Physiological mechanisms and adaptation strategies of *Lactuca sativa* L. in response to *Olea europaea* L. and *Ficus carica* L. allelochemicals

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A B S T R A C T

Agro-industrial wastes of *Ficus carica* L. and *Olea europaea* L. represent great sources of bioactive phenolic compounds that would be actively involved in sustainable development. Most of these wastes possess a valuable source of phytotoxic compounds that would be used as potential bioherbicides, but their function and mechanisms of action in cultivated crops remain far to be understood. In this study, we investigate the biochemical and physiological mechanisms of action of fig and olive allelochemicals extracts in lettuce as a model plant for weed species studies. Results revealed that these allelochemicals triggered an oxidative stress through cell membrane damage in lettuce roots and leaves, which was mitigated by various adaptive responses. Therefore, an intricate defense system was implicated by the increase of enzymatic and non-enzymatic antioxidants in lettuce tissues. This adaptive physiological response was highly correlated with the regulation of the phenylpropanoid pathway through the distinguished activation of phenylalanine ammonia-lyase by 98% and phenolic accumulation by 85% under olive and fig leaves aqueous extracts. The outcomes of this study will help understanding the response of cultivated crop to fig and olive phenolic compounds that can be selective in their actions, or the plants can be selective in their responses.

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1. Introduction

Climate change affects seriously the global food security for future generations in the entire globe (Campbell et al., 2016; Leisner, 2020). It has many negative facets, including changes in the plant's immune system, and could influence plant development (Munné-Bosch and Alegre, 2004). Therefore, plants have evolved several alternative defense strategies to overcome stress constraints through different metabolic processes (Bezemer and van Dam, 2005; Ladhari et al., 2014). Many of the reported studies have shown that the allelochemicals possess the major significance to counterbalance abiotic and biotic stresses, and subsequently involved in the adaptation of plant species (Ballhorn et al., 2009; Ladhari et al., 2020a). Some allelochemicals have multiple functions with impressive biological activities extending from antimicrobial, insecticidal, and herbicidal properties to highly important pharmaceutical activities (Stockigt et al., 1995; Irakli et al., 2018; Dayan and Duke, 2020). Despite their ecological importance, few studies have concerning their mechanisms of action or their response strategies by plants against different stress conditions (Bais et al., 2006; Ladhari et al., 2014). They presented incomparable structural diversity that could be attributed to their unique mode of action. Although this chemical diversity is slightly overlapped with synthetic herbicides, that could provoke approximately 20 modes of action (Duke, 2012; Dayan and Duke, 2020). Many studies have explained that the mode of action of allelochemicals interferes with several physiological cytological and biochemical processes in target species (Rasmann and Agrawal, 2009; Ladhari et al., 2014). Most of the discovered allelochemicals have been investigated to cause oxidative damage in different plant species, and subsequently trigger the antioxidant mechanism through a significant increase in free radical levels (Weir et al., 2004; Ladhari et al., 2014). In response to the overproduction of reactive oxygen species (ROS) levels, the plant cell structure was destructed, which was highly correlated with the marked change in enzymatic antioxidant activities including superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) (Ladhari et al., 2020b). Many kinds of allelochemicals reduced significantly plant root and shoot growths, which were correlated with the radical inhibition of the cell division (Sanchez-Moreiras et al., 2008; Ladhari et al., 2014), resulting depolarization of the root cell membrane thereby increasing the membrane permeability (Weir et al., 2004). The increase in membrane

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permeability and the decline in defensive abilities of plants in response to allelochemicals stress could affect the distribution in photosynthetic rate and the stratiform structure of the chloroplasts (Peng et al., 2004). In this context, some studies were performed to understand the response of target species to several allelochemicals extracts, which could be related to plant species and environmental factors.

Fig (*Ficus carica* L.) and olive (*Olea europaea* L.) trees are widely cultivated around the Mediterranean areas (Flaishman et al., 2008; Fao, 2013) and have high pharmaceutical, economic and ecological values (Amessis-Ouchemoukh et al., 2017; Ladhari et al., 2020c, 2021). However, their agro-industrial wastes are discarded as useless, which may cause serious environmental issues (Sud et al., 2008). Recently, alternative waste management strategies are investigated to obtain value-added products (Arun et al., 2020; Bharathiraja et al., 2020). Thus, according to our recent studies (Ladhari et al., 2020c, 2021), we have found that the agro-industrial wastes of olive mill wastewater and fig leaf aqueous extracts induced the main phyto-toxic effect on target species, which were suggested to be used for weeds management. This inhibitory effect was attributed to the occurrence of various levels of active allelochemicals in their extracts, especially the phenolic compounds that were highly correlated with the growth parameters of target species. The mechanism of action of these compounds may either be beneficial or detrimental to the plant development process. But the real wonder is how these compounds can suppress the growth and development of other plants that are until now relatively not clear. The study under scrutiny furnishes new insights into physiological and biochemical mode of action of fig and olive agricultural wastes extracts in lettuce, which is the most used model for weeds in allelochemicals studies because of its high sensitivity (Macías et al., 2000; Lotina-Mennsen et al., 2006; Ladhari et al., 2014, 2020c). Indeed, the most important consideration in selecting or emerging phytotoxic bioassay is the choice of the target species to reveal the specificity and the selectivity of the allelo-chemicals. Thus, to a deeper understanding of the allelochemicals mechanisms of fig and olive agricultural wastes in receptor plants may ultimately a perspicuous basis for a new mode of action design for safe and effective bioherbicides.

2. Material and methods

2.1. Plant culture and treatment assays

The treatment assays were investigated on lettuce which is the most widely used model species for allelochemical studies and was selected because of its fast germination and homogeneity (Macías et al., 2000; Lotina-Mennsen et al., 2006; Ladhari et al., 2014, 2020c). The cultivation and treatment of lettuce plants were assessed according to Ladhari et al. (2014).

The extracts were prepared from fig Khartoumi cultivar (leaf and twig) and olive wastes (olive mill wastewater (OMWW), leaves and olive mill solid waste (OMSW)) by soaking 30 g of each of the grinded dried plants materials in 100 mL of sterilized distilled water for 24 h. The aqueous extracts were filtered through a double layered muslin cloth followed by Whatman No. 1 filter paper and then passed through 0.22 μm micro-filter pore size to remove bacteria. The solu-tions form fig leaf and twig of Khartoumi cultivar were prepared at the concentration of 7.50 g/L and 8.75 g/L, respectively. The fig culti-var has been chosen because of its significant phytotoxic effect on let-tuce growth compared to the other tested cultivars (Ladhari et al., 2020c). While the aqueous extracts of OMWW, leaves and OMSW were respectively prepared at 2, 15 and 28.5 g/L (Ladhari et al., 2021). According to our previous studies (Ladhari et al., 2020c, 2021), the working solutions were prepared at the estimated IC$_{50}$ values.

On the other side, the uniform lettuce seedlings of seven-day-old were grown in hydroponic system containing a diluted Hoagland's medium (1:8 (w/w)) in a controlled greenhouse (16 h light/8 h dark at 20 °C). After three months of culture, the lettuce plants were treated with the prepared solutions. After three days of treatments, the plants were collected and divided into leaves and roots, then con-served at −80 °C until use for the determination of different parame-ters.

2.2. Metabolic activity

Fresh leaves and roots from treated and control lettuce seedlings (100 mg) were incubated for 4 h at 37 °C in 5 mL of triphenyl tetrazo-lium chloride (TTC) solution (0.2%, pH=7) in the dark. Then, 0.5 mL of sulfuric acid (1 M) was added in each sample. The different samples were washed with distilled water and quickly dried between filter, and subsequently ground in a mortar containing 3.5 mL of ethyl ace-tate in ice. The formazan content was measured at 485 nm and deter-mined according to Sampietro et al. (2006).

2.3. Membrane integrity

2.3.1. Electrolyte leakage

Electrolyte leakage (EL) was estimated according to Lutts et al. (1996). The different samples (roots and leaves) from con-trol and treated fresh lettuce were homogenized in tubes containing 25 mL of distilled water at room temperature in dark. Then, the initial electrical conductivity (L_1) of the solution, where the samples were immersed, was measured after 24 h using a digital conductivity meter. The samples were then autoclaved at 121 °C for 20 min in order to burst cell walls and liberate all electrolytes, and then cooled down to 25 °C, subsequently, incubated again in distilled water as indicated previously. After 24 h, a last conductivity reading (L_2) was obtained. The electrolyte leakage (EL) was measured as follows:

$$EL\,(\%) = (L_1/L_2) \times 100.$$

2.3.2. Lipid peroxidation

The amount of malondialdehyde (MDA) was measured to reveal the lipid peroxidation (Alia et al., 1995). Frozen samples were mixed with phosphate buffer 67 mM and polyvinylpolypyrrolidone (PVP) (1:1 (w/v)). Then, the solution was centrifuged at 2000 × g for 15 min at 4 °C, the obtained supernatant was equally added to the prepared mixture of thiobarbituric acid (0.5% (TBA)) of and trichloro-acetic acid (20% (TCA)), subsequently was heated for 10 min at 90 °C. The mixture was then centrifuged again, and the absorbance of the obtained supernatant was measured at 532 nm in order to determine the MDA content (nmol/g FW) (Doblinski et al., 2003).

2.4. Proline content

The powder of roots and leaves of lettuce (10 mg DW) were mixed in 3% (w/v) of sulphosalicylic acid (1.5 mL). The centrifuged solution was mixed equally with glacial acetic acid and ninhydrin reagent for 1 h at 100 °C. The products were extracted with 2 mL of toluene, and the absorbance was determined at 520 nm from the decanted upper phase. The proline (Pr) content was revealed through Bates et al. (1973) method.

2.5. Phenylalanine and tyrosine ammonia lyase activities

These activities were determined according to Ladhari et al. (2014). The samples were mixed with 20 mL of buffer borate (0.1 M), and then centrifuged at 15,000 g for 10 min at 5 °C. The supernatant was mixed with 1 mL of ʟ-phenylalanine (50 mM) for PAL assay, while ʟ-tyrosine (10 mM) for TAL assay. Then, the absorbances were measured at 270 nm and 333 nm, respectively.

The absorbances were determined again after incubation of the reactions during 90 min at 40 °C.

2.6. Estimation of photosynthetic pigments

The contents of carotenoids and chlorophylls a and b were revealed according to the protocol of Lichtenthaler and Wellburn (1983) with slight modification. The fresh leaf (100 mg) was homogenized with 80% acetone (5 mL), and after filtration, the absorbance was measured at 663 and 645 nm, respectively, for chlorophylls a and b; and 440 nm for carotenoids.

2.7. Biochemical responses

2.7.1. Total phenolics content

Fresh samples (1 g) were extracted with 80% methanol (10 mL) at room temperature. The prepared extract was then centrifuged for 10 min at 3000 × g. One hundred microliter of the supernatant was mixed with Folin-Ciocalteau reagent (500 μL). The mixture was added to 400 μL of 7.5% sodium bicarbonate, remained for 90 min at 30 °C, and then the total phenolic content was determined at 765 nm (Sineiro et al., 1996).

2.7.2. Total flavonoids content

The fresh lettuce roots or leaves (1 g) were extracted with 80% aqueous methanol (10 mL) and then centrifuged at 2000 × g for 10 min. The supernatant (0.5 mL) was mixed with the same volume of 2% aluminum chloride (AlCl$_3$). After 30 min of incubation in the obscurity, flavonoid content was revealed at 430 nm (Quettier et al., 2000).

2.7.3. Total tannin content

The previous prepared extracts form lettuce roots and leaves were mixed with 1% vanillin (3 mL) and sulfuric acid (0.5 mL). The absorbance of the mixture was revealed at 500 nm, and the total tannin was measured through the described method by Hagerman and Butler (1978).

2.8. Measurement of hydrogen peroxide (H$_2$O$_2$)

Hydrogen peroxide content in the treated leaves and roots of lettuce were measured using the protocol described in Velikova et al. (2000). Treated leaves were collected after 3 days, extracted with 5 mL of trichloroacetic acid (0.1%, w/v) in an ice bath, and the mixture was then centrifuged for 15 min at 12,000 × g. The supernatant (0.5 mL) was mixed with 1 mL of potassium iodide (1 M) and 0.5 mL phosphate buffer (pH 7). The absorbance of this mixture was measured at 390 nm, and the hydrogen peroxide content was expressed in nmol/g.

2.9. Preparation of enzyme extract

The enzymatic extraction was prepared from the frozen roots and leaves tissues of 2 g (from each treatment or control) through cold homogenization in 10 mL of sodium phosphate buffer (0.1 M). The obtained mixture was centrifuged for 15 min at 4 °C, then the supernatant was collected and used, as enzyme extract, for superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) analyses.

2.9.1. Activities of antioxidant enzymes

Superoxide dismutase was measured by using the method of Beauchamp and Fridovich (1971). The prepared enzyme extract (500 μL) was homogenized with 4 mL of a mixture of nitro blue tetrazolium chloride (NBT; 63 μM), methionine (13 mM), ethylene diamine tetra acetic acid (EDTA; 0.1 mM), riboflavin (13 μM), and sodium

carbonate (0.05 M). The reaction was remained successively under light and dark conditions for 20 min, and then the absorbance was measured at 560 nm. Catalase activity was assessed as per the method of Cakmak and Marschner (1992). The enzyme extract (200 μL) was mixed with 2 mL of the prepared solution (25 mM phosphate buffer (pH 7.0) and 10 mM H$_2$O$_2$), then the activity was calculated at 240 nm. The ascorbate reductase (APX) was examined by the explained method of Nakano and Asada (1981). The enzyme extract of 200 μL was mixed with 2 mL of the solution of phosphate buffer (25 mM), EDTA (0.1 mM), ascorbate (0.25 mM), and H$_2$O$_2$ (1.0 mM), the enzyme activity was revealed at 290 nm. All the determined antioxidant enzyme was expressed as enzyme units g/FW.

2.10. Statistical analysis

The laboratory bioassays in a complete randomized design were performed using IBM SPSS Statistics 20.0, to evaluate the mechanisms of action of fig and olive aqueous extracts over the control values. Experimental data were subjected to one-way analysis of variance (ANOVA) and a posthoc LSD tests, to determine significance differences among mean values at the probability level of 0.05. The data obtained for all parameters in accordance with all treatments were subjected to Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) using SPSS 20.0 software.

3. Results

3.1. Membrane integrity

The membrane damage of lettuce in response to fig and olive wastes aqueous extracts was assessed through relative electrolyte leakage and malondialdehyde production (Fig. 1). The electrolyte leakage was markedly influenced in lettuce roots more than in leaves under all treatment cases. The degree of cell membrane injury was revealed by an increase of the electrolyte leakage, which was
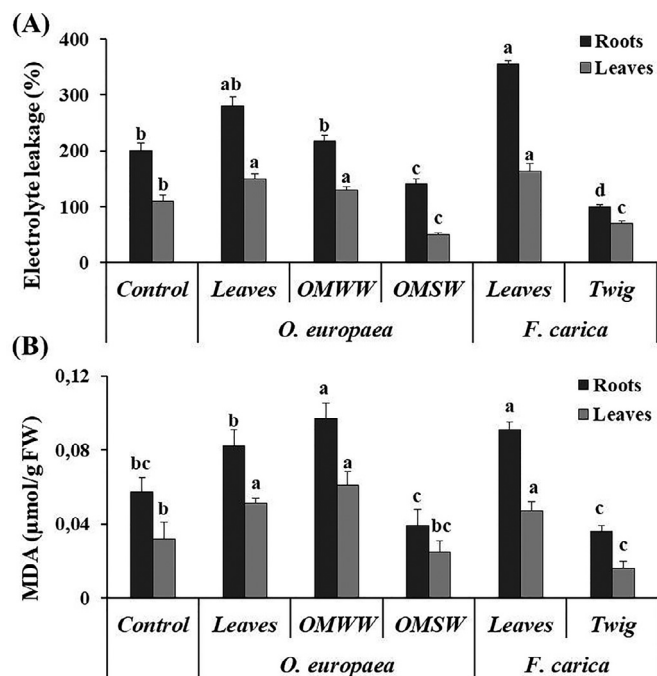
Fig. 1. Electrolyte leakage (%) (A) and Malondialdehyde (MDA) content (B) in lettuce roots and leaves in the absence (control) or the presence of olive (leaves (15 g/L); OMWW (2 g/L); OMSW (28.5 g/L)), and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts. The bars on each column show standard error. Values ($N = 5 \pm$ S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

simulated in lettuce roots by 76.7% and 48.2% in leaves under fig leaves aqueous extract. While the olive leaves and OMWW aqueous extracts improved slightly the ion leakage by an average stimulation of 25% in lettuce root and leaf compared to the control. Therefore, the depicted stimulation could trigger dramatic damage in the plant cells membranes. Contrariwise, the OMSW and twig of fig extracts did not affect the membrane permeability of lettuce organs, which was demonstrated by an average decrease of 43% compared to the control (Fig. 1A).

On the other side, the lettuce cells membranes displayed great sensitivity to allelochemicals stress present in fig and olive wastes aqueous extracts compared to control (Fig. 1B). In fact, OMWW aqueous extract induced the highest level of MDA level in lettuce organs by an average stimulation of 80.4%, while a medium stimulation of 52.4% was revealed under olive and fig leaves treatments. However, the twig and OMSW reduced the amount of MDA level in lettuce by an average decrease of 43.4% and 26.7%, respectively. Consequently, this reduction suggests the ability of lettuce to maintain the integrity of their membranes (Fig. 1B).

3.2. Hydrogen peroxide content

According to our recent studies (Ladhari et al., 2020c, 2021), we have speculated that the inhibitory effect of the fig and olive aqueous extracts could be mediated induction of oxidative stress and ROS generation in target species. To address this possibility, we next determined the extent of ROS generation quantitatively in terms of hydrogen peroxide content in lettuce roots and leaves tissues under control condition or after treatments with fig and olive aqueous extracts. As shown in Fig. 2, OMWW and fig leaves aqueous extracts raised the H_2O_2 content in lettuce roots more than in leaves by an average increase of 5.75-fold and 1.46-fold ($p \leq 0.05$), respectively. In addition, the olive leave extract enhanced the H_2O_2 content in roots lettuce by 3.63-fold and 1.84-fold in leaves lettuce compared to the control. On the other side, the lowest accumulation of H_2O_2 was depicted in lettuce leaves under the OMSW and fig twig aqueous extracts by an average level of 3.26 nmol/g FW and 4.74 nmol/g FW, respectively, compared to the control 3.66 nmol/g FW (Fig. 2).

3.4. Activation of antioxidant defensive enzymes in lettuce

The oxidative stress and ROS generation were rapidly stimulated the enzymatic and non-enzymatic antioxidant defense components in lettuce plants. In this study, the antioxidant enzyme activities, including SOD, CAT, POD, and APX, were significantly increased in lettuce under fig and olive aqueous extracts as compared to untreated

plants (Fig. 3). These enzymes were more prominently influenced by OMWW and leaf fig aqueous extracts than the other treatments, and thus were consistent with their phytotoxic potentials as obtained in our previous reports (Ladhari et al., 2020c, 2021).

The SOD activity was significantly enhanced in lettuce roots more than in leaves by fig leaves and OMWW aqueous extracts with an average stimulation of 118.4% and 82.7%, respectively. Regarding the other treatments, the SOD activity was moderately increased (an average stimulation of 53%) in lettuce plants, but the lowest activation of 3.7% was depicted by OMSW aqueous extracts in lettuce leaves.

The activities of ascorbate peroxidase and catalase were markedly enhanced by 123.5% in lettuce under fig leaves aqueous extract. The stress response induced by OMWW aqueous extract was also
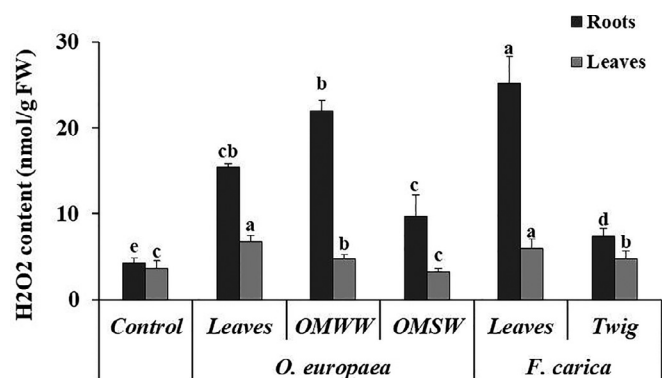
Fig 3. Effect of olive (leaves (15 g/L); OMWW(2 g/L); OMSW (28.5 g/L)) and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts on the activities of different enzymatic antioxidants of SOD, CAT, POD, and APX in lettuce roots and leaves. The bars on each column show standard error. Values ($N = 5 \pm$ S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

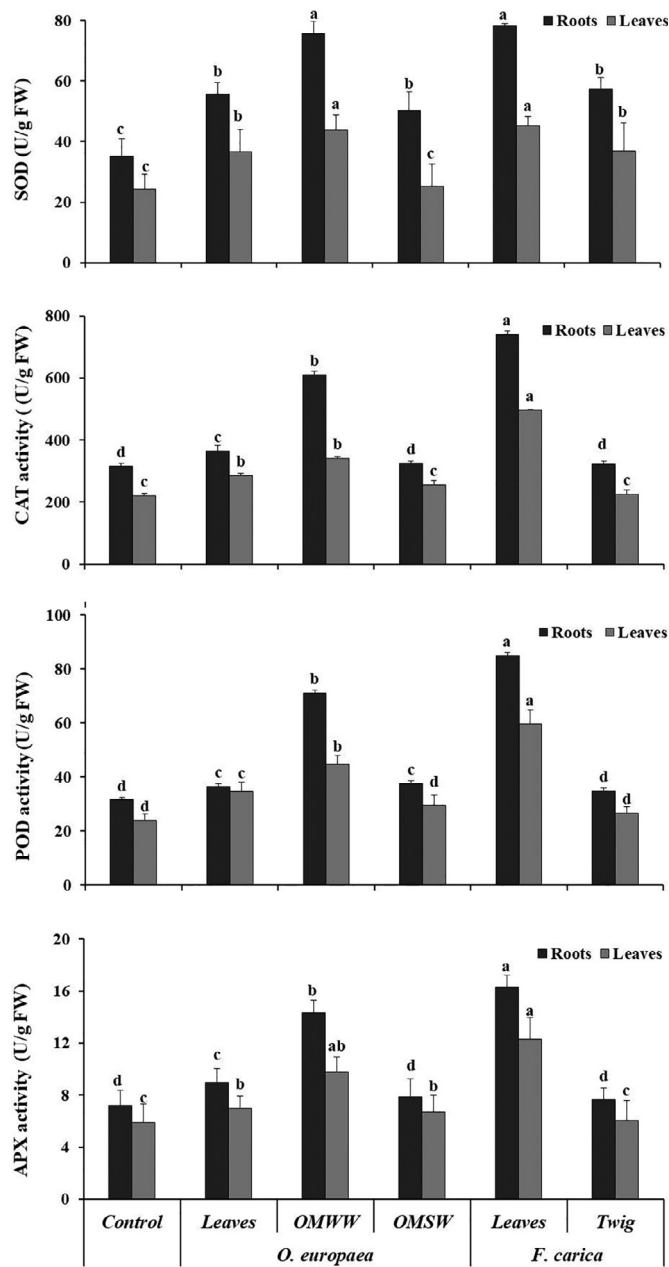**Fig. 2.** Effect of olive (leaves (15 g/L); OMWW(2 g/L); OMSW (28.5 g/L)) and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts on hydrogen peroxide production in roots and leaves of lettuce determined three days after treatments. The bars on each column show standard error. Values ($N = 5 \pm$ S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

depicted in lettuce roots and leaves by an average stimulation of 95.8% and 60.2%, respectively. While a slighter stimulation of 21.79% was induced by olive leaves extract and a lesser activation by 6.89% was revealed under OMSW and fig twig aqueous extracts.

Concerning the peroxidase activity, the fig leaves aqueous extract provoked the highest stimulation of 159.3% followed by OMWW (105.8%) and olive leaves (44.7%) aqueous extracts. While the lowest stimulation was induced by the fig twig and OMSW extracts with an average stimulation of 16% (Fig. 3).

3.5. Proline

The proline plays an important role as non-enzymatic antioxidant compounds for the detoxification of the harmful effects of ROS (Fig. 4). The oxidative damage was reflected by the raised level of proline in lettuce under all the treatments. Indeed, the highest accumulation was revealed in response to fig leaves aqueous extract with the level of 19.98 μmol g/DW and 17.61 μmol/g DW in lettuce leaves and roots, respectively, compared to the control (7.3 μmol g/DW and 11.23 μmol/g DW). However, less stress induction through the remained treatments, which demonstrated an average accumulation of proline by 15.15 μmol g/DW in lettuce roots more than in leaves (11.33 μmol g/DW) (Fig. 4).

3.6. Effect of olive and fig aqueous extracts on chlorophylls and carotenoids contents

The result showed a significant reduction in lettuce chlorophyll levels in response to fig and olive aqueous extracts (Fig. 5A). Parallel to chlorophyll a and chlorophyll b, there was a significant decrease in total chlorophyll of 53% in response to OMWW. Despite this considerable decrease, the slight effect was persuaded by the leaves of fig and olive aqueous extracts with an average reduction of 26.79%, while the lowest reduction was noted with OMSW and twig aqueous extracts (8.92%). Conversely, the carotenoid level increased significantly in response to olive aqueous extracts compared to the control (Fig. 5B). The greatest increase was achieved by OMWW with stimulation of 101.69%, while a lesser effect was recorded by 23.7% under OMSW and olive leave aqueous extract. However, fig leaves aqueous extract induced inhibition of 25.5% more than twig extract (9.6%).

3.7. Cell metabolic activity

The results showed that the formazan content reflects the mitochondrial metabolic activity in plant cells (Fig. 6). The formazan

production was significantly decreased in lettuce roots more than in leaves in response to fig and olive aqueous extracts compared to the control. It was noted that the fig aqueous extracts were depicted very stressful treatments compared to olive, as they induced a significant reduction of 71.33% and 55.43% in lettuce roots and leaves, respectively. The OMWW extract revealed a medium reduction of 53.7% in lettuce, while the OMSW and olive leaves aqueous extract possessed an average reduction of 22.65%.

3.8. Phenylalanine and tyrosine ammonia lyase activities

The phenylalanine and tyrosine ammonia-lyases activities in lettuce were markedly influenced after treatments with fig and olive aqueous extracts (Fig. 7). It was noted that the leaves of olive and fig aqueous extracts induced the greatest effect on the PAL activity by an average stimulation of 135.68% and 67.09% in lettuce roots and leaves, respectively. This enhancement in PAL activity was induced also by OMWW extracts by a respective stimulation of 61.12% and 28.55%. Despite the depicted enzymatic activation, the activity of PAL was notably declined in response to OMSW and fig twig aqueous extracts with an average reduction of 31.66% in lettuce (Fig. 7A).

The TAL activity was less influenced in comparison with PAL, it was more stimulated by olive aqueous extracts than the fig extracts (Fig. 7B). The olive leaves and OMWW extracts induced the TAL activity by an average stimulation of 104.63% and 57.79% in lettuce leaves and roots, respectively. Although, this activity was slightly enhanced in lettuce tissues by 16.4% in response to the fig aqueous extracts (Fig. 7B).

3.9. Phenylpropanoid compounds production in lettuce

The phenylpropanoid metabolism was markedly stimulated in lettuce roots more than in leaves by the fig and olive aqueous extracts (Table 1). Consequently, the total phenolic content was increased by 161.8% in lettuce roots under OMWW aqueous extracts, while the leaves of olive and fig extracts induced an average accumulation of 113%. Indeed, OMSW and fig twig aqueous extracts improved the total phenolic content by 80%. The total phenolic content in lettuce leaves was highly produced by olive leaves extract with a stimulation reached 149.3%. This level was moderately accumulated by OMWW and fig leaves (67%), but a slight accumulation of 11.5% was induced by OMSW and fig twig aqueous extracts. Regarding that, flavonoid content was obviously increased by 264.5% in lettuce under OMWW, while the leaves of olive and fig aqueous extracts induced respectively an accumulation of 126.1% and 186.3%. Similarly, the
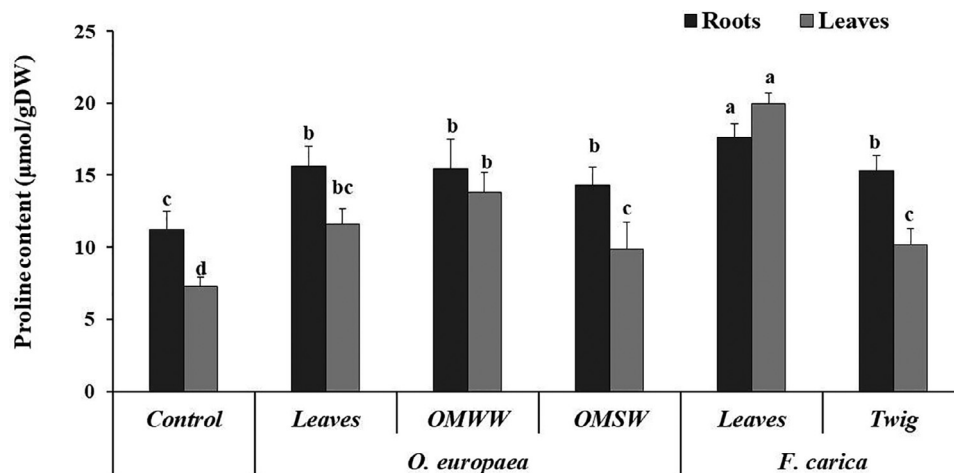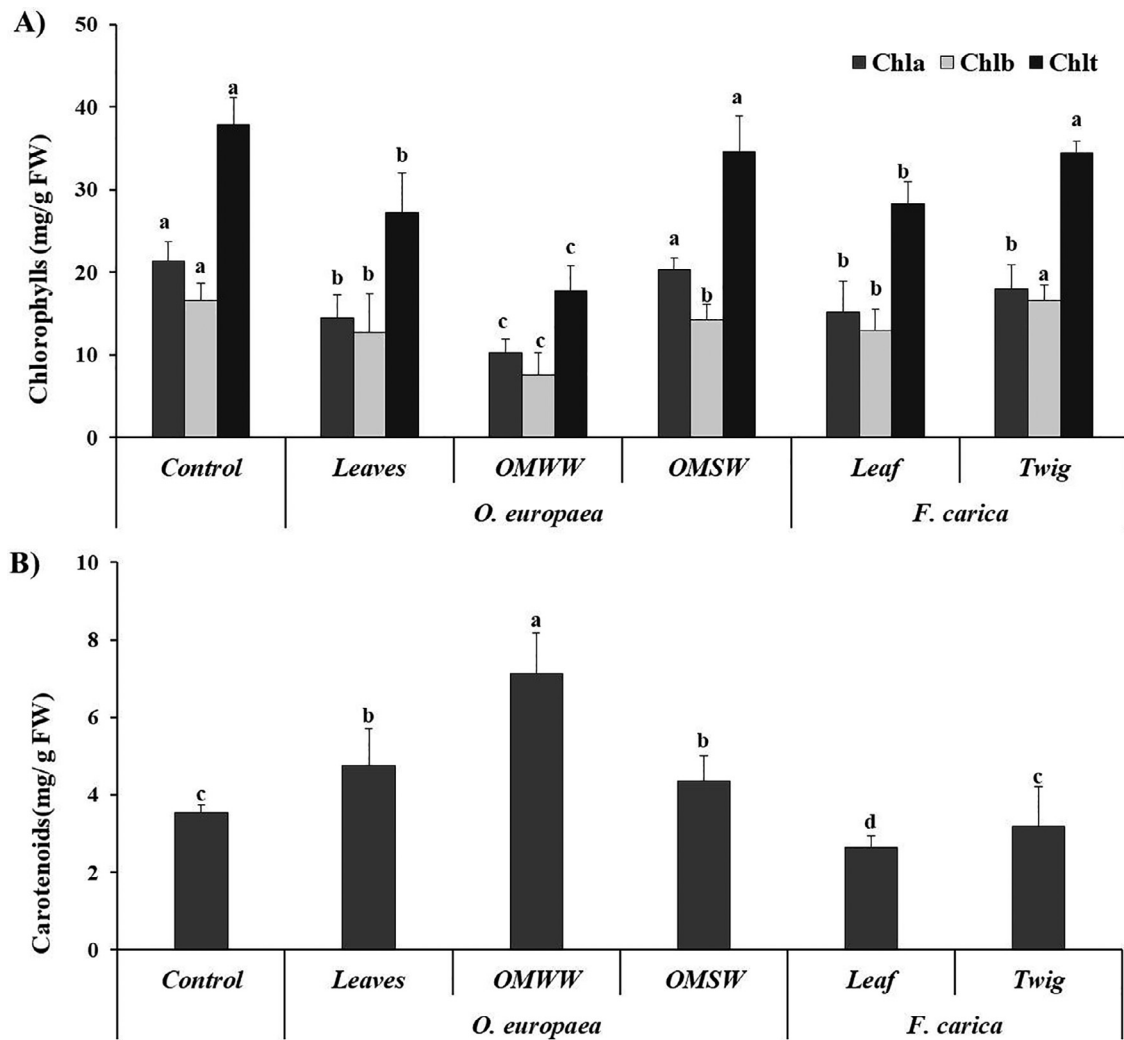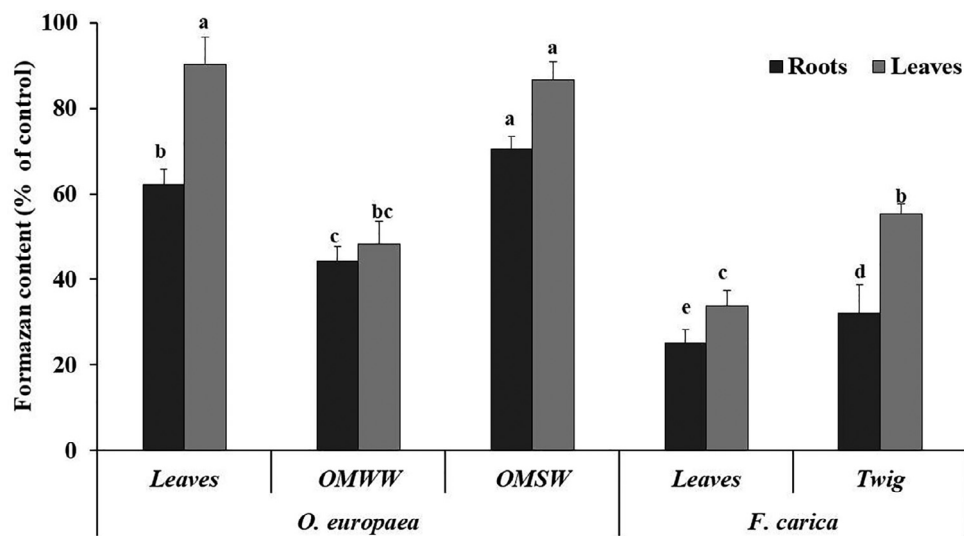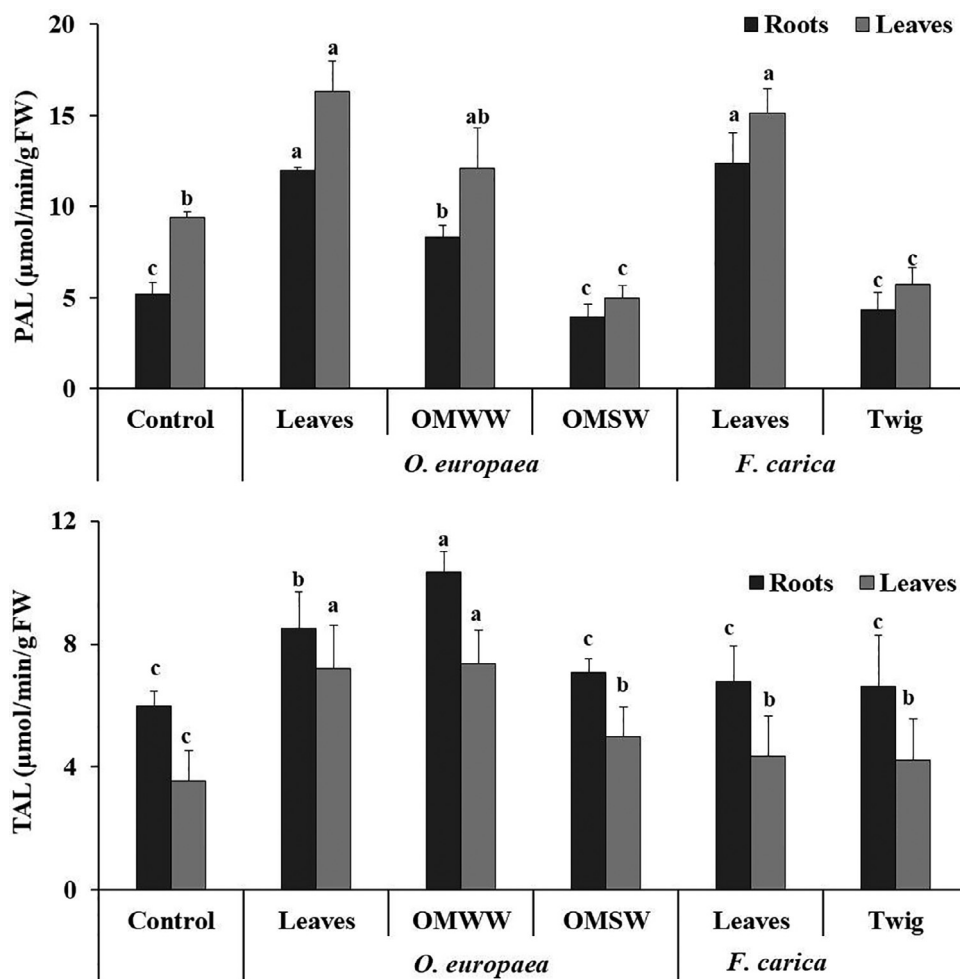

Fig. 4. Proline content (μmol /g DW) in lettuce tissues under olive (leaves (15 g/L); OMWW (2 g/L); OMSW (28.5 g/L)) and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts treatments. The bars on each column show standard error. Values ($N = 5 \pm$ S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

Fig. 5. Effect of olive (leaves (15 g/L); OMWW(2 g/L); OMSW (28.5 g/L)) and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts on chlorophylls (A) and carotenoids (B) contents (mg/g FW) in lettuce leaves. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters in columns indicate significant differences among treatments at P < 0.05 (LSD test).

Fig. 6. Formazan content (% control) in lettuce tissues treated with olive (leaves (15 g/L); OMWW(2 g/L); OMSW (28.5 g/L)) and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters in columns indicate significant differences among treatments at P < 0.05 (LSD test).

Fig. 7. Impact of olive (leaves (15 g/L); OMWW (2 g/L); OMSW (28.5 g/L)) and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts on phenylalanine ammonia-lyase (A) (PAL) and tyrosine ammonia-lyase (TAL) (B) activities (% of control) in lettuce roots and leaves. The bars on each column show standard error. Values ($N = 5 \pm$ S.E.). Different letters in columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

condensed tannins were significantly stimulated by 112.3% under OMWW and fig leaves aqueous extracts. However, this content was slightly improved by 34.1% and 28.4% under the fig twig and OMSW aqueous extracts, respectively (Table 1).

3.10. Correlation analysis

The correlation coefficients among the biochemical and physiological parameters analysed by Pearson's correlation are described in Fig. 8. It was noted that the parameters of membrane integrity (EL and MDA) and the antioxidant defense enzymes (H_2O_2, SOD, CAT, POD, and APX) showed significant positive correlations with each

other. In fact, the greatest positive correlation ($p < 0.01$) was observed among the antioxidant enzymes. These enzymes had significant positive correlations with the proline, flavonoids, and condensed tannins levels, but a negative correlation was recorded with the total chlorophyll (chlt), which was significantly displayed with POD and APX (-0.61, $p < 0.05$). The PAL and TAL enzymes were positively correlated with all the parameters, except with chlt, which was negatively correlated with each of them. Among the biochemical parameters, the phenolic and flavonoid contents revealed positive correlation with all physiological parameters, but significantly correlated with chlt and formazan levels (-0.908; $p < 0.01$).

Table 1
Total phenols (mg GAE/g FW), flavonoids (mg QE/g FW) and condensed tannins (mg TAE/g FW) contents under treated lettuce roots and leaves with olive (leaves (15 g/L); OMWW (2 g/L); OMSW (28.5 g/L)) and fig (leaf (7.5 g/L); twig (8.75 g/L)) aqueous extracts.

		Roots			Leaves		
		Total phenols	Flavonoids	Condensed tannins	Total phenols	Flavonoids	Condensed tannins
	Control	1.52 ± 0.09^c	0.085 ± 0.011^d	0.19 ± 0.012^d	2.36 ± 0.16^c	0.073 ± 0.001^d	0.042 ± 0.012^b
O. europaea	Leaves	3.32 ± 0.05^a	0.201 ± 0.037^b	0.37 ± 0.012^b	3.76 ± 0.21^b	0.150 ± 0.001^c	0.076 ± 0.012^a
	OMWW	3.98 ± 0.12^a	0.309 ± 0.001^a	0.43 ± 0.003^a	4.09 ± 1.03^a	0.256 ± 0.002^a	0.081 ± 0.001^a
	OMSW	2.66 ± 0.04^b	0.095 ± 0.021^d	0.23 ± 0.021^c	2.64 ± 0.01^c	0.083 ± 0.001^d	0.057 ± 0.001^b
F. carica	Leaves	3.15 ± 0.21^a	0.262 ± 0.002^b	0.43 ± 0.003^a	3.79 ± 0.25^b	0.193 ± 0.003^b	0.084 ± 0.001^a
	Twig	2.81 ± 0.31^b	0.105 ± 0.002^c	0.27 ± 0.023^c	2.62 ± 0.31^c	0.084 ± 0.002^d	0.053 ± 0.005^b

Different letters indicate significant differences between groups ($P < 0.05$); Values are given as mean $\pm$ SD of three assays ($n = 5$).
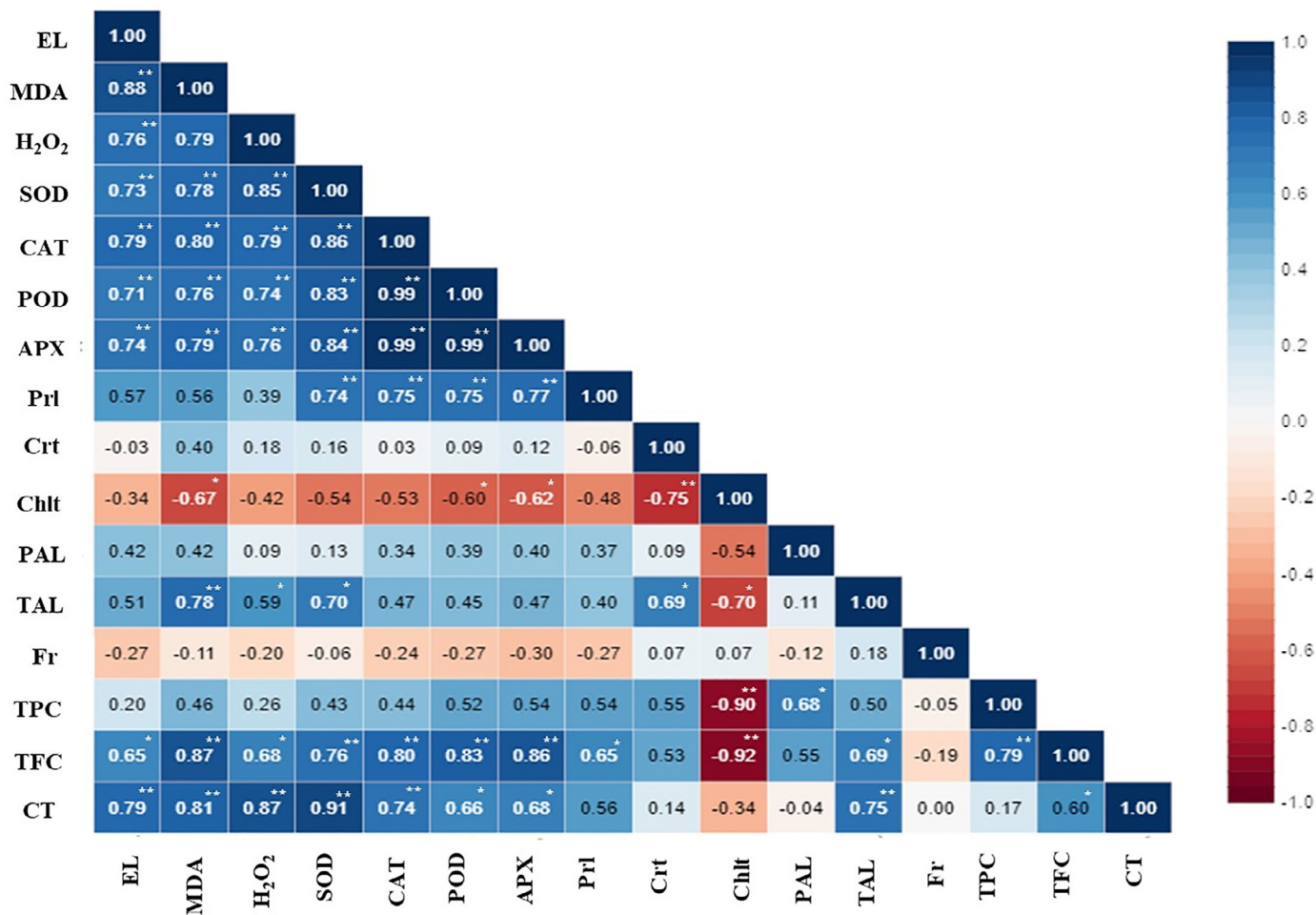
Fig. 8. Pearson's correlation coefficients among physiological and biochemical parameters in lettuce grown under fig and olive aqueous extracts. Each square indicates the Pearson's correlation coefficient of a pair of parameters. Correlation was significant at $^*p < 0.05$; $^{**}p < 0.01$. The deeper the color is, the greater the correlation coefficient.

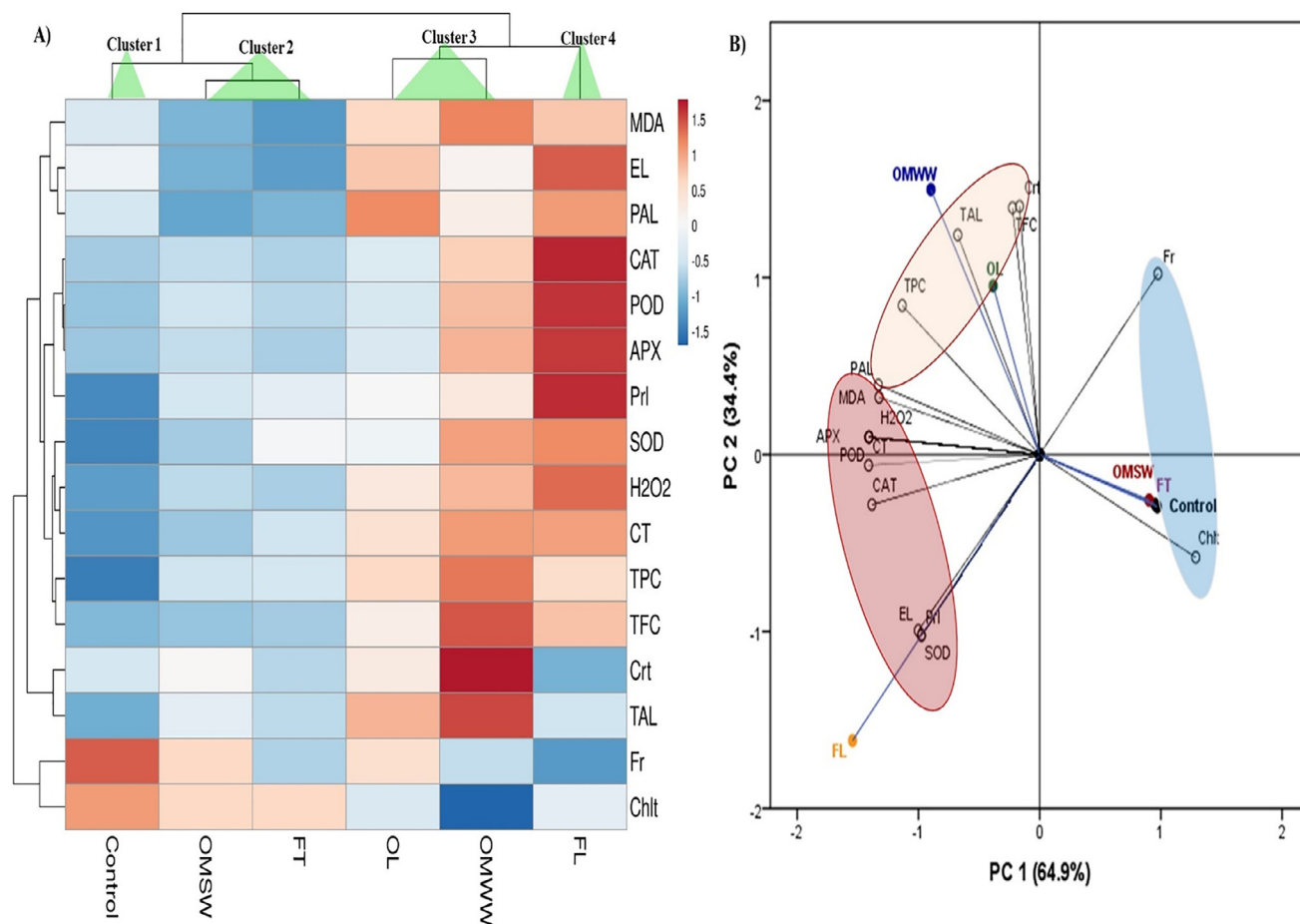### 3.11. Heat map and principal components analysis

Hierarchical clustering heat map and principal component analysis (PCA) were performed to understand treatment-variable relationships in treated plants (Fig. 9). The heat map identified four clusters and divided the fig and olive extracts differently compared to the control, due to their physiological and biochemical dissimilarity in response to the treatments. For instance, the control was completely separated from all the treatments but was grouped with one cluster including OMSW and fig twig extracts, which persuaded similar effects on plant biochemical and physiological parameters between each other. On the other side, the olive leaves and OMWW extracts were grouped together in cluster 3 because of their similar negative effects. It was noted also that the OMWW was markedly separated from the control due to its lower and higher levels of Chlt and Crt, respectively. In comparison to that, fig leaves extract was clustered separately from the other two treatments of cluster 3 due to its higher CAT, POD, APX, and Prl concentrations in lettuce plants, as well as its lower Crt and Fr levels. This cluster was completely separated from control by the highest level of Prl and H_2O_2 compared to the control (Fig. 9A). The results of PCA were presented by a biplot in order to gain insight into the physiological responses of lettuce to different treatments (Fig. 9B). The first two principal components explained 64.9% and 34.4% of the data variability, respectively. Almost all physiological and biochemical parameters, including MDA, EL, H_2O_2, SOD, CAT, POD, APX, Prl, Crt, PAL, TAL, TPC, TFC, and CT were negatively related to PC1, while the Fr and Chlt were positively related to PC1. While the Crt and TFC were positively related to PC2. The points of different treatments were separated along PC1. The fig leaves extract presented great antioxidant and non-antioxidant, and an important damage in membrane integrity and higher CT level, but lower level of Fr, Crt and Chlt. It was noted that the treated lettuce with OMWW characterized by high concentration of Crt, TAL, and TFC but with the lowest level of Chlt. Finally, the OMSW and fig twig aqueous extracts presented a similar level compared to the control, but the fig twig revealed by a low level of Fr. While the olive leaves extract characterized by the PAL and TAL activities (Fig. 9B).

4. Discussion

In response to environmental stress, the defense system of the plant induces scavenge toxic molecules and repair cellular homeostasis. In our results we noted that the phytotoxic molecules present in olive and fig wastes aqueous extracts significantly influenced the stability of the membrane of lettuce leaves and roots by troubling the entire cellular structure, and therefore perturb the provided suitable environment for all physiological and biochemical processes that occur in the cytoplasm. This alteration could explain the previous observed phytotoxic effect of fig (Ladhari et al., 2020c) and olive (Ladhari et al., 2021) wastes aqueous extracts. This phytotoxicity was caused mainly by the depicted phenolic compounds in the fig and olive extracts. The identified phenolic compounds belong mainly to different groups such as secoiridoids, phenyl alcohols, phenolic acids, phenylethanoid glycoside, and flavonoids. Among the identified

Fig. 9. Cluster heat map (**A**) and principal component loading plot and scores of principal component analysis (PCA) (**B**) of biochemical, physiological parameters of lettuce plants grown under olive (leaves "OL"; olive mill wastewater "OMWW"; olive mill solid waste "OMSW") and fig (leaves "FL"; twigs "FT") aqueous extracts. The Heat map was generated using the https://biit.cs.ut.ee/clustvis/ online program package with Euclidean distance as the similarity measure and hierarchical clustering with complete linkage. The variables included: EL= electrolyte leakage; MDA= lipid peroxidation; H_2O_2= hydrogen peroxide; SOD= Superoxide dismutase; CAT= catalase; POD= peroxidase; APX= ascorbate peroxidase; PAL= phenylalanine ammonia-lyase; TAL= tyrosine ammonia-lyase; crt= Carotenoids; chlt= Total chlorophyll; Prl= Proline; TPC= total phenol content; TFC= total flavonoid content; CT= Condensed tannins; Fr= formazan content.

phenolic compounds, ligstroside was detected in OMSW but was not noticed in the other olive extracts. While the hydroxytyrosol and oleuropein were the main components in olive leaf and OMWW extracts, respectively (Ladhari et al., 2021). Moreover, the phenolic compounds such as the quercetin-3-O-rutinoside (rutin) and kaempferol 3-O-glucoside (astragalin) were identified as the major compounds in fig Khartoumi cultivar of leaf and twig, respectively (Ladhari et al., 2020c). The main identified phenolic allelochemicals in fig and olive extracts may influence the biochemical and physiological processes of lettuce. The hypothetical scheme for the mode of action of fig and olive allelochemicals extant in their aqueous extracts is revealed in Fig. 10.

The oxidative markers (EL and MDA) were highly accumulated under fig leaves and OMWW aqueous extracts compared to the other treatments, and consequently affect the membrane integrity in lettuce roots more than in leaves. This positive correlation between EL and MDA was in accordance with several studies on other plant species (Ladhari et al., 2014; Cheng and Cheng, 2015). It was revealed that the cell membranes of cucumber and sorghum roots were influenced by allelopathic compounds, which was relative to lipid peroxidation determined as MDA content (Zeng et al., 2008). Some phenolic acids raised the MDA level in the roots of pea, radish, and maize (Gmerek and Politycka, 2011). The destructed membrane was revealed by the loss of electrolytes, and consequently provoked necrosis in lettuce roots (Ladhari et al., 2014). In contrast to fig leaves and OMWW extracts, fig twigs and OMSW did not affect the

membrane integrity, which was displayed by a decrease in the electrolyte leakage and MDA production, suggesting that lettuce roots and leaves had the ability to maintain membrane integrity and/or repair the membrane damage caused by the allelopathic stress. In fact, less lipid peroxidation (low MDA) leading to a greater ability to maintain membrane integrity (lower electrolyte leakage). The recorded decrease of MDA content in lettuce plants by OMSW and fig twig aqueous extracts could be explained by mitigation of allelopathic-induced oxidative stress through an increase of the antioxidant status to maintain membrane integrity. Indeed, the balance between generation and elimination of reactive oxygen species (ROS) plays an important role in resisting oxidative stress under allelopathic stress (Cheng and Cheng, 2015). The lipid peroxidation and enzymatic activity in soybeans were markedly correlated under phenolic extract of *Brassica napuse* (Haddadchi and Gerivani, 2009).

The increase of membrane permeability leads easily to the entrance of the allelochemicals into the cells and can raise their concentration in the cytosol, and subsequently trigger the antioxidant enzymatic activities. Many studies have shown that allelochemicals inhibit the antioxidant system activity with a significant increase of free radical levels. Free radicals damage membrane lipids, which are the most abundant macromolecules in the cell, and lead to the peroxidation of these molecules. As well as increasing the concentration of hydrogen peroxide in both leaves and roots of lettuce under all the tested aqueous extracts is the effect of olive and fig allelochemicals. The occurrence of the highest concentration of H_2O_2 in cell walls and
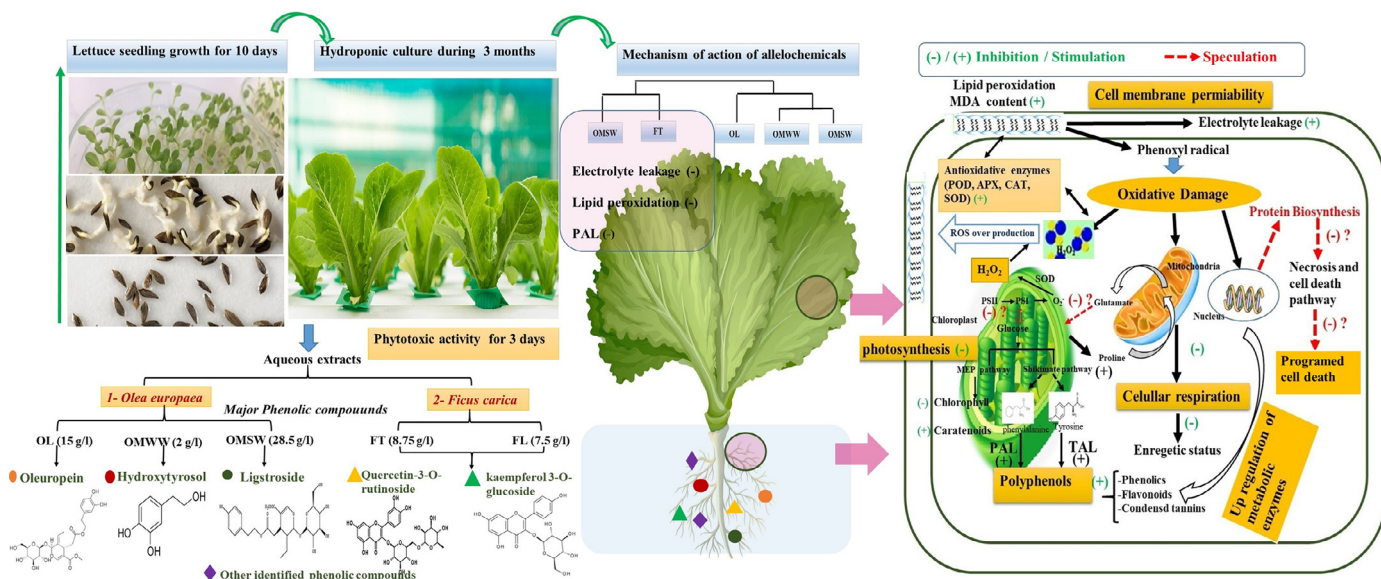
Fig. 10. Hypothetical and schematized of defense strategies adapted by lettuce in response to fig an olive allelochemicals. Interaction of the phenolic compounds with the polar part of the membrane induced the oxidative damage, which was triggered by an intricate defense system in lettuce.

along the plasma membrane suggested that both the cell wall and the plasma membrane may be the major source of H_2O_2 (Ranieri et al., 2003). Some phenolic compounds are the cofactor for specific enzymes, resulting an important morphological and physiological changes in target plants (Haddadchi and Gerivani, 2009). One of the most important enzyme complexes affected by the allelopathic phenomenon is the H^+-ATPase in the plasma membrane. The remarkable point is that impairment in the function of this enzyme ultimately causes a change in the physiological pH and affects the activity of other enzymes. According to Lee et al. (2004), a correlation was recorded between H_2O_2 level and H^+-ATPase activity in the isolated plasma membrane. Several natural plant products, such as sorgoleone from Sorghum spp (Hejl and Koster, 2004a), juglone from Juglans spp. (Hejl and Koster, 2004b), and prehelminthosporol from *B. sorokiniana* (Olbe et al., 1995) interfere with plant growth by inhibiting plasma membrane H^+-ATPase, which are essential in maintaining water uptake and cell turgor for plant growth and development.

Thus, it was noted that the allelochemical stress-induced excessive oxidative damage and lipid peroxidation in lettuce plants. To reduce the impact of the induced oxidative stress, plants have activated a complex system of enzymatic antioxidants (SOD, CAT, POD, and APX) which they were highly accumulated in plants under stress conditions (Farhoudi and Lee, 2013). In fact, antioxidants enzymes have different roles in protecting cells by maintaining membrane structures and are responsible for neutralizing the deleterious effects of ROS (Willekens et al., 1995). The recorded increase of antioxidant enzyme activities under allelochemical stress has been shown previously in other plant species (Oracz et al., 2007). In maize, the H_2O_2 content and the activity of CAT, SOD, and APX enzymes were increased under allelochemical stress of walnut husk (Ciniglia et al., 2015). The antioxidant enzyme activities, including peroxidase (POD) and catalase (CAT), were increased in cucumber after treatment by allelochemical *p*-hydroxybenzoic acid (*p*HBA) (Huang et al., 2020). Cruz-Ortega et al. (2007) showed that the allelochemicals released by endemic weed Sicyos deppei G. Don (Cucurbitaceae) caused oxidative damage through an increase in reactive oxygen species (ROS) and activation of antioxidant enzymes. According to Sakihama et al. (2002), plant phenolic compounds induced the formation of ROS and thus produce pro-oxidant compounds such as the phenoxy groups that could be detoxified through enzymatic and non-enzymatic reactions. The production of ROS was elucidated by

blocking the electron-carrying chain, and electrons become free, which react easily with O_2 to form superoxide (Tigre et al., 2015). The induced oxidative imbalance could assist in the phytotoxic phenomenon (Ladhari et al., 2020a, b), which was explained by the mitodepressive effect (Gaaliche et al., 2017).

On the other side, the enzyme activities were markedly inhibited or activated under allelochemicals stress in different plant tissues. PAL and/or TAL activation plays a fundamental role in plant adaptation to its environment and the two enzymes are involved in the biosynthetic pathway of phenylpropanoids in lettuce plants. It was noted that the olive and fig aqueous extracts influence the metabolic pathway through the regulation of PAL and TAL enzymes in roots and leaves of lettuce. Indeed, this result revealed that the activation of PAL and TAL depends on plant parts which are more triggered by olive than fig aqueous extracts. This finding was in agreement with Alqarawi et al. (2018), who revealed a significant increase of PAL activity in *Salsola villosa* (Chenopodiaceae) when exposed to aqueous extracts of *Rhazya stricta* (Apocynaceae). The improvement in PAL activity by the aqueous extracts may be involved in divergent allelochemical biosynthetic pathways. This amplification was markedly depicted in the roots of soybean in response to ferulic acid (Dos Santos et al., 2004). In contrast, Sato et al. (1982) revealed that the ferulic acid reduced the PAL activities in pea (*Pisum sativus)* and sweet potato (*Ipomea batatas*). The decline in PAL activity was detected also in our study under OMSW and fig twig aqueous extracts. The drastic fall in PAL activity could be explained by the involvement of TAL in phenolic compound synthesis (Dogbo et al., 2007). In our results, the TAL activity was more stimulated by all the treatment cases and was less influenced than the activity of PAL compared to control. Due to the few studies on these enzymatic activities, the PAL activity has an unclear contribution in allelochemical mechanisms that could be explained by its ability to act as a special enzyme or to receive tyrosine as a substrate. Besides that, the simultaneous activation of the two enzymes was correlated with increased production of phenylpropanoid compounds (Dogbo et al., 2012). Consequently, it is essential to uncover the metabolic biochemical processes in plants under allelochemical stress. According to our findings, the secondary metabolites were highly accumulated in lettuce roots more than in leaves in response to fig and olive wastes aqueous extracts. This inequality of accumulation could be explained by the translocation of active compounds from biosynthesis site to

storage site, while the important levels could be an indicator of high expression of genes and metabolic pathway for its biosynthesis in plant cells (Yang et al., 2018). The physiological process of lettuce was influenced by olive and fig allelochemicals, which was highly correlated with the increased levels of some phenolic compounds. In addition, the enhancement of the flavonoids contents in lettuce was markedly induced by OMWW and olive leaves aqueous extracts compared to the other treatments. A similar effect was noted by Garrido et al. (2012), who revealed that the olive-mill residue aqueous extract induced a significant inhibition on root length of sunflower, which was positively correlated with the accumulation of flavonoid components. The phytotoxic effect of brown seaweed (Ascophyllum nodosum) extracts increased the flavonoid content in cabbage (Lola-Luz et al., 2013). While the extent allelochemicals in leaves leachate of eucalyptus enhanced the total phenolic contents in sorghum and bean (Djanaguiraman et al., 2005).

Correspondingly, the proline level was induced significantly by OMWW aqueous extract in lettuce leaves more than in roots. This result agrees with Wang et al. (2009) who depicted the significant accumulation level of proline in Tagetes erecta treated with the leachate of Jatropha curcas. Similarly, this increase was recorded in sorghum under the effect of eucalyptus leaves leachate (Djanaguiraman et al., 2005), in wheat leaves treated with the aqueous extract of leaves of maize (Ibrahim et al., 2013), in Pisum sativum in response to phenolic compounds (Batish et al., 2007), and in the roots of Cicer arietinum treated with α- pinene (Singh et al., 2006). Regarding the recent findings, the significant phytotoxic effect of olive and fig aqueous extracts (Ladhari et al., 2020c, 2021) could be correlated to the accumulation of proline. This relationship was confirmed by Chiapusio et al. (2015), who revealed that the drastic phytotoxic effect of 2-benzoxazolinone (BOA) at 10^{-3} M was closely associated with the rapid accumulation of proline. Proline was reported as an exceptional osmolyte due to its essential acts during stress, such as antioxidative defense and signaling molecule (Hayat et al., 2012).

Besides the enhancement of the biochemical process, lettuce plants were faced with an expected reduction in chlorophylls and stimulation of carotenoids, which are the most important pigments in the photosynthetic pathway. The decrease in chlorophyll content recorded in this study, especially with the application of fig aqueous extract, is consistent with those of the previous literature. The decline in photosynthetic pigments was noted also in rice (O. sativa L.) and amaranth (A. cruentus L.) in response to Tithonia diversifolia (Asteraceae) aqueous extract (Ilori et al., 2007). Similarly, chlorophyll and protein contents were reduced in L. esculentum, M. indicus, T. alexandrium, T. pyramidal, and C. sativa under C. murale extracts (Ahmed et al., 2004). Therefore, the allelochemicals present in the tested extracts exerted a marked phytotoxic effect on lettuce growth through a reduction in chlorophyll synthesis, which is the result of cell damage (Jaleel et al., 2008; Ladhari et al., 2014). In fact, plant photosynthesis could be affected by allelochemicals mainly through influencing the function of PSII. During oxidative stress, allelochemicals may indirectly influence the photosynthetic process by declining the energy and electron transfer due to reducing ATP synthesis activity (Achigan-Dako et al., 2014).

In contrast to chlorophyll, plant carotenoid concentration was enhanced in lettuce tissues under the treatment with olive extracts markedly by OMWW, but a reduction was depicted by fig aqueous extracts. This discrepancy in carotenoid level was depicted by Ahrabi et al. (2011), who revealed that the concentration of carotenoid was influenced in plants under biotic and abiotic stresses. They explained also that the recorded increase could be involved in oxidative stress resistance through the activation of the xanthophyll cycle, which is participating in removing the free radicals. Ibrahimet al. (2013) revealed that the aqueous extract of Zea mays induced that the carotenoid content was significantly decreased in

wheat. It was reported that the decrease of carotenoids biosynthesis was explained by the inhibition of hydroxyphenyl pyruvate dioxygenase activity with a marked deficiency of plastoquinone (Meazza et al., 2002). The varied response among target species under different plant extracts was attributed to the interference of allelochemicals present in aqueous extracts. Among phenolic compounds, usnic acid inhibited the phytoene desaturase, which transforms phytoene into carotenoids (Romani et al., 2000).

The level of formazan is an indicator of mitochondrial respiration. Results of this study showed that mitochondrial respiration decreased in the roots of lettuce more than in leaves under fig aqueous extracts compared to olive extracts. The decrease of metabolic activity in lettuce cells was depicted also by Rashid et al. (2010), who revealed that the aqueous and methanol extracts from Pueraria montana (Fabaceae) reduced the formazan level in the roots of lettuce and radish. It was concluded previously that some allelochemicals may affect plant growth by influencing the respiration process. According to Sampietro et al. (2006), the isolated phenolic acids from the leachate of Saccharum officinarum (Poaceae) reduced the formazan rate in the roots of lettuce. This decline could be explained by the reduction in oxygen intake, inhibition of ATP synthesis in the mitochondria, and ultimately disturb the oxidative phosphorylation in plants (Cheng and Cheng, 2015). In addition, the inhibition of the electron transfer chain, as well as the reduction of the mitochondrial membrane potential, has been demonstrated as the main effect of active compounds on respiratory metabolism (Abrahim et al., 2003).

5. Conclusion

Shedding some light on the correlation between the mode of action and oxidative stress of olive and fig allelochemicals appears to be one of the most interesting aspects of this study. Their allelochemicals have different modes of action markedly by targeting membrane which could be highly useful for weed management. The lettuce cell membrane was damaged through the increase of electrolyte leakage, which lead to influence the photosynthetic system by disturbing the electron transport chain, and then rising ROS production with a decline of ATP levels as a source of energy in a plant cell. The protein biosynthesis was speculated to be influenced by the induced oxidative damage, which causes cell death through alteration of meristematic cell proliferation. Moreover, some secondary metabolites were enhanced with a notable increase of proline levels in lettuce leaves and roots. Overall, fig and olive extracts can be suggested as a potential eco-friendly herbicide with a distinguished mode of action that will gain more popularity in the near future.

Authors' contributions

All authors have contributed equally to this manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

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