of the conidial suspension was deposited in the center of a microscope coverslip together with a five µl drop of pomegranate or carob extracts, or Cu sulphate, at the different doses tested (*see* 2.1); a five µl drop of the conidial suspension mixed with a five µl drop of SDW was used as control (dose = 0 mg L^{-1}). Coverslips were incubated at 24 ± 2ºC in the dark for 16 h and evaluated as described above.

A factorial design was used with 18 treatments [two pathogens (*C. godetiae* and *C. nymphaeae*), three products (two plant extracts and Cu sulphate), and three doses] and a positive control for each pathogen (dose = 0 mg L^{-1}) as independent variables and three replicate coverslips per treatment combination or controls ($20 \times 3 = 60$ coverslips in total). The experiment was repeated twice. After the incubation period, 100 conidia were randomly observed per slide, and ungerminated and germinated conidia, and those with or without appressoria were counted as described above. The percentages of conidia germination inhibition (CGI; %) and appressoria formation inhibition (AFI; %) were estimated relative to the control as described above.

2.4. Effect of plant extracts on disease progression in detached olive fruits

Healthy detached olive fruits [onset of ripening; color class 2–3 (Barranco et al., 2017)] of cv. Arbequina (moderately susceptible; Moral et al., 2017) were collected from the World Olive Germplasm Bank of the University of Cordoba (Spain). Once in the laboratory, the fruits were manually selected for uniformity in size and color, discarding those with damage. They were then washed in a 0.02 % (v/v) Tween 20 aqueous solution and surface disinfected first in a 70 % (v/v) ethanol solution for 2 min and then in a 20 % (v/v) sodium hypochlorite solution (5 g of Cl L^{-1}) for 7 min. Subsequently, the fruits were rinsed under running tap water and allowed to dry on sterile filter paper for 30 min. Pomegranate or carob extracts, or Cu sulphate, were applied at the maximum doses indicated in the 2.1., by spraying 160 µl of dosed product in a SDW solution per olive fruit. Treatment applications were conducted twice at 96 and 24 h before inoculation with *C. godetiae* (preventive application); or at 72 h after pathogen inoculation (curative application) (Supplementary Fig. S1). Fruit inoculation with *C. godetiae* was carried out by spraying the olives with a conidial suspension adjusted at 10^5 conidia mL^{-1} as described above, at a rate of 160 µl of the adjusted suspension per fruit. Plastic humidity chambers with fruits sprayed with only SDW (dose = 0 mg L^{-1}) or with *C. godetiae* were included as negative or positive control, respectively. The fruits were incubated at 20 ± 2ºC in the dark and 100 % RH for one month after pathogen inoculation.

For each type of application (preventive or curative), a randomized complete block design was used with three products (two plant extracts and Cu sulphate) and two controls (positive and negative) as independent variables with three humidity chambers (blocks) per treatment combination or controls and 20 replicate olive fruits per block ($2 \times 5 \times 3 \times 20 = 600$ olives in total). The experiment was repeated twice.

Disease severity (DS) was assessed every three days using a rating 0–4 scale adapted from Moral et al. (2021): 0 = olives without symptoms, 1 = 25 %, 2 = 50 %, 3 = 75 % and 4 = 100 % of affected olive surface. The area under the disease progression curve (AUDPC) was calculated using trapezoidal integration according to the following formula (Campbell and Madden, 1990):

$$AUDPC = \sum_{i=1}^{n} \frac{(DS_i + DS_{i+1})}{2} \times (t_{i+1} - t_i)$$

where n is the number of observations, DS_i is the DS value for observation number i, and t_i is the number of days between planting and observation i.

Subsequently, AUDPC and DS were expressed as relative percentage (RAUDPC; RDS; %) with respect to the pathogen-inoculated and untreated fruits (positive control). At the end of the experiment, the disease incidence (DI; number of total symptomatic plants) and the number of total rotten fruit were counted and expressed as percentage (%).

2.5. Effect of plant extracts on disease progression in olive plants and assessment of plant defence response

2.5.1. Effect on disease progression

Nine-month-old healthy potted olive plants of cv. Arbequina with attached fruits were obtained from a commercial nursery. Pomegranate or carob extracts, or Cu sulphate, were applied at maximum doses indicated above, by spraying 10 mL of the dosed product in a SDW solution per plant in two moments, at 96 and 24 h before inoculation with the pathogen (Supplementary Fig. S2A). Plant inoculation with

C. godetiae was carried out by spraying a conidial suspension (10^5 conidia mL^{-1}) onto plants at a rate of 10 mL per plant. Plants sprayed with only SDW (dose = 0 mg L^{-1}) or the pathogen (10^5 conidia mL^{-1}), were included as negative or positive control, respectively. Plants were incubated in a growth chamber at 20 ± 2ºC in the dark and 100 % RH for 5 days after inoculation. The RH was then maintained at 80 % and the photoperiod at 8 h day/16 h night with photosynthetically active radiation (10,000 lux). Plants were watered four times a week.

There were four groups of plants: 1) noninoculated and treated with pomegranate or carob extracts, or Cu sulphate; 2) pathogen-inoculated and treated with pomegranate or carob extracts, or Cu sulphate; 3) noninoculated and untreated (negative control); and 4) pathogen-inoculated and untreated plants (positive control).

A factorial randomized complete block design (four blocks) was used with eight treatments [($n = 4$; SDW, pomegranate or carob extracts, or Cu sulphate), and inoculation with *C. godetiae* ($n = 2$; pathogen-inoculated or noninoculated plants)] as independent variables and three replicate olive plants per treatment and block ($4 \times 4 \times 2 \times 3 = 96$ plants in total). The experiment was conducted twice.

Disease incidence was assessed every week and area under the incidence progress curve (AUIPC) was calculated using the formula described previously by substituting DS for DI. The AUIPC was expressed as relative percentage (RAUIPC; %) with respect to the pathogen-inoculated and untreated plants (positive control). At the end of the experiment, the DI (%) and the percentage of total rotten fruit were estimated.

To assess plant defence response, leaf samples from each group of plants were collected following the protocol described by Silva et al. (2023), at 0, 3, 7, and 24 h after inoculation with *C. godetiae* or SDW (control). For each time point and treatment, three samples were obtained, each consisting of ten leaves from replicated plants. Samples from two experiments were then combined, resulting in three final samples of 20 leaves each (Supplementary Fig. S2B). All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.5.2. Hydrogen peroxide quantification

The H_2O_2 content in leaf samples was determined according to Baptista et al. (2007). To this, 70 mg of each sample was weighed and homogenized in 2 mL of 0.1 % (w/v) trichloracetic acid (TCA; Scharlab, Barcelona, Spain) in an ice bath. The solution was centrifuged at 12000×*g* for 20 min at 4ºC and 0.5 mL of the supernatant was transferred to 0.5 mL of potassium phosphate buffer (Sigma-Aldrich, MO, USA) 10 mm (pH 7.0) and 1 mL of 1 M KI (Honeywell, NC, USA). The absorbance was measured at 390 nm with UV-1280 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). A calibration curve was performed using solutions with known H_2O_2 concentrations (Panreac Química SLU, Barcelona, Spain) to quantify the content in the samples expressed in μmol per gram of fresh weight of leaf sample (FW).

2.5.3. Phenolic compounds analysis

Phenolic compounds extraction from leaf samples was determined according to Vinha et al. (2005). Samples were freeze-dried using Scanvac CoolSafe (Labogene, Lillerød, Denmark). 200 mg of lyophilized leaf sample was mixed with 20 mL of methanol ≥ 99.8 % (Honeywell, NC, USA) and shaken at room temperature for 3 h. The methanolic extract was filtered through Whatman paper and evaporated at 40ºC using the Rotavapor Stuart® RE300 (Bibby Scientific, Staffordshire, UK). The extract was redissolved in methanol at a concentration of 50 mg mL^{-1} and filtered with a 0.2 μm Whatman membrane filter. Subsequently, phenolic compounds were determined by high-performance liquid chromatography with photodiode array detection (DAD-HPLC) with a system controller (SCL-40), a degassing unit (DGU-405), a solvent delivery module (LC-40D XS), an auto sampler (SIL-40C XS), a column oven (CTO-40C) and DAD (SPD-M40, Shimadzu). Compounds separation was performed on a C18 reversed-phase column (250 mm×4 mm id, 5 μm, Spherisorb) (Nucleosil, Macherey-Nagel, Düren, Germany) at 30°C. A gradient of water/formic acid (19:1) (A) and methanol (B) was used at a flow rate of 0.9 mL min^{-1}. Peak identification was carried out with LabSolutions software by comparing retention times and UV/Vis spectra (200–600 nm) with pure standards analyzed with the same protocol, for the following compounds: phenolic acids (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, hydroxytyrosol, oleuropein, tyrosol, verbascoside) and flavonoids (apigenin, apigenin-7-O-glucoside, luteolin-7-O--glucoside, rutin). Wavelengths were set at 254 nm (oleuropein); 280 nm (gallic acid, hydroxytyrosol, tyrosol); 320 nm (apigenin, caffeic acid, chlorogenic acid, ferulic acid, rutin, verbascoside); and 350 nm (apigenin-7-O-glucoside, luteolin-7-O-glucoside). Results were expressed as mg of compound per kg of dry weight of leaf sample (DW).

2.6. Data analysis

The data obtained from the two repetitions of each experiment were combined after verifying that there were no significant differences between them ($P \geq 0.05$). Homogeneity of variances and data normality were tested prior to performing ANOVAs. Logarithmic transformations of the data were performed when necessary.

A factorial ANOVA was performed for the evaluated parameters as dependent variables, and pathogens, products (two plant extracts and Cu sulphate), doses, and/or mode of application, and/or time point and their respective interactions as independent variables. Data from Cu sulphate treatments were excluded from the analysis when this treatment reached 100 % inhibition. Because significant differences were observed for the independent variables and their interactions ($P \leq 0.05$; *see results sections for further details*), the comparison of the means was performed for the interactions according to Fisher's protected least significant difference (LSD) test at $P = 0.05$ (Steel and Torrie, 1985). In cases where only two treatments were compared, Student's *t*-test was used at $P = 0.05$. In addition, data of DI (%) and percentage of total rotten fruit from both detached and attached olive fruit assays were analyzed by means of multiple comparison tests for proportions at $P = 0.05$ (Zar, 2010). Statistix 10 software (Analytical Software, 2013) was used for data analysis.

Heatmaps were generated to show the mean Log2 Fold Change in H_2O_2 content and total phenolic compounds production of each treatment (pomegranate, or carob extracts, or Cu sulphate) in leaves of plants inoculated with the pathogen with respect to untreated and inoculated plants (positive control) at each time point (0, 3, 7 and 24 h). R v.4.4.1 and RStudio v. 2024.12.0 + 467 (R Core Team, 2024) software was used to perform this analysis using the pheatmap function of the 'pheatmap' package (Kolde, 2019).

To identify the phenolic compounds that best discriminate the different treatments (pomegranate or carob extracts, or Cu sulphate) in leaves of pathogen-inoculated and noninoculated plants at each time point (0, 3, 7 and 24 h), a principal component analysis (PCA) was performed. R v.4.4.1 and RStudio v. 2024.12.0 + 467 (R Core Team, 2024) software was used to perform this analysis using the pca function of the 'FactoMineR' package (Lê et al., 2008). The PCA biplot was then plotted using the fviz_pca_biplot function from the 'factoextra' package (Kassambara and Mundt, 2020). The PCA arrows represent the contribution of each phenolic compound to the two components (arrow length), and the color of the specific gradient indicates its contribution to explaining the largest variance in the data set. Pearson correlation analysis was performed to determine the correlation between phenolic compounds production and RAUIPC. R v.4.4.1 and RStudio v. 2024.12.0 + 467 (R Core Team, 2024) software was used to perform this analysis using the cor_mat and corrplot functions of the 'corrplot' package (Wei and Simko, 2024).

3. Results

3.1. In vitro effect of plant extracts

3.1.1. Effect of plant extracts on mycelial growth of Colletotrichum spp. on amended culture media, and their influence on conidial production and viability

For both *C. godetiae* and *C. nymphaeae* isolates, there were significant differences in the effect on mycelial growth inhibition (MGI) among products, doses, and their interaction ($P \leq 0.0001$ in all cases). The extracts could not significantly inhibit the mycelial growth of the two pathogens regardless of the doses even they induced mycelial growth with respect to the control in some cases. However, the reference product (Cu sulphate) was highly effective in inhibiting the mycelial growth for both *Colletotrichum* species at medium and high doses, with the MGI values ranging from 96.1 % to 100 % (Table 1).

For both *Colletotrichum* species, there were significant differences in the effect on conidia production inhibition (CPI) among products, doses, and their interaction ($P \leq 0.0001$ in all cases). All the products were highly effective in inhibiting *C. godetiae* conidia production regardless of the doses, with the CPI values ranging from 75.0 % to 100 %. In this case, the most effective treatments were the two extracts applied at 3000 and 300 mg L^{-1} and the Cu sulphate at all the doses. For *C. nymphaeae*, only pomegranate extract at 3000 mg L^{-1} (CPI = 93.2 ± 2.5 %) and Cu sulphate at 2000 and 500 mg L^{-1} (CPI = 100 ± 0.0 % in both cases) significantly inhibited conidia production of the pathogen, followed by pomegranate extract at 300 and 30 mg L^{-1} (CPI = 61.7 ± 2.3 % and 61.6 ± 9.1 %, respectively). Carob extract had a significant less effect on inhibiting conidia production regardless of the doses than the rest of the treatment combinations (Table 1).

The viability of the conidia produced on the fungal colonies growing on amended PDA with the products at the different doses tested was also evaluated by estimating the percentage of germinated conidia and appressoria formation. The two extracts did not have any effect in inhibiting the germination and appressoria formation on the conidia obtained from the *Colletotrichum* colonies grown on PDA amended with the extracts at 300 or 30 mg L^{-1}; while they were effective when applied at 3000 mg L^{-1}. However, it was not possible to evaluate these parameters for Cu sulphate since this product inhibited totally the mycelial growth of both *Colletotrichum* species regardless the dose, and it was not possible to collect conidia from the fungal colonies. Thus, only the results on the effect of pomegranate and carob extracts at 3000 mg L^{-1} on conidia germination (CGI) and appressoria formation (AFI) inhibition for both *Colletotrichum* species are shown in Fig. 1. For *C. godetiae*, both pomegranate and carob extracts had a similar effect on CGI (15.9 ± 2.7 % and 35.5 ± 14.5 %, respectively); while pomegranate extract was significantly more effective on AFI (98.8 ± 0.7 %) than carob extract (87.2 ± 1.8 %) (Fig. 1A). For *C. nymphaeae*, both pomegranate and carob extracts had a similar significant effect on CGI (18.0 ± 3.0 % and 25.0 ± 3.3 %, respectively) and AFI (64.9 ± 8.1 % and 55.0 ± 7.6 %, respectively) (Fig. 1B).

Table 1
Effect of pomegranate or carob extracts, or Cu sulphate on mycelial growth (MGI; %) and conidia production (CPI; %) inhibition of *Colletotrichum godetiae* and *C. nymphaeae*, with respect to the control.

Product	Dose (mg L^{-1})	Mycelial Growth Inhibition (MGI; %)[a]		Conidia Production Inhibition (CPI; %)[a]	
		C. godetiae	*C. nymphaeae*	*C. godetiae*	*C. nymphaeae*
Pomegranate extract	3000	3.1 ± 6.1 bc	−2.0 ± 2.6 d	96.4 ± 1.0 ab	93.2 ± 2.5 a
	300	−1.6 ± 3.1 cd	2.3 ± 2.1 cd	98.8 ± 0.6 a	61.7 ± 2.3 b
	30	−10.2 ± 2.5 d	3.4 ± 1.9c	75.0 ± 2.8c	61.6 ± 9.1 b
Carob extract	3000	−0.4 ± 3.1c	−0.6 ± 1.7 cd	99.8 ± 0.1 a	36.3 ± 12.9c
	300	0.1 ± 5.3c	3.1 ± 1.5 cd	98.3 ± 0.3 a	38.5 ± 6.2c
	30	−0.9 ± 3.2 cd	3.7 ± 1.7c	91.9 ± 3.6 b	30.4 ± 4.7c
Cu sulphate	2000	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a	100 ± 0.0 a
	500	96.1 ± 0.6 a	98.4 ± 0.2 a	100 ± 0.0 a	100 ± 0.0 a
	125	11.3 ± 4.9 b	16.8 ± 4.7 b	97.4 ± 1.1 a	36.9 ± 5.2c

[a] For each product and dose combination, data represent the mean of two experiments with four replicated Petri dishes each ± standard error of the means. Means in a column followed by a common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).

3.1.2. Effect of plant extracts on conidia germination and appressoria formation of Colletotrichum spp.

There were significant differences between CGI and AFI among products, doses, and their interaction for both *Colletotrichum* species ($P \leq 0.0001$ in all cases). Cu sulphate was the most effective product inhibiting conidia germination of both species regardless of the doses, with CGI values ranging from 99.2 ± 0.8–100 ± 0.0 %. Due to the absence of conidia germination, it was not possible to evaluate the effect on appressoria formation for Cu treatments. For *C. godetiae*, pomegranate or carob extracts at 3000 mg L^{-1} were the next most effective treatments, even the CGI values (51.5 ± 7.2 and 40.5 ± 3.6 %) were significantly lower than those observed for Cu sulphate treatments. However, the appressoria formation was 100 % inhibited in the conidia subjected to these two extracts at the maximum dose tested. The remaining treatments were ineffective. For *C. nymphaeae*, the effect of pomegranate extract at 3000 mg L^{-1} on CGI (89.4 ± 4.8 %) did not differ significantly compared to Cu sulphate treatments. Carob extract at 3000 mg L^{-1} was the next most effective treatment after those, even the CGI (44.6 ± 11.0 %) was significantly lower than Cu sulphate. The appressoria formation was 100 % inhibited in the conidia subjected to these two extracts at the maximum dose tested. The remaining treatments were ineffective (Table 2).

3.2. Effect of plant extracts on disease progression in detached olive fruits

There were significant differences for all the disease related parameters among products, type of application and their interaction ($P \leq 0.05$). Both pomegranate and carob extracts were significantly more effective when they were applied as curative (after inoculation), whereas Cu sulphate was more effective in preventive application (before inoculation). Thus, separate ANOVA were conducted for each type of application to determine the effect of the products on disease development in detached fruit.

For preventive application, there were significant differences in RAUDPC and final RDS among products ($P \leq 0.0001$ in both cases). Only Cu sulphate had a significant effect on reducing disease progression and final RDS (RAUDPC = 8.4 ± 2.3 %; RDS = 14.1 ± 6.4 %) compared to the positive control (RAUDPC = 100 ± 11.2 %; RDS = 100.0 ± 5.9 %). Likewise, Cu sulphate was the only product that significantly reduced the DI (18.3 %) and the percentage of total rotten fruit (8.3 %) compared to the positive control (DI = 93.3 %; rotten fruits = 76.7 %) (Table 3).

For curative application, there were significant differences in RAUDPC and RDS among products ($P \leq 0.0001$ in both cases). Pomegranate and carob extracts showed a moderate effect on reducing disease progression compared to the positive control (RAUDPC = 100 ± 12.7 %) with RAUDPC ranging from 66.1 ± 1.2–66.9 ± 5.3 %, respectively. Only Cu sulphate significantly reduced DI and final RDS (80.0 % and 73.9 ± 4.3 %, respectively) compared to the positive control (DI = 100 % and RDS = 100.0 ± 1.7 %) (Table 3).

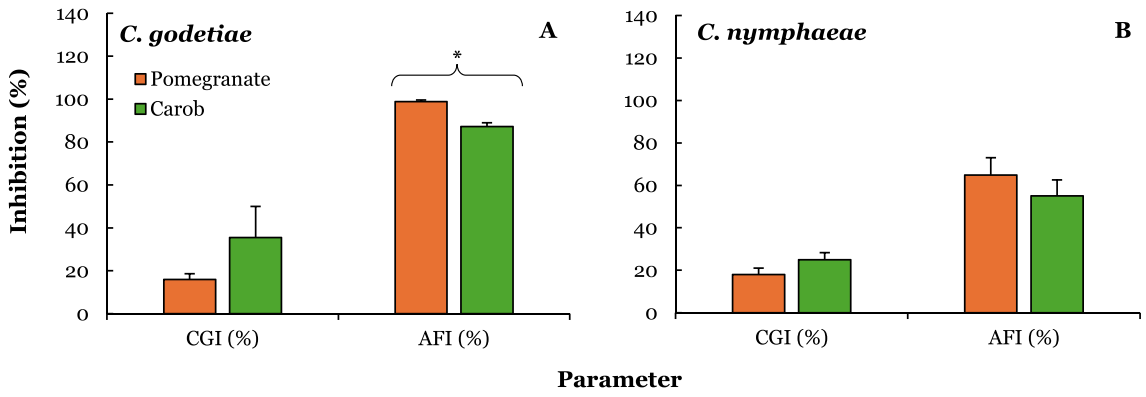

Fig. 1. Effect of pomegranate (orange columns) or carob (green columns) extracts on the conidia germination (CGI, %) and appressoria formation (AFI, %) inhibition, with respect to the control, from conidial suspensions obtained from the margin of the active growing colonies of *Colletotrichum godetiae* (A) and *C. nymphaeae* (B) grown on PDA adjusted at high doses (3000 mg L^{-1}). Columns represent the mean of two experiments with four replicate counts each, and vertical bars the standard error of the means. For each graph and dependent variable (CGI or AFI), columns with asterisk differ significantly according to Student's *t*-test at $P = 0.05$.

Table 2
Effect of pomegranate or carob extracts, or Cu sulphate on conidia germination (CGI; %) and appressoria formation (AFI; %) inhibition of *Colletotrichum godetiae* and *C. nymphaeae*, with respect to the control.

Product	Dose (mg L^{-1})	Conidia Germination Inhibition (CGI; %)[a]		Appressoria Formation Inhibition (AFI; %)[a]	
		C. godetiae	*C. nymphaeae*	*C. godetiae*	*C. nymphaeae*
Pomegranate extract	3000	51.5 ± 7.2 b	89.4 ± 4.8 a	100 ± 0.0 a	100 ± 0.0 a
	300	28.5 ± 6.5c	3.2 ± 2.7c	−1.7 ± 1.2 b	80.7 ± 8.9 b
	30	26.3 ± 10.8c	5.1 ± 4.2c	−5.8 ± 4.0 bc	26.6 ± 7.6 d
Carob extract	3000	40.5 ± 3.6 bc	44.6 ± 11.0 b	100 ± 0.0 a	100 ± 0.0 a
	300	4.5 ± 4.5 d	−2.6 ± 2.4 d	−3.6 ± 3.1 bc	91.2 ± 3.0 ab
	30	−2.3 ± 1.8 e	−3.0 ± 1.7 d	−11.1 ± 1.2c	46.5 ± 6.0c
Cu sulphate	2000	100 ± 0.0 a	100 ± 0.0 a	n/d	n/d
	500	99.2 ± 0.8 a	99.3 ± 0.7 a	n/d	n/d
	125	100 ± 0.0 a	100 ± 0.0 a	n/d	n/d

[a] For each product and dose combination, data represent the mean of two experiments with three replicated coverslips each ± standard error of the means. Means in a column followed by a common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985). n/d = not determined due to total conidia germination inhibition.

3.3. Effect of plant extracts on disease progression in olive plants and assessment of plant defence response

3.3.1. Effect on disease progression

Olive fruits from pathogen-inoculated and untreated plants (positive control) showed typical disease symptoms approximately one week after inoculation and reached a DI of 100 % one month after inoculation. The first symptoms observed were small sunken necrotic lesions on the fruit, sometimes culminating in complete rotting of the olives. As the disease progressed, orange conidial masses, produced by the asexual fruiting bodies (acervuli) of the fungus, could be observed on the fruit. In some cases, loss of moisture led to mummification of the fruit and its adherence to the plant (Fig. 2).

There were significant differences in RAUIPC among products ($P = 0.0123$). Cu sulphate followed by carob extract resulted in the most effective products in reducing the disease incidence progression since they showed significant less RAUIPC values (35.6 ± 8.3 % and 65.1 ± 15.4 %, respectively) compared to the positive control (RAUIPC = 100 ± 6.5 %). However, the effect of pomegranate extract on disease incidence progression (RAUIPC = 89.1 ± 15.4 %) did not differ

Table 3
Disease-related parameters evaluated on detached olive fruits of cv. Arbequina inoculated with conidial suspensions of *Colletotrichum godetiae* and treated with pomegranate or carob extracts, or Cu sulphate by preventive or curative applications.

Product	Preventive application			
	Incidence (%)[c]	Total rotten fruit (%)[c]	Final RDS* (%)[d]	RAUDPC* (%)[d]
Pomegranate extract	98.3 a	88.3 a	110.5 ± 1.2 a	101.3 ± 8.2 a
Carob extract	98.3 a	71.7 b	100.6 ± 4.3 a	91.9 ± 9.7 a
Cu sulphate	18.3 b	8.3c	14.1 ± 6.4 b	8.4 ± 2.3 b
Negative control[a]	0.0c	0.0 d	0.0 ± 0.0c	0.0 ± 0.0 b
Positive control[b]	93.3 a	76.7 ab	100.0 ± 5.9 a	100 ± 11.2 a
Product[a]	**Curative application**			
	Incidence (%)[c]	Total rotten fruit (%)[c]	Final RDS* (%)[d]	RAUDPC* (%)[d]
Pomegranate extract	98.3 a	78.3 a	95.4 ± 2.8 a	66.1 ± 1.2 b
Carob extract	98.3 a	66.7 b	89.2 ± 7.8 a	66.9 ± 5.3 b
Cu sulphate	80.0 b	56.7 b	73.9 ± 4.3 b	48.6 ± 2.5 b
Negative control[a]	0.0c	0.0c	0.0 ± 0.0c	0.0 ± 0.0c
Positive control[b]	100 a	85.0 a	100.0 ± 1.7 a	100 ± 12.7 a

[a] Negative control: noninoculated and untreated olive fruits.
[b] Positive control: inoculated and untreated olive fruits.
[c] In each column, data are the mean of three blocks with 20 replicated fruits ± standard error of the means. Means followed by a common letter do not differ significantly according to multiple comparisons tests for proportions at $P = 0.05$ (Zar, 2010).
[d] In each column, data are the mean of three blocks with 20 replicated fruits ± standard error of the means. Means followed by a common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).
* Final RDS: Relative final disease severity; RAUDPC: Relative area under the disease progress curve.

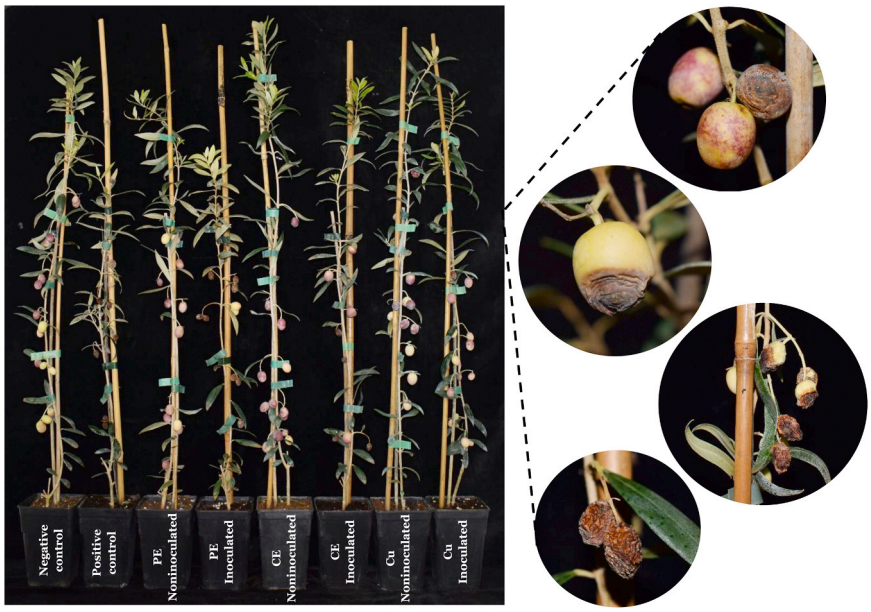

Fig. 2. Disease symptoms on attached olive fruits in plants of cv. Arbequina at one month after inoculation with conidial suspensions of *Colletotrichum godetiae* and treated with pomegranate (PE) or carob (CE) extracts, or Cu sulphate by preventive applications. Negative control: noninoculated and untreated olive plant; Positive control: inoculated and untreated olive plant.

significantly compared to the positive control (Table 4). Likewise, a similar pattern was observed for the final DI values, with Cu sulphate and carob extract showing significantly less DI values (91.7 % in both cases) than the positive control (100 %) or pomegranate extract (95.8 %). Regarding the effect of the products on final fruit rot, carob extract showed significantly lower total rotten fruit percentage (8.3 %) compared to the remaining treatments, with the percentages of the total rotten fruit ranging from 12.5 for Cu sulphate to 25.0 % for the positive control (Table 4).

3.3.2. Hydrogen peroxide quantification

There were significant differences in H_2O_2 content among products (two plant extracts, Cu sulphate, or SDW), inoculation (inoculated or noninoculated), time point and their interaction ($P \leq 0.05$). Thus, separate two-way factorial ANOVA were conducted for each time point to determine the effect of the products and inoculation treatment combinations in the H_2O_2 content. For each time point, there were significant differences among products, inoculation, and their interaction ($P \leq 0.05$ in all cases) (Supplementary Table S1).

Inoculated plants treated with carob extract showed the highest H_2O_2 content at 0 h (40.0 ± 0.9 μmol gFW^{-1}) among all treatments, suggesting a strong and immediate oxidative response. After that, the H_2O_2 levels gradually declined over time but remained significantly higher at 3 (32.4 ± 0.4 μmol gFW^{-1}) and 7 h after inoculation (32.3 ± 2.6 μmol gFW^{-1}) than those of the positive control (27.6 ± 0.2 and 27.2 ± 1.4 μmol gFW^{-1}, respectively). Pomegranate extract also enhanced H_2O_2 accumulation in inoculated plants, albeit to a slightly lesser extent. Inoculated plants treated with pomegranate extract showed significantly higher H_2O_2 levels at 3 (33.7 ± 2.7 μmol gFW^{-1}) and 7 h after inoculation (32.0 ± 1.3 μmol gFW^{-1}) than the positive control (Fig. 3A-B; Supplementary Table S1; Supplementary Fig. S3A). Inoculated plants treated with Cu sulphate showed similar H_2O_2 levels at all time points compared to the positive control, indicating that Cu sulphate may not strongly induce oxidative defences in infected plants (Fig. 3C; Supplementary Table S1).

In noninoculated plants, neither carob nor pomegranate extracts strongly triggered H_2O_2 accumulation (Fig. 3A-B; Supplementary Table S1). However, both extracts caused a significant increase in the H_2O_2 levels at 0 and 3 h and additionally at 7 h after inoculation for carob extract than the noninoculated and untreated plants (negative control). However, noninoculated plants treated with Cu sulphate showed higher H_2O_2 levels at all time points ranging from 36.1 ± 0.6–38.2 ± 2.7 μmol gFW^{-1}, compared to the negative control, suggesting that Cu sulphate has a potential stress-inducing effect in the absence of the pathogen.

Table 4
Disease-related parameters evaluated on attached olive fruits in plants of cv. Arbequina inoculated with conidial suspensions of *Colletotrichum godetiae* and treated with pomegranate or carob extracts, or Cu sulphate by preventive applications.

Product	Incidence (%)[c]	Total rotten fruit (%)[c]	RAUIPC* (%)[d]
Pomegranate extract	95.8 ab	20.8 ab	89.1 ± 15.4 ab
Carob extract	91.7 b	8.3 b	65.1 ± 15.4 bc
Cu sulphate	91.7 b	12.5 ab	35.6 ± 8.3c
Negative control[a]	0.0c	0.0c	0.0 ± 0.0 d
Positive control[b]	100 a	25.0 a	100 ± 6.5 a

[a] Negative control: noninoculated and untreated olive fruits.
[b] Positive control: inoculated and untreated olive fruits.
[c] In each column, data are the mean of two experiments with 12 replicated plants. Means followed by a common letter do not differ significantly according to multiple comparisons tests for proportions at $P = 0.05$ (Zar, 2010).
[d] In each column, data are the mean of two experiments with 12 replicated plants ± standard error of the means. Means in a column followed by a common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).
* RAUIPC: Relative area under the incidence progress curve.

3.3.3. Phenolic compounds analysis

A total of 12 phenolic compounds were identified and quantified: phenolic acids (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, hydroxytyrosol, oleuropein, tyrosol, verbascoside) and flavonoids (apigenin, apigenin-7-O-glucoside, luteolin-7-O-glucoside, rutin). There were significant differences in total phenolic compounds production among products (two plant extracts, Cu sulphate, or SDW), inoculation (inoculated or noninoculated), time point and their interaction

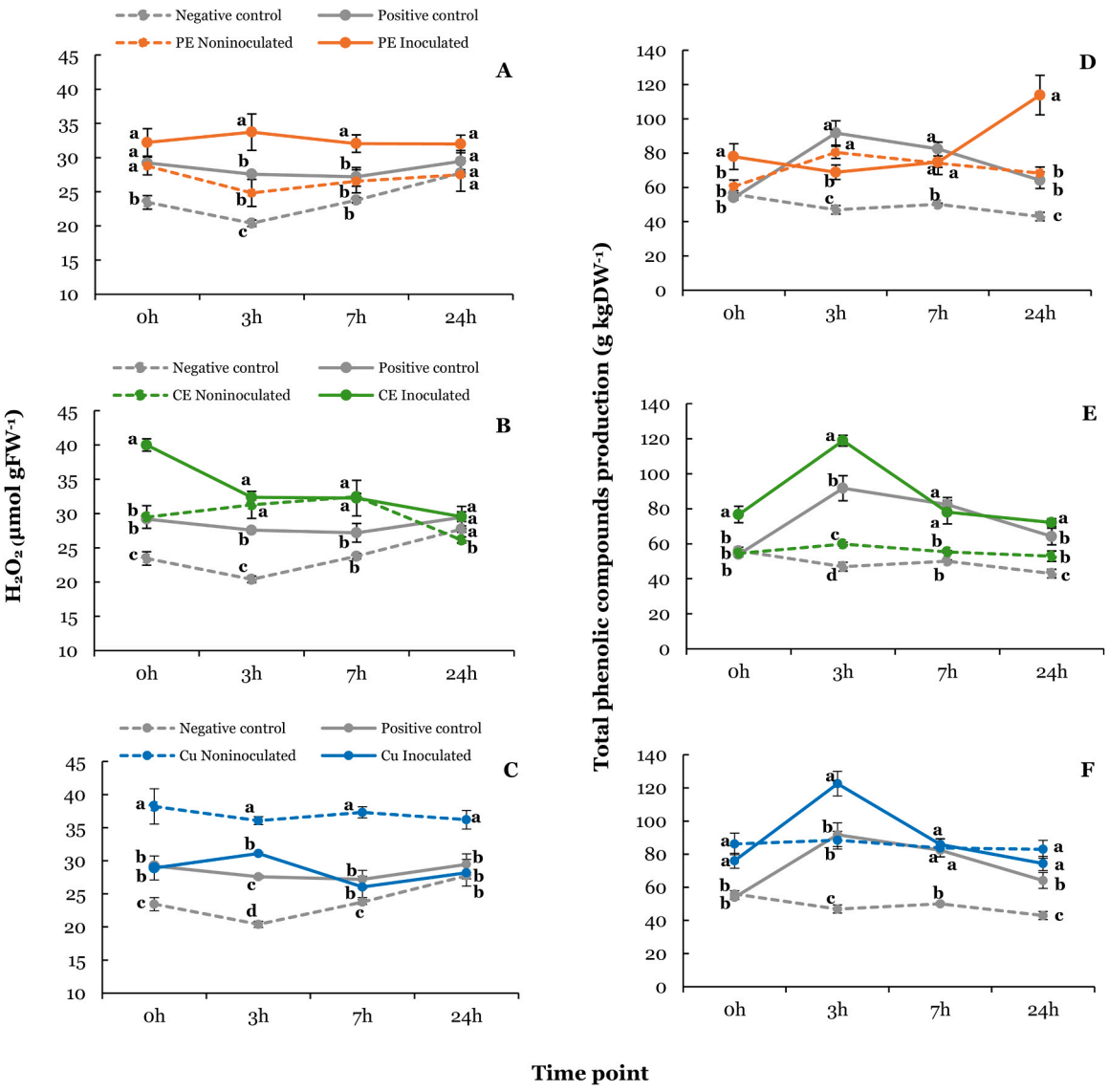

Fig. 3. Hydrogen peroxide H_2O_2 content (µmol gFW^{-1}) (A-C) and total phenolic compounds production (g $kgDW^{-1}$) (D-F), estimated in olive leaves from plants of cv. Arbequina, under various treatments: noninoculated and untreated (negative control), inoculated with *Colletotrichum godetiae* and untreated (positive control), treated with pomegranate (PE) or carob (CE) extracts, or Cu sulphate, each under both noninoculated and inoculated conditions. Samples were collected at 0 h, 3 h, 7 h, and 24 h after inoculation. Data points represent the mean of three samples with 20 leaves each, and vertical bars the standard error of the means. For each graph and time point, means with different letters differ significantly according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).

($P \leq 0.0001$). Thus, separate two-way factorial ANOVA were conducted for each time point to determine the effect of the products and inoculation treatment combinations in the total phenolic compounds production. For each time point, there were significant differences among products, inoculation, and their interaction ($P \leq 0.05$ in all cases) (Supplementary Table S2).

Both pomegranate and carob extracts and Cu sulphate induced the total phenolic compounds production in inoculated plants (Fig. 3D-F; Supplementary Table S2; Supplementary Fig. S3B). Inoculated plants treated with pomegranate extract showed significantly higher total phenolic levels at 0 (78.0 ± 7.5 g $kgDW^{-1}$), 3 (68.8 ± 4.2 g $kgDW^{-1}$) and 24 h after inoculation (113.8 ± 11.6 g $kgDW^{-1}$) than the positive control (54.0 ± 1.3; 91.7 ± 7.1 and 91.7 ± 7.1 g $kgDW^{-1}$, respectively). Inoculated plants treated with carob extract showed significantly higher total phenolic levels at 0 (76.7 ± 4.7 g $kgDW^{-1}$) and 3 h after inoculation (118.8 ± 3.1 g $kgDW^{-1}$) than the positive control. Finally, inoculated plants treated with Cu sulphate showed significantly higher total phenolic levels at 0 (76.0 ± 4.5 g $kgDW^{-1}$) and 3 h after inoculation (122.5 ± 7.4 g $kgDW^{-1}$) compared to the positive control.

Noninoculated plants treated with pomegranate extract showed significantly higher phenolic levels at 3, 7 and 24 h after inoculation, compared to the negative control, suggesting some elicitation even in the absence of the pathogen. In contrast, noninoculated plants treated with carob extract showed total phenolic content closer to the negative control, suggesting minimal phytotoxic or elicitor effect without pathogen presence (Fig. 3D-E; Supplementary Table S2). Cu sulphate triggered consistently high phenolic content across all time points when compared to the negative control (Fig. 3F; Supplementary Table S2).

To identify which phenolic compounds were most relevant to discriminate between treatments, four PCAs were performed (one for each time point), based on the content of 12 phenolic compounds identified in the leaves (Fig. 4). The PCAs showed variances of 55.4, 64.0, 64.0 and 57.2 %, respectively for each time point (0, 3, 7 and 24 h after inoculation) considering the two principal components (Dim1 and Dim2), with clustering according to treatment (Fig. 4). Inoculated plants treated with carob extract were distinguished by the early production

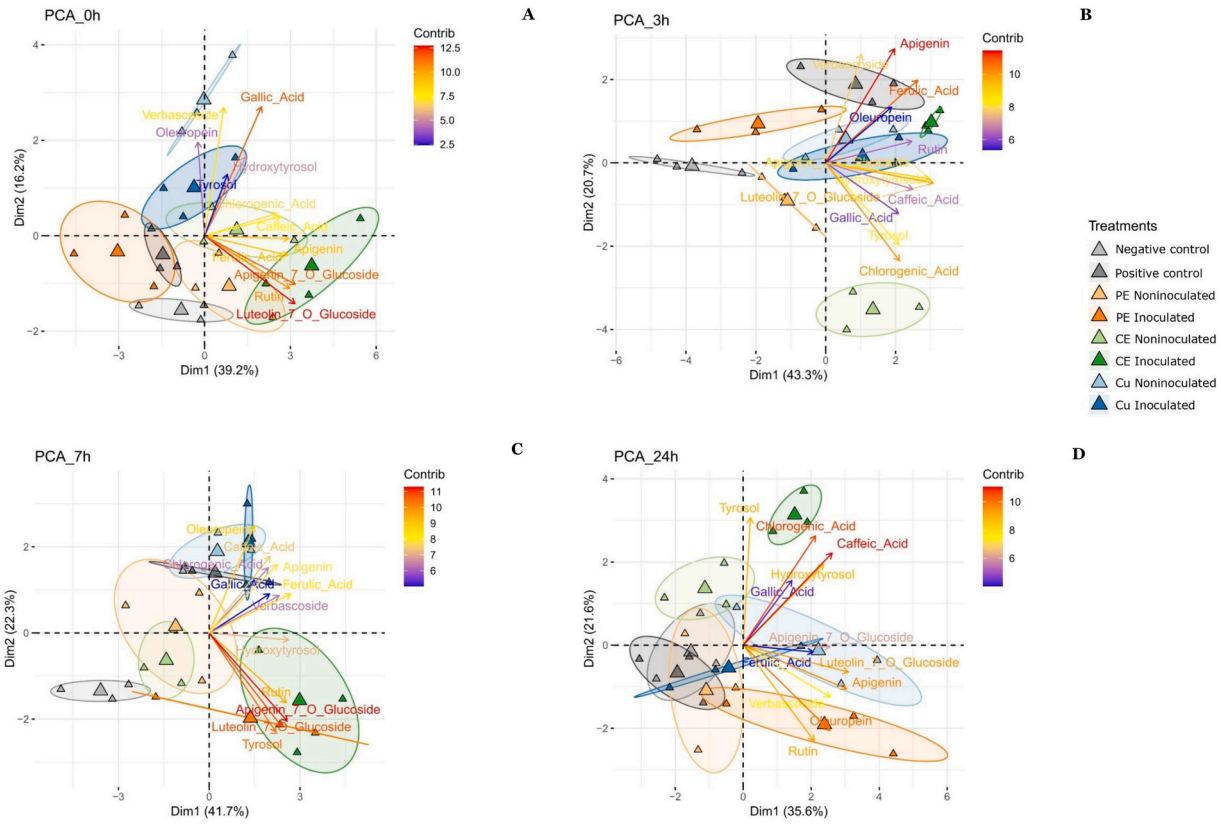

Fig. 4. Principal component analysis (PCA) plots obtained from phenolic compounds in olive leaves from plants of cv. Arbequina, under various treatments: noninoculated and untreated (negative control), inoculated with *Colletotrichum godetiae* and untreated (positive control), treated with pomegranate (PE) or carob (CE) extracts, or Cu sulphate, each under both noninoculated and inoculated conditions. The analysis was performed in samples collected at 0 h (A), 3 h (B), 7 h (C), and 24 h (D) after inoculation. Triangles of the same colour represent the same treatment, with the largest triangle being the average. The colour of the gradient represents the contribution of each phenolic compound to the explanation of the largest variance in the data set.

(0 h) of all the studied flavonoids and ferulic acid; of rutin at 3 h after inoculation; of luteolin-7-O-glucoside, apigenin-7-O-glucoside, rutin, tyrosol and hydroxytyrosol at 7 h after inoculation; and of caffeic and chlorogenic acids, tyrosol and hydroxytyrosol at 24 h after inoculation. Inoculated plants treated with pomegranate extract were characterized by high amounts of luteolin-7-O-glucoside, apigenin-7-O-glucoside, rutin and tyrosol at 7 h after inoculation, like inoculated plants treated with carob extract, and of oleuropein, luteolin-7-O-glucoside, apigenin and rutin at 24 h after inoculation. Inoculated plants treated with Cu sulphate were distinguished by an increase in gallic acid production at 0 h, which remained high throughout the time points, as well as caffeic and chlorogenic acids at 7 h after inoculation. It should be noted that the inoculated and untreated plants also produced phenolic compounds, although to a lesser extent than the inoculated and treated plants, except for verbascoside, which was produced in greater quantities at 3 h after inoculation in these plants than in the other treatments (Fig. 4).

One of the objectives of the phenolic profile study was to identify which specific phenolic acids or flavonoids produced in inoculated plants treated with plant extracts or Cu sulphate were involved in triggering a defensive response in olive trees against *C. godetiae*. Accordingly, Pearson's correlation analysis was performed to determine the association between the whole individual phenolic compounds and RAUIPC at each time point (Fig. 5). The results revealed significant negative correlations at 0 h between RAUIPC and the levels of chlorogenic acid (r = -0.6038; $P < 0.01$), tyrosol (r = -0.5297; $P < 0.01$), and caffeic acid (r = -0.4749; $P < 0.05$) (Fig. 5A). Similarly, at 24 h after inoculation, both caffeic acid (r = -0.4688; $P < 0.05$) and chlorogenic acid (r = -0.4523; $P < 0.05$) were still significantly negative correlated with RAUIPC (Fig. 5D). At 3 h after inoculation, although no statistically significant negative correlations were observed, tyrosol, chlorogenic acid, and gallic acid were the phenolic compounds most associated with RAUIPC reduction (Fig. 5B). No statistically significant negative correlations were detected at 7 h after inoculation (Fig. 5C). Also noteworthy was the significant positive correlation between chlorogenic acid and caffeic acid ($r > 0.6588$; $P < 0.001$) as well as between apigenin-7-O-glucoside and luteolin-7-O-glucoside ($r > 0.7875$; $P < 0.001$), across all time points, suggesting a possible co-regulation or shared pathways in phenolic metabolism during the defence response (Fig. 5).

4. Discussion

The present study aimed to assess the effect of pomegranate peel and carob leaf extracts against olive anthracnose and to elucidate their mode of action by determining their antioxidant activity and the phenolic compounds production. These two extracts were selected since they were highly effective against Verticillium wilt of olive (Antón-Domínguez et al., 2024b; 2025). Therefore, these previous and the present studies confirm the potential bioprotective effect of pomegranate and carob extracts against two major olive diseases. Indeed, this study is innovative since the effectiveness of carob and pomegranate extracts is evaluated for the first time against the main causal agents of OA in both Spanish and Portuguese olive growing areas, determining their mode of action as potential resistance inducers as well.

Regarding the results from the *in vitro* sensitivity tests, both extracts showed a low effect on MGI for both *Colletotrichum* species tested. For instance, pomegranate extract did not show significant mycelial growth inhibition of *P. digitatum*, *P. expansum* or *B. cinerea* (Glazer et al., 2012). However, significant CPI, CGI and AFI were observed in *C. godetiae* and *C. nymphaeae* colonies grown on PDA amended with the extracts, as well as CGI and AFI in suspensions of both species when mixed with the

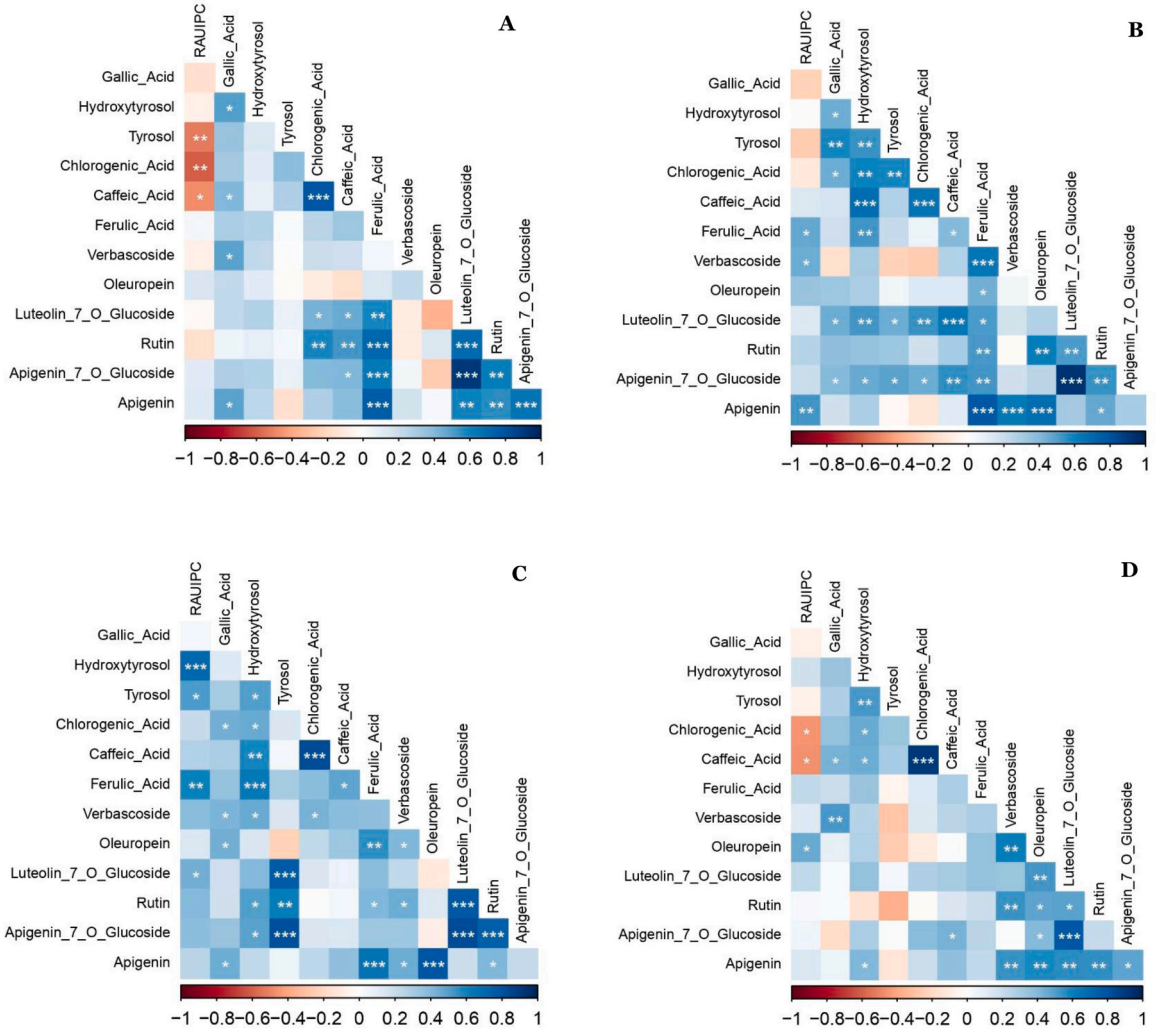

Fig. 5. Pearson correlation between relative area under the incidence progress curve (RAUIPC, %) and phenolic compounds production (g $kgDW^{-1}$) at different time points 0 h (A), 3 h (B), 7 h (C), and 24 h (D) after inoculation. Blue boxes represent positive correlations (max. = 1) and red boxes negative correlations (min. = - 1). Asterisks indicate statistically significant correlations at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

treatments. Furthermore, to our knowledge, this is the first study to investigate the effect of pomegranate and carob extracts on the inhibition of *Colletotrichum* spp. appressoria. The results of conidia germination inhibition are consistent with those described by Pangallo et al. (2017a), where it was observed that pomegranate extract completely reduced the *C. acutatum* conidia germination. Cu sulphate showed an inhibitory effect on all structures of both *Colletotrichum* species, consistent with previous studies (Moral et al., 2018). Both extracts have been shown to be effective in reducing conidia germination of other plant pathogenic fungi. In this sense, carob extract reduced the germination of *Geotrichum candidum* arthrospores by 33 % compared to the control (Talibi et al., 2012). Similarly, pomegranate extract completely inhibited the conidia germination of *F. oxysporum* f. sp. *lycopersici* and almost completely inhibited those of *B. cinerea* (Nicosia et al., 2016; Rongai et al., 2015). These two extracts also significantly inhibited the *V. dahliae* conidia germination by more than 95 % compared to the control (Antón-Domínguez et al., 2024b).

Assays in detached olives performed in the present study showed that both extracts were ineffective in reducing the development of olive anthracnose when they were applied preventively. However, they reduced the disease progression by 33.1–33.9 % compared to the positive control when applied curatively (after pathogen inoculation) but did not reduce incidence or final severity. Oppositely, Pangallo et al. (2017a) reported that both preventive and curative application with pomegranate extract reduced the anthracnose incidence on olives cv. Leccino. However, the experimental conditions differed substantially (mode of fungal inoculation, fungal species, fruit cultivar, number, and timing of extract application). Elsherbiny et al. (2016) demonstrated that the methanolic extract of pomegranate was more effective against *Fusarium* dry rot on potato tubers when applied curatively than preventively. In that study, curative applications at a dose of 20,000 mg L^{-1} inhibited the disease by 88.9 %, while there was no significant inhibition compared to the control at a dose of 2500 mg L^{-1}, like our study. In contrast, Cu sulphate proved to be more effective when applied preventively. These results are in contrast with those from Moral et al. (2018), who showed that preventive applications with Cu sulphate at 12 h before inoculation with *C. godetiae* did not significantly reduce the disease in olives of cvs. Hojiblanca and Arbequina with respect to the control. These discrepancies could be since only one preventive application was carried out by Moral et al. (2018) while two preventive treatments were done in our study. However, the limited effect observed when applied preventively suggests that these extracts may primarily act as resistance inducers, with their protective effect more evident in whole plants than in detached fruits, which lack the active physiological responses of a living system. Under this premise, preventive applications were carried out *in planta* assays to verify this. Carob extract was the most effective in reducing the incidence progression of the disease in attached olive fruit cv. Arbequina, reducing it by 35 % compared to the

positive control, with no significant differences with Cu sulphate. Although there were no significant differences in the disease incidence progression between inoculated plants treated with pomegranate extract and inoculated and untreated plants, pomegranate extract reduced the disease incidence progression by 11 % compared to the control. Our results are in contrast with those obtained by Pangallo et al. (2017a); (2022), where pomegranate extract applied preventively under natural infection conditions in the field at a dose of 3000 mg L^{-1} reduced the incidence of olive anthracnose in olive plants of cvs. Arbosana and Ottobratica by approximately 72 and 52 %, respectively. It is important to note that the conditions of both studies differed, since in our study all cv. Arbequina plants were homogeneously inoculated with 10^5 conidia mL^{-1} of *C. godetiae*, whereas in the other study, the infection occurred naturally in the field on other olive varieties. In addition, the controlled and homogeneous inoculation in a culture chamber allowed us to determine the mechanisms of action of these extracts.

In recent studies, the efficacy of both extracts in reducing Verticillium wilt in olive trees and their involvement in the activation of the salicylic acid and abscisic acid pathways and in the expression of enzymes such as polyphenoloxidase were demonstrated (Antón-Domínguez et al., 2024b; 2025). The potential role of both extracts in protecting olive trees against anthracnose via the induction of plant defence was assessed by analyzing the levels of H_2O_2 and phenolic compounds in olive leaves from the different treatments, given their well-established role in defence signaling and antimicrobial properties (Anjali et al., 2023; Baptista et al., 2007; Llorens et al., 2017; Nag et al., 2024; Sahu et al., 2022). Overall, our results reveal that both extracts, particularly carob extract, induce an oxidative defence in plants inoculated with *C. godetiae* by increasing H_2O_2 content in their leaves. This increase in H_2O_2 levels in extract-treated plants occurred in the first 7 h after inoculation with the pathogen, suggesting a rapid defence response. Afterwards, H_2O_2 levels decreased by 24 h after pathogen inoculation, reflecting the plant's ability to regulate its oxidative response. This quick reduction in H_2O_2 levels is important as prolonged high levels can be detrimental to the plant (Sahu et al., 2022). Previous studies have similarly shown the ability of plant extracts, such as *Mimosa tenuiflora* or *Quercus robur*, to activate biochemical resistance mechanisms in *Lactuca sativa* against *Sclerotinia sclerotiorum* through the increase in ROS production (Llorens et al., 2019). Likewise, Pangallo et al. (2017b) observed an increase in ROS activity in grapefruit treated with pomegranate extract and inoculated with *P. digitatum*, which reached its peak after 24 h. Interestingly, in our study was detected higher levels of H_2O_2 in plants treated with Cu sulphate and noninoculated than in those treated and inoculated with *C. godetiae*. This may be due to the fungistatic nature of Cu, which might reduce pathogen load in inoculated plants, diminishing the need for the plant to produce H_2O_2 upon infection. In the absence of the pathogen, copper appears to induce an oxidative stress by promoting H_2O_2 accumulation, indicating possible phytotoxic or stress-related effects on the plant, as reported previously (Drążkiewicz et al., 2004; Thounaojam et al., 2012).

Our results reveal that both carob and pomegranate extracts increase the production of total phenolic compounds in plants inoculated with *C. godetiae* when compared to the positive control (i.e. untreated and inoculated plants), corroborating their role in inducing plant defence. This effect was more pronounced in plants treated with pomegranate extract although at later times (peak phenolic levels at 24 h), than those treated with carob extract. The ability of plant extracts to induce defence responses upon pathogen infection has been demonstrated previously (Abo-Elyousr et al., 2022; Antón-Domínguez et al., 2025; Llorens et al., 2019; Naz et al., 2021). This effect has been ascribed to the composition of plant extracts on phenolic acids and flavonoids, which play a major role in the enhancement of the innate plant defences by reducing oxidative stress in plants (Anjali et al., 2023; Báidez et al., 2006; Borges et al., 2018; Del Río et al., 2003; Llorens et al., 2017; Nag et al., 2024). Specifically, the effect observed in our study may be attributed to the phenolic composition of the pomegranate and carob extracts, which contain large amounts of gallic acid, catechins, chlorogenic acid, kaempferol, hesperidin and quercetin (Antón-Domínguez et al., 2024b; Darwish et al., 2021; Elsherbiny et al., 2016; Ismail et al., 2021; Meziani et al., 2015). Moreover, phenolic compounds naturally present in olive leaves have also been associated with reduced incidence of other important foliar diseases in olive, such as olive leaf spot caused by *Venturia oleaginea* (Gomes et al., 2023), further supporting their potential defensive role. A significant increase in total phenolic compounds concentration was also observed in plants treated with Cu sulphate after inoculation with the pathogen, compared to the positive control. However, these results contrast with those reported by Ferreira et al. (2007), who observed a decrease in phenolic compound levels in olive leaves 44 days after Cu treatment, compared to the untreated plants. This may be due to the phytotoxic effects associated with the prolonged Cu accumulation and the consequent inhibition of secondary metabolism. Therefore, the effect of this compound should continue to be studied in long-term experiments.

In our study, carob extracts led to an early accumulation (0–7 h) of flavonoids in plants inoculated with *C. godetiae,* particularly apigenin-7-O-glucoside, luteolin-7-O-glucoside and/or rutin, followed by increased levels of phenolic acids like chlorogenic acid, tyrosol, and caffeic acid at later stages (24 h). In contrast, pomegranate extract induced a delayed response, with low flavonoid levels at 0–3 h that increased by 7 h, and a later accumulation of phenolic acids, particularly oleuropein, at 24 h. These results suggest that each extract activates plant defence through distinct metabolic pathways, with carob extract promoting an earlier flavonoid-based response compared to pomegranate. Moreover, both treatments seem to exhibit differentiated metabolic signatures, particularly at the later stages of infection (24 h). To our knowledge, there is currently no literature describing distinct metabolic responses triggered by these extracts, and this trend warrants further investigation to better understand the underlying mechanisms.

From the phenolic compounds detected in olive tree leaves in the present study, chlorogenic acid, caffeic acid and tyrosol (phenolic acids), showed significant negative correlation with RAUIPC, suggesting that their accumulation may contribute to the plant's defence against *C. godetiae*. In previous studies, these compounds have been shown to have antifungal activity against plant pathogens (Báidez et al., 2006; Del Río et al., 2003; Martínez et al., 2017). Specifically, in the study conducted by Del Río et al. (2003), they observed an increase in the content of tyrosol, catechin and oleuropein, among others, in leaves and stems of olive trees treated with a natural commercial product, which subsequently proved to significantly reduce the growth of *Phytophthora* sp. Although no significant negative correlations with disease were found, several phenolic compounds identified in our work, such as oleuropein, have been reported to exhibit fungicidal activity against *Colletotrichum* spp. (Miho et al., 2024). On the other hand, a positive significant correlation was observed between caffeic and chlorogenic acids ($r > 0.6588$; $P < 0.0001$), possibly due to their biosynthetic relationship, since chlorogenic acid results from the combination of caffeic acid and quinic acid. Similarly, apigenin-7-O-glucoside and luteolin-7-O-glucoside showed a significant positive correlation ($r > 0.7875$; $P < 0.0001$), as both belong to the same type of flavone (flavonoid subclass).

5. Conclusions

This work represents a significant advance in bioprotection against olive anthracnose using plant extracts. In particular, the promising results obtained with the carob extract, which showed efficacy in reducing olive anthracnose incidence and inducing plant defence responses, suggest it as a potential candidate for its control. However, at this point, further studies are needed to optimize the extraction method of the extracts and to clarify their efficacy under natural field infection conditions against olive anthracnose, as a prelude to a possible industrial scale-up.

CRediT authorship contribution statement

Begoña I. Antón-Domínguez: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luiza Sánchez-Pereira:** Methodology, Investigation, Data curation. **Sandra Lamas:** Methodology, Investigation. **Nuno Rodrigues:** Methodology, Investigation, Formal analysis. **Paula Baptista:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization. **Carlos Agustí-Brisach:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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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. The manuscript is approved by all authors for publication.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.121653.

Data Availability

Data will be made available on request.

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