

# Lignification, phenols accumulation, induction of PR proteins and antioxidant-related enzymes are key factors in the resistance of *Olea europaea* to *Verticillium* wilt of olive

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Received: 20 September 2016/Revised: 21 December 2016/Accepted: 29 December 2016/Published online: 3 January 2017  
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**Abstract** To determine whether differences in olive susceptibility to *Verticillium dahliae* are accompanied by differential biochemical and physiological defense responses, we monitored the changes in fungal DNA concentration, membrane conductivity, total polyphenols, orthodihydroxyphenols, lignin and defense-related enzymes in the stems and roots of two olive cultivars differing by their susceptibility to *V. dahliae*. Fungal DNA was detected in the roots at 4 dai and attains a pick of 3.614 and 2.475 ng/100 ng of total DNA, respectively, in the susceptible and resistant cultivars. *V. dahliae* DNA in the stems was detected from 15 dai and raised slowly to attain a pick of 0.231 ng/100 ng of total DNA in the susceptible cultivar. Correlation tests between the biochemical and physiological parameters revealed that high membrane conductivity, together with early activation of peroxidase (POX) and polyphenol oxidase (PPO), resulted in a reduced root rot and wilt symptoms in the resistant cultivar Sayali when compared to the susceptible cultivar Chemlali. Monitoring of chitinase and  $\beta$ -1,3-glucanase genes' expression levels indicates that early and simultaneous upregulation of both genes is correlated

with reduced susceptibility in the resistant cultivar Sayali. Correlation tests performed between the different studied parameters, revealed a clear association between, POX activity and lignin content ( $r = 0.873$ ), polyphenols and lignin contents ( $r = 0.886$ ), PPO activity and OD phenols content (0.795), chitinase and  $\beta$ -1,3-glucanase activities (0.878) in the resistant cultivar Sayali. By contrast, lower correlation coefficients were obtained for the susceptible cultivar Chemlali. Overall, this study provides new insights into the improvement of olive genetic resources.

**Keywords** Membrane conductivity · Pathogenesis-related proteins · Cultivar susceptibility · Root resistance · OD-phenols · Soluble lignin · Peroxidase

## Introduction

As native to the Mediterranean region, olive cultivation is one of the predominant agricultural activities in countries belonging to this region. In Tunisia, culture of this crop extends over 1.5 million hectares, producing around 350.000 tons per year (National Olive Office 2015). However, olive production is influenced by the occurrence of many pests and diseases, which can affect both the quality and the yield on olive oil (Gharbi et al. 2015a). *Verticillium* wilt of olive (VWO) provoked by the pathogen *Verticillium dahliae* is one of the major diseases infecting this crop in Tunisia and worldwide. However, the control of VWO represents a serious challenge for olive growers since no efficient treatment is established nowadays. Nevertheless, despite its persistence in soil as microsclerotia, *V. dahliae* could be effectively controlled by combining several control measures such as, soil inoculum reduction, employment of adequate cultural practices, and the use of tolerant cultivars in areas

Communicated by L Bavaresco.

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with high risk of infection (Morello et al. 2015). Among these measures, the selection of tolerant olive cultivars is a promising alternative to fight against *V. dahliae*, given its low cost, sustainability and safety for the environment (Trapero et al. 2015; Chaari Rkhis et al. 2016). This method has attracted many researchers aiming to select performant cultivars using bioassay infection models and monitoring of wilt symptoms over time (Trapero et al. 2015; Arias-Calderón et al. 2015). Although this method provided useful information for olive breeders and farmers, it does not allow the identification of the true resistance given that a tolerant cultivar could be sheltered by high pathogen population, which in turn increase the soil inoculum and, therefore, increase the risk of infection (Roca et al. 2016). Consequently, an accurate evaluation of resistance to VWO as well as the understanding of mechanisms that modulate the response of olive plants to fungal attack requires further molecular and physiological analyses (Gómez-Lama Cabanas et al. 2015). However, most of the previous reports investigating the olive response to *V. dahliae* were generally descriptive and do not report any relevant information about the mechanisms underlying resistance to this disease (Lopèz-Escudero et al. 2004). Thus, only few studies have reported a detailed investigation of the biochemical and physiological defense responses of olive plants, upon infection by *V. dahliae*. For instance, interaction between the tolerant Spanish cultivar Frantoio and *V. dahliae* was studied at the transcriptome level, through suppression subtractive hybridization cDNA and sequencing of 585 positively regulated and 381 negatively regulated genes (Gómez-Lama Cabanas et al. 2015). In addition, the role of antioxidant enzymes and polyphenol compounds in the differential response of olive cultivars upon *V. dahliae* infection was previously reported (Baidez et al. 2007; Markakis et al. 2010).

To defend themselves against pathogen attacks, plants have developed various defense mechanisms to restrict the pathogen invasion. In fact, to successfully invade the host, the pathogen must cross the mechanical defense layer composed of lignin and suberin depositions and suppress the activity of secondary metabolites and antimicrobial compounds (Pusztahelyi et al. 2015). Thus, an increase in cell permeability is generally linked to host response during pathogen attack, and the extent of that raise is noticeable during the hypersensitive response (HR) following pathogen infection (Pottosin et al. 2014). The role of membrane conductivity, orthodihydroxyphenols (OD-phenols), lignin polymers and enzymes such as chitinase and  $\beta$ -1,3-glucanase has not been yet studied. In many other pathosystems, these factors are potentially involved in plant defense against pathogen attacks. OD-phenols become highly reactive once oxidized to quinones, which act either, by direct toxic effect on the pathogen or by inactivation of cell wall hydrolytic enzymes secreted by fungal pathogens (Daayf et al. 2012).

However, an exhaustive biochemical and physiological study of olive resistance against *V. dahliae* was not performed. Thus, the current study attempts to investigate the biochemical basis of resistance in the cultivar Sayali possessing root resistance in contrast to the susceptible one Chemlali (Gharbi et al. 2016). The current study aimed to define the role of biochemical factors such as, variation in membrane conductivity, total phenols content, OD-phenols, lignin polymers and enzymes like peroxidase,  $\beta$ -1,3-glucanase and chitinase in the response of olive plants upon their infection by *V. dahliae*. To achieve that, these parameters were monitored in two olive cultivars that differ by their susceptibility to *V. dahliae* to disclose potential correlation with disease resistance.

## Materials and methods

### Plant material

Two olive cultivars with different susceptibility levels to *V. dahliae* were used to perform this study. They were selected from a collection of two resistant (Meski and Sayali) and two extremely susceptible cultivars (Chemlali and Chetoui). Among these four cultivars, Meski is cropped for olive table production, while the three remaining are used for olive oil production. To make comparison, we decided to use only olive oil cultivars, since they occupy more than 95% of orchards and given the fact that the cultivar Chemlali represents more than 80% of these orchards. Therefore, the cultivar Sayali, as the sole olive oil cultivar resistant to *V. dahliae*, and the cultivar Chemlali were selected to conduct this study (Gharbi et al. 2016). Plants were kindly provided by the nursery of the Olive Tree Institute (OTI). All olive plants were obtained by stem-cuttings method from genetically authenticated mother plants. The rooted olive cuttings were grown and maintained in sterile substrate mixture (50% soil:50% peat) in polyethylene bags under greenhouse conditions and irrigated weekly until subsequent use.

### Pathogen

The highly pathogenic Vms 2 isolate maintained in the OTI fungal collection was used for plant inoculation. This isolate belongs to the non-defoliating pathotype and the vegetative compatibility group VCG2A and was previously selected among 42 *V. dahliae* isolates infecting olive trees in Tunisia on the basis of pathogenicity levels (Gharbi et al. 2015a). The Vms 2 isolate was sub-cultured on potato dextrose agar medium (PDA) for 7 days at 25 °C and then used to produce fungal inoculum in potato dextrose broth

medium (PDB) for artificial inoculation as previously described by Gharbi et al. (2015a).

### Plant inoculation

The infection bioassay was conducted following the method described by Gharbi et al. (2015a). Plants of each cultivar were divided into three groups (98 inoculated plants; 18 non-inoculated plants; 98 mock-inoculated plants). Plant roots were thoroughly cleaned with tap water and immediately soaked for 1 h in the conidia suspension previously adjusted to  $10^6$  conidia/mL. At the same time, mock infection of both cultivars was performed using sterile distilled water (SDW) instead of the pathogen inoculum. Inoculated olive plants were planted in new sterile substrate (50% soil:50% peat). The experiment was designed in a randomized complete block and set up in a greenhouse at a temperature of  $23 \pm 2$  °C, high relative humidity, and a 16/8-h photoperiod. The inoculation procedure was conducted in triplicate and repeated twice using the same experimental design described above.

### Disease severity rating

The disease severity rating was started from 15 days after inoculation using a scale from 0 to 4 according to the percentage of affected plants tissues (Gharbi et al. 2015b). The estimated area under disease progress curve (AUDPC) was determined following the formula previously described by Gharbi et al. (2015b). The final average severity of wilt symptoms and the percentage of dead plants (PDP) were also determined. The stem vascular colonization was determined in each of the inoculated olive plants by isolating the pathogen on PDA medium. The intensity of stem vascular colonization was determined by calculation of stem colonization index (SCI) following the formula described by Jiménez-Fernandez et al. (2016). The extent of stem colonization by *V. dahliae* in each plant was assessed in roots and lower, middle, and upper parts of the stem using the real-time q-PCR and isolation in PDA medium at the end of the experiment.

### Tissue sampling

After inoculation, root and stem tissues were harvested at 1, 2, 4, 8, 15, 21 and 30 days after inoculation (dai) and subjected to biochemical and molecular analyses. For each sampling time, 10 plants from each cultivar were uprooted and examined for wilting and root rot symptoms and then photographed using a digital camera. Similarly, 10 plants were sampled at each time point from the mock-inoculated groups. The remaining 18 inoculated plants and the 18 non-inoculated plants were preserved to monitor the disease

development. All plant tissues were pre-chilled in liquid nitrogen and stored at  $-80$  °C until subsequent use, except those used for the membrane conductivity, which were kept fresh. From the stem samples, the lower, middle and upper parts were collected separately and each part was divided into 0.5 cm pieces, washed using SDW, disinfested in 1% sodium hypochlorite for 5 min, washed twice in SDW, and dried under laminar hood. Subsequently, half of the stem pieces were plated onto PDA medium for pathogen re-isolation and the remaining half was kept at  $-80$  °C until used for q-PCR analyses.

### Molecular analyses

#### Total DNA extraction

The total DNA was recovered from 100 mg of dried mycelium using the ZR Fungal/Bacterial DNA mini prep D6005 Kit (Zymo Research, Irvine, CA, USA) following the instructions given by the manufacturer. The recovered DNA will be serially diluted to construct standard curves for the q-PCR. The total DNA was also recovered from 100 mg of freeze-dried root and stem tissues following the method previously described by Gharbi et al. (2015b) and kept in  $-20$  °C until used. Both recovered DNA extracts were assayed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and analyzed in a 0.8% agarose gel to ensure the DNA quality.

#### Quantification of fungal DNA by q-PCR

The q-PCR assays were performed on a CFX96 real-time PCR cycler using the primers VertB-F and VertB-R adopted by Attalah et al. (2007). The q-PCR were conducted in a final reaction volume of 25  $\mu$ L containing 12.5  $\mu$ L of Syber Green Premix (TAKARA), 0.2  $\mu$ M of each primer, and 5  $\mu$ L of total plant DNA (20 ng/ $\mu$ L). The reaction volume was finally adjusted using diethylpyrocarbonate (DEPC)-treated H<sub>2</sub>O. The best PCR efficacy was achieved using a PCR program that included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each PCR assay included a negative control containing 5  $\mu$ L SDW and a positive control containing 5  $\mu$ L of *V. dahliae* DNA (20 ng/ $\mu$ L). Samples were run in triplicate and the analysis was repeated twice. Furthermore, the DNA quality was checked using the primer pair Act-F/Act-R that amplifies a 120 bp from the gene "Actin". To make the protocol quantitative, a DNA standard curve was constructed using a tenfold dilution ranging from 10 ng/ $\mu$ L to 0.1 pg/ $\mu$ L of fungal DNA. The standard curve was generated by plotting the DNA concentration against the corresponding Ct values

through which the DNA quantity in the different samples is estimated.

#### RNA extraction and cDNA synthesis

The total RNA was recovered from plant tissues with Trizol reagent (Invitrogen, USA) from 200 mg of plant tissue collected at different time points and treated by DNase I (Invitrogen) to remove DNA contaminants. The RNA quality was checked on a 1% agarose gel and assayed using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). In addition, RNA was also recovered from the control samples at the same time points. The cDNA synthesis was carried out using the superscript III reverse transcription kit (Invitrogen, USA) following the protocol provided by the manufacturer. The cDNA concentration was estimated using a Nanodrop ND-1000 spectrophotometer and stored in  $-20^{\circ}\text{C}$  until used.

#### Q-PCR analysis of olive defense genes

The effect of *V. dahliae* infection on the expression levels of selected genes was determined using q-RT-PCR. Oligonucleotide sequences were designed based on the previously nucleotide sequences deposited in GenBank and the UniPort databases (Table 1). The gene actin was employed as internal control during q-RT-PCR assays. A CFX96™ real-time PCR cycler (Biorad) was used for the PCR assays in a total reaction volume of 25  $\mu\text{L}$ , including, 12.5  $\mu\text{L}$  of Syber Premix Ex Taq II with ROX (TAKARA), 0.15  $\mu\text{M}$  of each primer, 5  $\mu\text{L}$  of cDNA (20 ng/ $\mu\text{L}$ ), and adjusted to the final volume with SDW. The optimum PCR results were achieved using a cycling profile with an initial denaturation step at  $95^{\circ}\text{C}$  for 30 s, followed by 45 cycles of 10 s at  $95^{\circ}\text{C}$  and 30 s at  $60^{\circ}\text{C}$ . To avoid cross amplification with any contaminating DNA, a negative control containing 5  $\mu\text{L}$  (DEPC)-treated  $\text{H}_2\text{O}$ , instead of DNA was included in all q-PCR sets. All cDNA

samples were run in triplicate and q-RT-PCR analysis was repeated twice for each gene. The Ct values obtained with the internal control in the same plate were used to normalize the results using the  $\Delta\Delta\text{Ct}$  method. The fold change of each tested gene in both cultivars was calculated using the equation:  $\Delta\Delta\text{Ct} = \Delta\text{Ct}(\text{sample}) - \Delta\text{Ct}(\text{calibrator})$ .

#### Biochemical analyses

##### Determination of the membrane conductivity

Monitoring of the change in membrane conductivity was performed using leaves and root samples collected at different time points of the experiment. Each sample was cut into small pieces of 1 cm long approximately, and incubated overnight in 25 mL SDW, at ambient temperature (Vandana et al. 2014). Samples were removed from the SDW and used to estimate the conductivity using Orion Versa Star conductivity meter (Thermo Scientific). Values were measured using a microsiemens (mS) device at  $20^{\circ}\text{C}$ . The measurement of membrane conductivity was conducted in triplicate and the experiment was repeated twice.

##### Extraction and estimation of total polyphenols and OD phenols contents

Total polyphenols concentration was assayed in the stems and roots of each cultivar using the Folin–Ciocalteu method as adapted by Sofó et al. (2004). The total polyphenols concentration was estimated by measuring the absorbance of the extract at 760 nm. For quantification, a standard curve was constructed with different concentrations of gallic acid (GA) (Sigma) ranging from 1 to 200 mg/L. The final total polyphenols concentration was determined by calculating the mean of three measures and expressed as (GAE) mg/100 g of FW tissue. The quantification of polyphenol compounds was conducted in triplicate and the experiment was repeated twice.

**Table 1** List of primers used for q-RT-PCR analysis

Gene name	Accession no	Gene description	Primer sequence (forward/reverse)	Product size (bp)
POX	JX266210.1	Peroxidase	ACGAATATCGAAGGCCAGTG GATGGGGCAAATGCTTCTTA	250
PPO	JX266194.1	Polyphenol oxidase	CGCAGCAAAGACAACCTACA CTCGACCCGCTGAGTAAAAG	250
$\beta$ -1,3-Glucanase	AJ810085.1	Beta-1,3-glucanase	TGATGGAACACGGTACCAGA AGCCTTTCAAGTGCTGCAT	70
Chitinase	JQ429794.1	Chitinase	GTGCCTGGTTATGGTGTCGT TTCGAACCTTTACCGCATTC	65
Actin	AY788899.1	Actin	CAGCCTTCAATGATCGGAAT GCGCTGTAATTCCTTGCTC	172

OD phenol contents were estimated as previously described by Vandana et al. (2014). In fact, 0.2 mL of polyphenols extracts were homogenized with 1 mL of phosphate buffer (0.1 M; pH 6.5) and 2 mL of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O solution (5%). The mixture was gently homogenized and incubated for 15 min at ambient temperature. Subsequently, OD phenols contents were estimated by measuring the absorbance at 350 nm. The quantification was performed using a standard curve generated with serial dilutions of caffeic acid (CA). The experiment was conducted in triplicate and repeated twice. The OD phenols content was expressed as mg of CA equivalents based on a calibration curve (CAE/100 g of FW).

#### Determination of soluble lignin content

Lignin content was estimated in the root and stem samples of each cultivar. Plant tissues were first washed with SDW and dried at room temperature. Prior to lignin quantification, all samples were treated to remove proteins and other UV-absorbing materials following the protocol adapted by Moreira-Vilar et al. (2014). The resulting dry matter was used for lignin content estimation using the acetyl bromide method developed by Moreira-Vilar et al. (2014). 0.25 g of powdered samples was suspended in 2 mL of acetyl bromide–glacial acetic acid mixture [1:3 v/v containing 0.08 mL perchloric acid (70%)]. The mixture was incubated at 70 °C for 30 min and then transferred to 50-mL conical flasks containing 5 mL of sodium hydroxide (2 M) and 12 mL of acetic acid. The absorbance was measured at 280 nm and lignin monomers content was estimated using the specific absorption coefficient of lignin, 20 g<sup>-1</sup> cm<sup>-1</sup>. Lignin content assay was conducted in triplicate and the experiment was repeated twice.

#### Statistical analysis

The final mean severity of different cultivars were subjected to statistical analysis of variance using SPSS software (IBM Software) and were compared using Fisher's protected least significance difference (LSD) at  $P < 0.05$  to determine the variability between the two cultivars. Significant differences in the gene expression levels between the two cultivars were assessed using Graph Pad Prism software (San Diego, CA) at each time point by multiple comparisons of means using the Tukey HSD test at a significance level of  $P$  value  $< 0.05$ . Pearson correlation coefficients between the different tested parameters were determined using Graph Pad Prism (Table 2).

## Results

### Symptoms and disease severity assessment

Plants were artificially inoculated with *V. dahliae* and samples were collected from 1 to 30 dai. At each time point, plants were examined for wilting and root rot symptoms. Phytosanitary evaluation of roots revealed that until 15 dai there was no evidence of root infection on any of the inoculated plants, which suggests a minimal period of 15 days for *V. dahliae* to colonize the roots. The symptoms of disease appeared at 21 dai on the susceptible cultivar Chemlali, when the roots exhibit rot symptoms and the extent of lesions rapidly increased (Fig. 1a). By contrast, roots of the resistant cultivar Sayali remained healthy until 45 dai and did not display any symptoms of disease (Fig. 1b). At 45 dai, plants belonging to the susceptible cultivar Chemlali were almost dead with occurrence of high defoliation phenomenon, while plants of the resistant cultivar Sayali remained healthy, although there was occurrence of wilt signs on the leaves. After three months of inoculation, the isolation frequency of *V. dahliae* from affected roots was around 86% from the susceptible cultivar against only 29% from the resistant one. In addition, the frequency of *V. dahliae* isolation from the lower, middle and upper parts of the stem was about 91, 75 and 68%, respectively, collected from the susceptible cultivar. By contrast, *V. dahliae* was isolated from 56, 43 and 21% from the lower, middle and upper parts of the stems collected from the resistant cultivar (Table 3).

### Quantification of fungal DNA in planta

The q-PCR quantification has allowed the amplification of a unique band of 115 bp without any non-specific amplification products. The specificity of the q-PCR assay was checked by melt curve analysis, which indicates the presence of a single melt peak at 87 °C. Fungal DNA was detected from 4 dai in the roots of the susceptible cultivar and from 8 dai in the roots of the resistant one. The highest fungal DNA concentration was determined at 15 dai. At that time, fungal DNA quantities ranged from  $2.475 \pm 0.158$  in the roots of resistant cultivar, to  $3.614 \pm 0.331$  ng/100 ng of total DNA in the roots of the susceptible one. The fungal DNA in the roots was not detectable after 30 dai. Statistical analysis of the q-PCR data revealed that fungal DNA concentration is significantly lower in the resistant cultivar compared to the susceptible one ( $P < 0.05$ ). At the end of the experiment, *V. dahliae* was detected in 90% of the plants belonging to the susceptible cultivar compared to 37% of those belonging to the resistant one (Fig. 2a).

**Table 2** Pearson correlation coefficients between the resistance level and different biochemical and physiological parameters

Physiological and biochemical factors	Organ	Correlation coefficients ( $r^2$ )	
		Resistant cultivar Sayali	Susceptible cultivar Chemlali
Polyphenols	Roots	0.88***	0.73**
	Stem	0.82**	0.77**
OD-phenols	Roots	0.91***	0.71**
	Stem	0.83***	0.69*
Lignin	Roots	0.94***	0.78**
	Stem	0.69*	0.66*
POX	Roots	0.86***	0.73**
	Stem	0.82**	0.69*
PPO	Roots	0.91***	0.74**
	Stem	0.89***	0.73**
Chitinase	Roots	0.88***	0.79**
	Stem	0.82**	0.75**
$\beta$ -1,3-Glucanase	Roots	0.81**	0.76**
	Stem	0.79**	0.72**

Asterisks indicate significant change in the expression levels regarding to the reference genes (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; \*\*\*\*  $P < 0.0001$ ). Data were compared using Pearson correlation test



**Fig. 1** Symptoms of leaf wilting, stem discoloration, and root rot disease revealed in the resistant cultivar Sayali (a) and the susceptible cultivar Chemlali (b) after *V. dahliae* inoculation

The *V. dahliae* DNA in the stems remain undetectable until 15 dai. Fungal DNA quantities were raised from 21 dai and ranged from  $0.048 \pm 0.00113$  to

$0.029 \pm 0.0134$  ng/100 ng of total DNA. At 30 dpi, the concentration of fungal DNA attained a maximum of  $0.231 \pm 0.0114$  and  $0.117 \pm 0.0165$  ng/100 ng,

**Table 3** Classification of olive cultivars based on the AUDPC, PDP and SCI parameters

Disease severity parameters	Olive cultivars	
	Sayali	Chemlali
AUDPC	27.36 <sup>a</sup>	72.66 <sup>b</sup>
PDP (%)	00.00 <sup>b</sup>	75.00
SCI (%)		
Lower stem	56.00 <sup>b</sup>	91.00 <sup>a</sup>
Middle stem	43.00 <sup>b</sup>	75.00 <sup>a</sup>
Upper stem	21.00 <sup>b</sup>	68.00 <sup>a</sup>

Values denoted by the same letter are not significantly different according to LSD test;  $\alpha \leq 0.05$

respectively, in the susceptible and resistant cultivars (Fig. 2b). At last sampling point, *V. dahliae* was detected in the lower (77%), middle (89%) and upper (96%) parts of the stems collected from the susceptible cultivar (Fig. 2b–d).

### Change in membrane conductivity

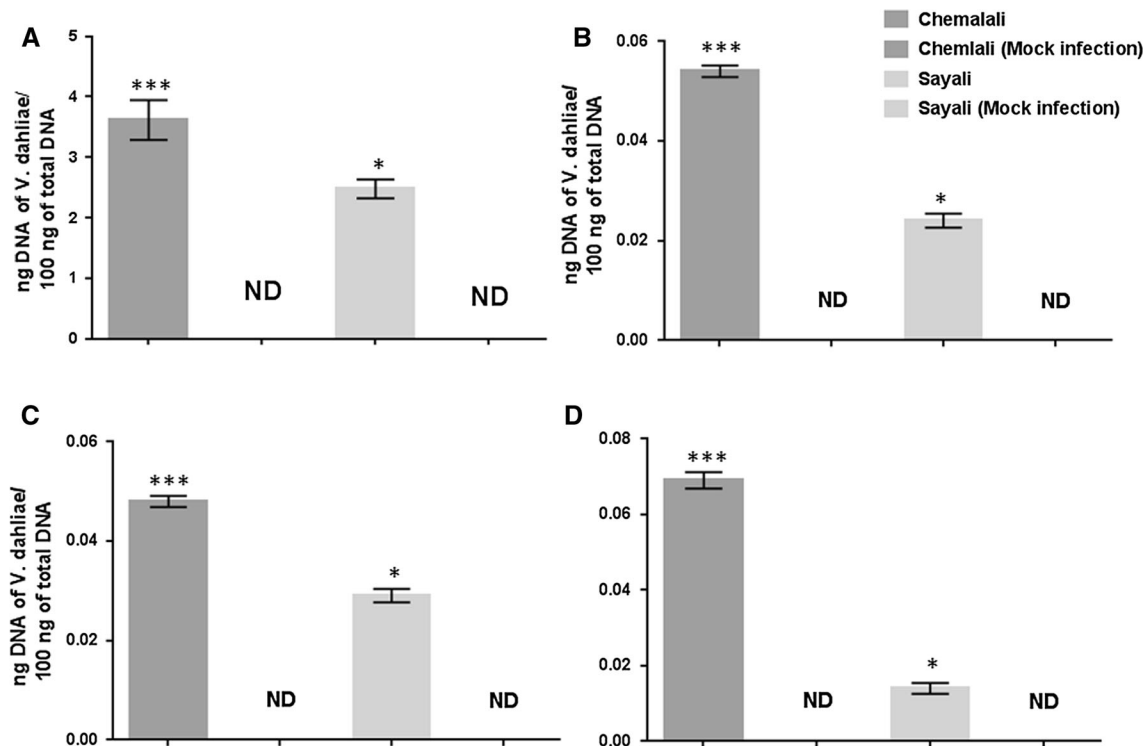
Before *V. dahliae* inoculation, differences in membrane conductivity between the two tested cultivars were not significant ( $P > 0.05$ ). After pathogen inoculation, the

membrane conductivity was significantly increased from 2 dai in the resistant cultivar Sayali and from 4 dai in the susceptible one. Thus, a maximum increase in membrane conductivity was recorded at 8 dai in the resistant (~1.82-fold) and susceptible cultivars (~1.32-fold) (Fig. 3a).

Similarly, at the stem level, differences in membrane conductivity between the two cultivars were not significant under uninoculated condition ( $P > 0.05$ ). After pathogen inoculation, the membrane conductivity was significantly increased from 8 dai in the resistant cultivar, and from 15 dai, in the susceptible one. The maximum membrane conductivity was recorded at 15 dai in the resistant cultivar and at 21 dai in the susceptible one (Fig. 3b).

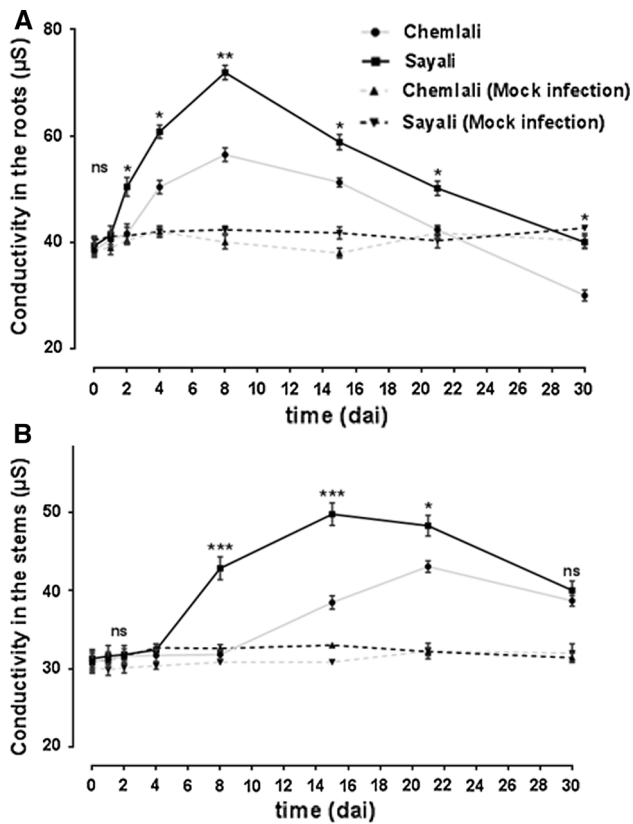
### Total polyphenols content

During the time course, the accumulation of polyphenols followed similar trends in the roots of both cultivars. Until 2 dai, polyphenols content showed low but non-significant changes. Subsequently, their content increased by 1.45-fold in the susceptible cultivar Chemlali and by 1.79-fold in the resistant one. At 8 dai, polyphenols content strongly increased by 2.25-fold in the resistant cultivar Sayali and became significantly higher than that observed in the



**Fig. 2** Comparison of fungal DNA amounts in the roots and different stem parts of resistant and susceptible olive cultivars (**a** fungal DNA concentration in the roots; **b** fungal DNA concentration in the lower part of the stem; **c** fungal DNA concentration in the middle part of the stem; **d** fungal DNA concentration in the upper part of the stem).

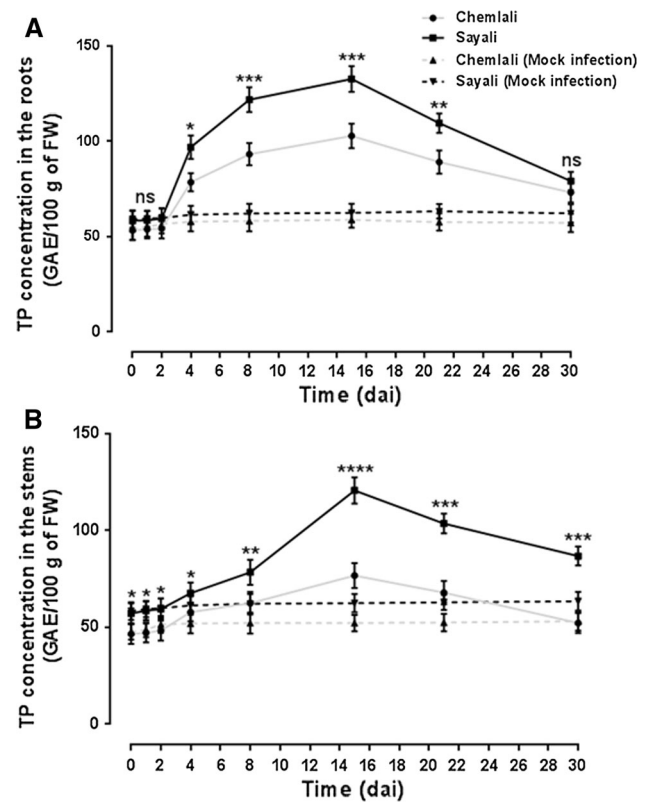
Error bars indicate standard deviation (SDs) calculated for (3 × 2) replicates. Asterisks indicate significant change in the expression levels regarding the reference genes (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ). Data were compared using Tukey HSD test



**Fig. 3** Changes in membrane conductivity in the resistant and susceptible cultivars after *V. dahliae* inoculation. **a** Evolution of membrane conductivity in the roots of both cultivars. **b** Evolution of membrane conductivity in the stems of both cultivars. Asterisks indicate significant change in proline concentration among the different cultivars (*ns* not significant,  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ;  $****P < 0.0001$ ). Data were compared using Tukey HSD test

susceptible one (1.75-fold). At 15 dai, the polyphenols content reached a peak of 2.45-fold and 1.93-fold, respectively, in the resistant and susceptible cultivars. At 30 dai, the polyphenols content decreased in the roots of both cultivars but remained significantly higher than that recorded in the uninoculated plants (Fig. 4a). The statistical analysis revealed that high polyphenol contents were strongly correlated with the resistance level of each cultivar (Table 2).

Polyphenol contents were estimated in the stems of both cultivars. Before inoculation, polyphenol content was slightly higher in the resistant cultivar Sayali (~1.23-fold). Under inoculation condition, a significant raise in polyphenol contents was recorded in both cultivars from 4 dai. Subsequently, polyphenols content was strongly increased by 2.11-fold in the resistant cultivar, and became significantly higher than that observed in the susceptible one (1.78-fold). At 30 dai, polyphenols content was slightly decreased in the resistant cultivar (1.81-fold) compared to 1.45-fold only in the susceptible cultivar (Fig. 4b).



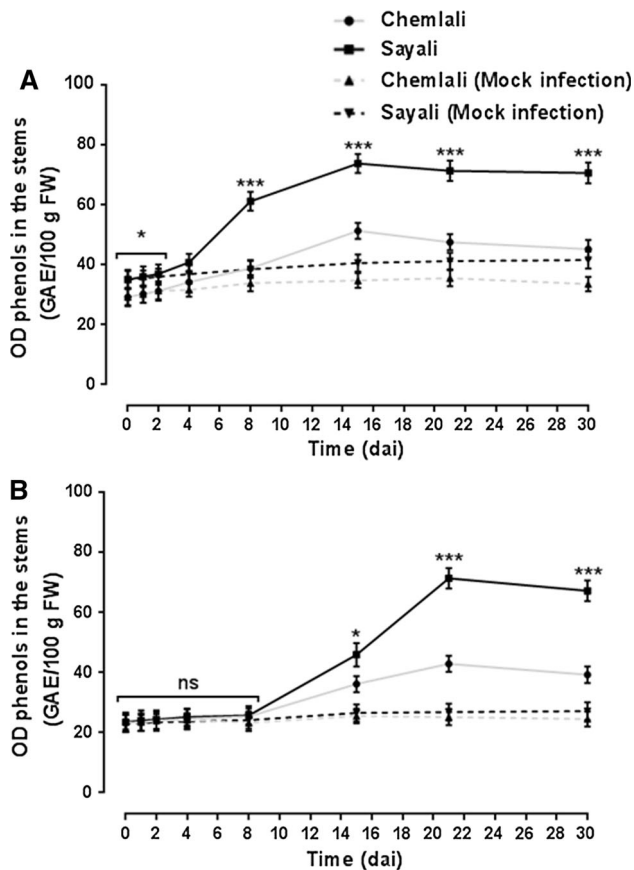
**Fig. 4** Monitoring of polyphenols content in the susceptible and resistant cultivars after *V. dahliae* inoculation. **a** Evolution of polyphenols content in the roots of both cultivars. **b** Evolution of polyphenols content in the stems of both cultivars. Asterisks indicate significant change in polyphenols content among the two cultivars ( $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ;  $****P < 0.0001$ ). Data were compared using Tukey HSD test

### OD phenols content

Under no inoculation condition, the OD phenols content in the roots was slightly higher in the resistant cultivar Sayali (1.20-fold). After pathogen inoculation, the OD phenols content significantly raised from 4 dai in both cultivars. At 8 dai, OD phenols content rapidly increased by 1.75-fold in the resistant cultivar against 1.33-fold increase in the susceptible one. The maximum increase in OD phenol contents was recorded at 15 dai, with peaks of 2.11-fold and 1.76-fold, respectively, in the resistant and susceptible cultivar (Fig. 5a). The comparison of the two cultivars at each sampling point revealed that high OD phenols content are positively correlated with resistance level of each cultivar (Table 2).

Similar results were recorded when OD phenols were estimated at the stem level. In fact, under no inoculation condition, both cultivars exhibit similar OD phenols content ( $P > 0.05$ ). After pathogen inoculation and until 8 dai, OD phenols content slightly but not significantly increased in both cultivars. Subsequently, OD phenols content



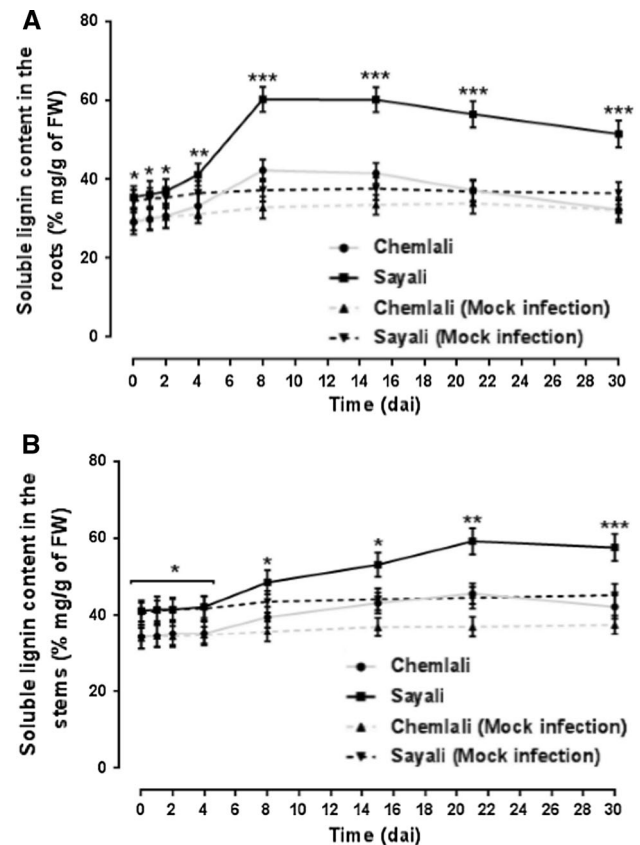


**Fig. 5** Monitoring of OD phenols content in the susceptible and resistant cultivars after *V. dahliae* inoculation. **a** Evolution of OD phenols content in the roots of both cultivars. **b** Evolution of OD phenols content in the stems of both cultivars. Asterisks indicate significant change in OD phenols content among the two cultivars (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ). Data were compared using Tukey HSD test

rapidly increased by 1.96-fold and 1.56-fold, respectively, in the resistant and susceptible cultivars. At 21 dai, the OD phenols content reaches a maximum increase of 1.85-fold and 2.18-fold, respectively, in the susceptible and resistant cultivars (Fig. 5b). The statistical analysis revealed that OD phenols content in the stem is positively correlated with the resistance level of each cultivar (Table 2).

### Soluble lignin content

The soluble lignin content was estimated in the roots and stems of the two cultivars. In fact, under no inoculation condition, the soluble lignin content in the roots was significantly higher in the resistant cultivar Sayali (1.2-fold) than that observed in the susceptible cultivar Chemlali. Under inoculation condition, a significant raise was recorded from 4 dai in both cultivars. At 8 dai, a maximum raise of 1.45-fold and 1.70-fold were, respectively, recorded in susceptible and resistant cultivars (Fig. 6a). Overall,



**Fig. 6** Monitoring of soluble lignin content in the susceptible and resistant cultivar after *V. dahliae* inoculation. **a** Evolution of soluble lignin content in the roots of both cultivars. **b** Evolution of soluble lignin content in the stems of both cultivars. Asterisks indicate significant change in soluble lignin among the two cultivars (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ). Data were compared using Tukey HSD test

soluble lignin content in the roots was positively correlated with the resistance level of each cultivar ( $P < 0.05$ ).

Before inoculation, the soluble lignin content was slightly higher (~1.2-fold) in the stems of the resistant cultivar when compared to the susceptible cultivar Chemlali. After inoculation, a significant raise in soluble lignin content was recorded in both cultivars from 8 dai. Subsequently, lignin content continues increasing in both cultivars and reaches a peak of 1.44-fold and 1.33-fold, at 21 dai, respectively, in the resistant and susceptible cultivars (Fig. 6b). The comparison of the two cultivars based on their soluble lignin content in the stems indicates a weak correlation coefficient between the resistance level and lignin content (Table 2).

### Relative expression of defense-related enzymes

#### *Peroxidase (POX) expression level*

Under uninoculated and inoculated condition, the resistant cultivar Sayali exhibits high expression level of POX gene.

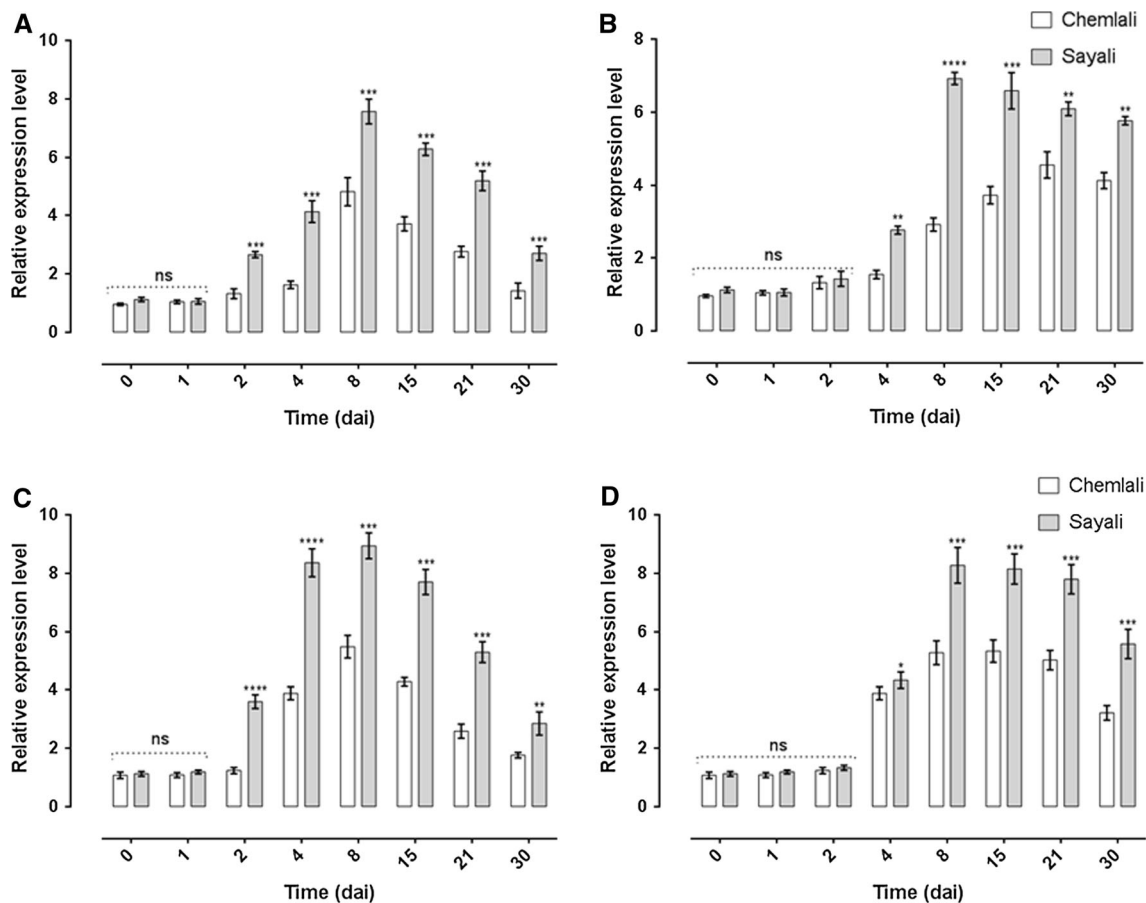
Inoculated plants of both cultivars showed a significantly higher expression level of POX when compared to the uninoculated control plants. Significant increase in the expression level of POX was recorded from 2 dai in the resistant cultivar Sayali (2.55-fold), and from 4 dai in the susceptible cultivar Chemlali (1.55-fold). The highest expression level was recorded at 8 dai in both cultivars, with approximately 7.75-fold and 4.85-fold, respectively, in the resistant and susceptible cultivars (Fig. 7a).

At the stem level, POX expression level was not significantly different between uninoculated plants of the two cultivars. Under inoculation condition, a significant raise was observed from 4 dai in resistant cultivar Sayali (2.2-fold) and from 8 dai in the susceptible cultivar Chemlali (1.33-fold) when compared to the uninoculated control plants. The maximum expression level was recorded at 15 dai in the resistant cultivar (6.55-fold) and at 21 dai in the susceptible cultivar (4.41-fold) (Fig. 7b). The

correlation test revealed that high POX activity is strongly correlated with the resistance level of each cultivar (Table 2).

#### Polyphenol oxidase (PPO) expression level

The gene coding for PPO enzyme was differentially upregulated in the two cultivars. First, PPO activity remained stable until 1 dai in both cultivars. Subsequently, a significant increase was recorded at 2 dai in the resistant cultivar and at 4 dai in the susceptible one. PPO activity continues increasing in both cultivars until reaching a peak of 8.78-fold and 5.47-fold, at 8 dai, respectively, in the resistant and susceptible cultivars. Subsequently, levels of PPO activity were maintained significantly high in both cultivars until 15 dai, and then gradually decrease to reach values close to those recorded in the control plants (Fig. 7c).



**Fig. 7** Comparison of POX and PPO gene expression levels in the roots and stems of resistant and susceptible olive cultivars after *V. dahliae* inoculation, at different time intervals (**a** expression level of POX in the roots; **b** expression level of POX in the stem; **c** expression level of PPO in the roots; **d** expression level of PPO in the stem). Gene expressions were expressed as the ratio to the average

expression of the control group (no infection) including actin as reference gene. Error bars indicate standard deviation (SDs) calculated for three replicates. Asterisks indicate significant change in the expression levels regarding the reference genes (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ). Data were compared using Tukey HSD test

At the stem level, similar results were obtained when PPO expression level was monitored in the stems of both cultivars. Indeed, a significant increase was recorded in both cultivars from 4 dai. Subsequently, the PPO expression level became significantly higher in the resistant cultivar and attains a maximum of 8.25-fold against only 5.24-fold in the susceptible one (Fig. 7b). Statistical significant changes were obvious when both cultivars were compared at each sampling point of the experiment (Table 2).

#### *β-1,3-Glucanase expression level*

Under uninoculated condition, roots of both cultivars showed similar expression patterns of β-1,3-glucanase. After inoculation, significant raise in the expression level of β-1,3-glucanase was recorded in the resistant cultivar from 2 dai, but not in the susceptible cultivar, for which, a significant increase was recorded only from 4 dai. Subsequently, the highest maximum expression level of β-1,3-glucanase was recorded in the resistant cultivar Sayali at 8 dai (8.58-fold). In the susceptible cultivar Chemlali, the highest raise was recorded at 15 dai (6.26-fold) and was significantly lower than that recorded in the resistant cultivar (Fig. 8a).

At the stem level, the two cultivars have the same expression level of β-1,3-glucanase before inoculation. Subsequently, the pathogen inoculation induced a significant raise in the expression level of β-1,3-glucanase in both cultivars from 4 dai. The highest β-1,3-glucanase expression level was observed at 8 dai in the resistant cultivar Sayali (8.45-fold) and at 15 dai in the susceptible cultivar Chemlali (5.11-fold) (Fig. 8b). Correlation analysis revealed that high β-1,3-glucanase expression level is strongly correlated with resistance to *V. dahliae* (Table 2).

#### *Chitinase expression level*

Under no inoculation condition, the expression level of chitinase gene was not significantly different between the two cultivars. After pathogen inoculation, chitinase activity significantly induced from 2 dai in the resistant cultivar and from 4 dai, in the susceptible one. The highest chitinase activity was recorded at 8 dai in resistant cultivar Sayali (7.85-fold) and at 15 dai in the susceptible cultivar Chemlali (5.36-fold) (Fig. 8c).

At the stem level, no significant variations in chitinase activity were recorded before inoculation. Upon pathogen inoculation, chitinase activity increased in both cultivars from 4 dai, and reached its maximum at 15 dai in the resistant cultivar Sayali (7.11-fold) and the susceptible cultivar Chemlali (4.65-fold) (Fig. 8d). The correlation analysis demonstrated that high chitinase activity is

positively correlated with the resistance level of each cultivar (Table 2).

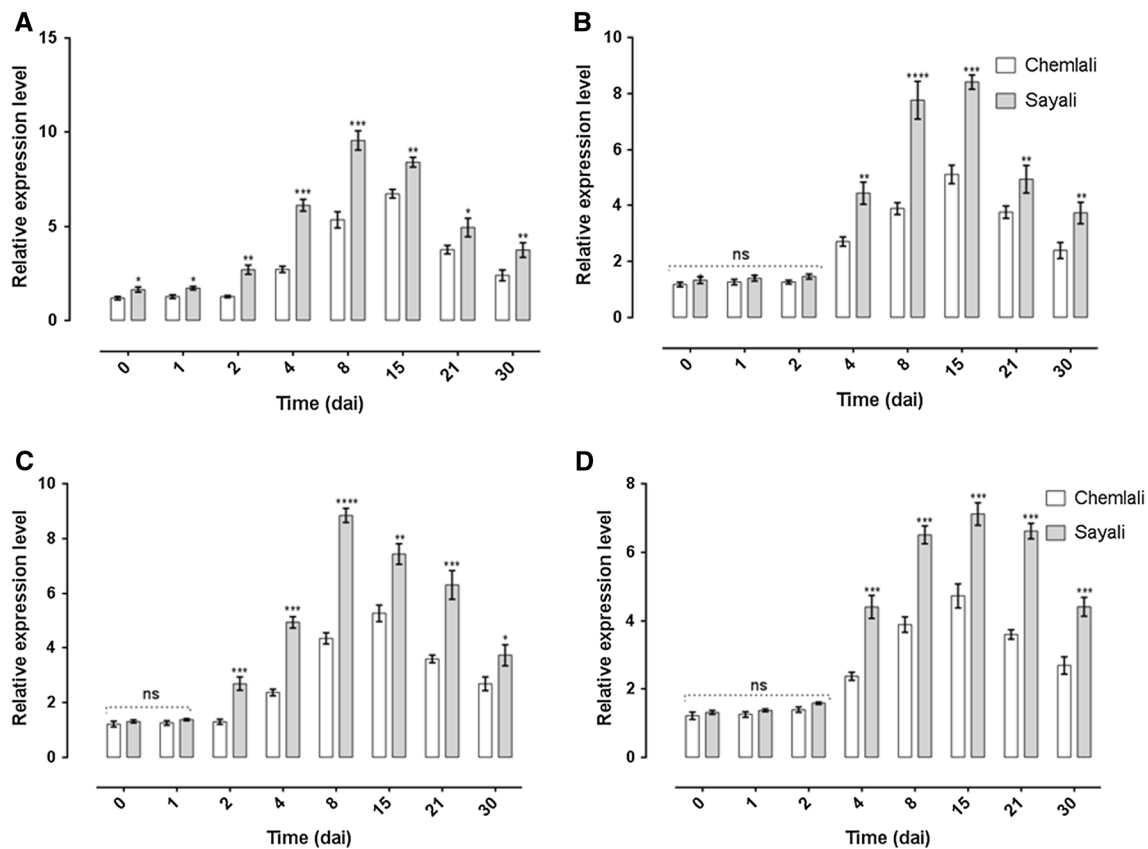
### **Correlation analyses between the physiological and biochemical parameters ( $P < 0.05$ )**

A strong correlation coefficients between peroxidase and lignin content (0.873), peroxidase and polyphenols content (0.832), polyphenols content and lignin content (0.886), OD phenols content and PPO activity (0.795), OD-phenols content and lignin (0.664), chitinase and β-1,3-glucanase expression levels (0.878) were obtained in the roots of the resistant cultivar Sayali. The corresponding correlation coefficients in the roots of the susceptible cultivar were 0.712, 0.734, 0.663, 0.675, 0.59 and 0.534, respectively. In stem of resistant cultivar, a strong correlation between peroxidase and polyphenols content (0.847), peroxidase and lignin content (0.813), OD phenols and PPO (0.856), chitinase and β-1,3-glucanase (0.853) was found. The corresponding correlation coefficients in the stems of susceptible cultivar were 0.712, 0.684, 0.751, and 0.789, respectively.

### **Discussion**

Despite the development of several alternative strategies to manage VWO, the selection of olive genetic resources exhibiting resistance to the pathogen remain the best biologically, environmentally and commercially reasonable alternative, which would be efficient in restricting this disease (Morello et al. 2015). In this study, a comparative approach was performed to disclose the importance of biochemical factors in the defense of two olive cultivars differing by their susceptibility to the pathogen *V. dahliae* and to correlate these changes with the resistance level of each cultivar.

Results obtained during the infection bioassay (AUDPC, PDP), the frequency of pathogen isolation from roots and stems of both cultivars revealed that roots of the susceptible cultivar Chemlali were more rapidly invaded by the pathogen, which indicates that this cultivar does not exhibit root resistance to *V. dahliae*. By contrast, roots of the resistant cultivar Sayali remained healthy until 45 dai without severe damage, indicating that this cultivar possess a strong root resistance to *V. dahliae*. Indeed, during field prospections, similar root resistance was recorded when natural infection occurred (Chaari Rkhis et al. 2016). In fact, unlike the cultivar Chemlali, which is cropped in regions near to other susceptible hosts, the cultivar Sayali is occasionally cropped and, therefore, very little confronted with *V. dahliae*, which could restrict the capacity of



**Fig. 8** Comparison of chitinase and  $\beta$ -1,3-glucanase genes expression levels in the roots and stems of resistant and susceptible olive cultivars after *V. dahliae* inoculation, at different time intervals (**a** expression level of  $\beta$ -1,3-glucanase in the roots; **b** expression level of  $\beta$ -1,3-glucanase in the stem; **c** expression level of chitinase in the roots; **d** expression level of chitinase in the stem). Gene expressions

were expressed as the ratio to the average expression of the control group (no infection) including actin as reference gene. Error bars indicate standard deviation (SDs) calculated for three replicates. Asterisks indicate significant change in the expression levels regarding the reference genes (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ ). Data were compared using Tukey HSD test

the pathogen to modulate its pathogenicity towards a favorable infection over this cultivar (Alkher et al. 2009). The employment of the q-PCR confirmed the previous results and revealed that the roots and stems of the susceptible cultivar are significantly more colonized by the pathogen. This result indicates that the employment of pathogen quantification allows for more refined classification of cultivars based on their susceptibility levels to *V. dahliae* (Mercado-Blanco et al. 2003; Atallah et al. 2007). In addition, the pathogen quantification has the advantage of discrimination between the resistance phenomenon, which restricts fungal invasion and growth and the tolerance phenomenon, which does not restrict pathogen invasion, but instead reduces or offsets its virulence mechanisms. In this study, cultivar-dependent changes were recorded for metabolites accumulation, such as lignin, phenolics compounds and the expression levels of defense-related genes. These coordinated physiological and molecular changes led us to hypothesize that root resistance to *V. dahliae* is quantitative and roughly related to the strength of the innate defense system as well as its ability to

restrict the pathogen invasion (St Clair 2010). This hypothesis is supported here by the results obtained from the physiological and molecular analyses performed on roots and stems of both cultivars. In fact, before inoculation, we observed that roots of the resistant cultivar Sayali exhibits higher polyphenols content, lignin content and peroxidase activity as compared to those recorded in the susceptible cultivar Chemlali. In addition, these factors are roughly correlated with each other, which demonstrate that they are determinants in the defense mechanisms of olive plants against pathogens. Indeed, the correlation analysis confirmed that resistance observed in the cultivar Sayali is mainly quantitative since it is under the control of multiple biochemical and molecular factors. Therefore, this cultivar could be a reliable alternative for improving the genetic traits of susceptible cultivars, which provide them durable resistance (Palloix et al. 2009).

After plant inoculation, a significant raise in membrane conductivity was observed earlier in the resistant cultivar Sayali, as compared to the susceptible one. In fact, the correlation tests indicate that the increase in membrane

conductivity is associated with high disease resistance, which is in agreement with results obtained with other pathosystems such as pepper (Vandana et al. 2014), and *Arabidopsis* (Cheng et al. 2013). In fact, it was reported that during the hypersensitive reaction (HR), increase in membrane conductivity, through modification of ion fluxes are the earliest responses of the host cell to fungal elicitors. In addition, the extent of this induction is a strong determinant for the activation of a set of metabolic changes, leading to the installation of mechanical and chemical barriers that restrict the pathogen invasion (Wan et al. 2008). In this study, an early increase (<2 dai) in membrane conductivity was accompanied by simultaneous increase in POX activity in the resistant cultivar Sayali, while a delayed increase in membrane conductivity was accompanied by low activity of this enzyme in the susceptible cultivar Chemlali. These observations indicate that successful resistance to *V. dahliae* is time dependent, especially during the HR, where variation in membrane conductivity leading to calcium and proton influx and potassium and chloride efflux appears to be essential for oxidative burst initiation, defense genes induction and metabolites production. All of these features are similar to the effects of pathogen elicitors in higher plants, in which the HR has been investigated (Mohanraj et al. 2003).

In this study, the total polyphenols and lignin contents were significantly higher in roots and stems of the resistant cultivar Sayali. Indeed, the major differences between the two cultivars could be summarized in the following ways. First, the correlation between total polyphenols, OD phenols and root resistance revealed the importance of these compounds in the tolerance of *V. dahliae* infection. Thus, when comparing the polyphenols, OD phenols and lignin contents with the phytosanitary status of roots we concluded that the extent of lesions and rotting is low and occurred only after 45 dai in the cultivar Sayali, while a severe rotting occurred earlier in roots of the susceptible cultivar. The differential metabolic changes observed in the roots and stems of resistant cultivar were accompanied at the same time by strong upregulation of genes coding for POX and PPO. By contrast, a delayed upregulation of these two enzymes was related to lower lignin and polyphenols contents in the roots of the susceptible cultivar. These findings suggests that resistant olive cultivars possess a multicomponent defense system, in which, early and durable upregulation of POX and PPO plays a central role in the restriction of pathogen invasion and growth, through induction of polyphenols and lignin accumulation at the site of infection during the HR. In fact, polyphenol compounds together with lignin could have a direct effect on pathogen growth and invasion since they play a critical role in the mechanical defense against pathogen attacks (Daayf et al. 2012). Similar result was obtained with transgenic

*Arabidopsis* plants overexpressing the gene *UGT72E2*, which accumulates lignin and soluble phenylpropanoid coniferin, was more resistant to *Verticillium longisporum*. These findings confirm that not only lignin monomers but also phenolic compounds play a critical role in the plant defense mechanisms against fungal invasion (Konig et al. 2014). In fact, polyphenol compounds also act as precursors in the synthesis of lignin (Daayf et al. 2012) and suberin (Ngadze et al. 2012), which are implicated in the installation of mechanical barriers that can restrict the spread of plant pathogens. In line with these results, Lewis et al. (1998) reported a differential accumulation of polyphenols compounds and upregulation of PPO activity in potato cultivars with different resistance levels. In addition, upregulated PPO activity is required at early stage of infection for successful defense against pathogen invasion (Kumar et al. 1991).

The oxidation process, activated by PPO and POX enzymes is also an important defense strategy of plant hosts against fungal pathogens (War et al. 2012), through accumulation of toxic compounds like quinones, which were shown to inhibit the cell wall-degrading enzymes of plant pathogens (Khan et al. 2001). In addition, PPO is known to induce the oxidation of phenolic compounds and create unfavorable conditions for pathogen growth (Khan et al. 2001). Indeed, OD phenols play critical role in enhanced resistance against fungal infection and onset of defense mechanisms such as the HR (Vandana et al. 2014).

Lignification of the host cell wall plays a central role in HR upon pathogen attacks and peroxidase is one of the enzymes involved in this process as they catalyze the production of lignin. In this study, higher lignin content was positively correlated with the root resistance observed in the cultivar Sayali. Several reports have shown variations in lignin content after fungal attacks, and most of these studies have sufficiently confirmed the importance of lignin during the invasion process (Xu et al. 2011a). For instance, analysis of quantitative resistance of cotton cultivars to the hemibiotrophic pathogen *V. dahliae* indicated an association between high lignin content in the stems after inoculation and resistance against VWO (Xu et al. 2011b). In a similar study, the overexpression of cotton *DIRIGENT1* gene enhances the lignification process and restricts the invasion by *V. dahliae* (Shi et al. 2012).

The stimulation of  $\beta$ -1,3-glucanase and chitinase enzymes occurs in several plant species in response to pathogen invasion (Hanselle and Barz 2001). These two enzymes were simultaneously induced in the stems and roots of resistant cultivar after 2 dai. By contrast, a delayed upregulation of  $\beta$ -1,3-glucanase was recorded in the susceptible cultivar (>4 dai). These results suggest that early co-induction of PR proteins in resistant cultivar after pathogen attack restricts the fungal growth by hydrolysis of

its cell wall and leads to no disease development. By contrast, a delayed induction of PR proteins to a required level and absence of synergism between the two enzymes in the susceptible cultivar facilitate the pathogen entry and lead to severe tissue damage. In fact, these enzymes are crucial components of the plant resistance to biotic diseases (Funnell et al. 2004). For instance, it was that overexpression of genes coding for these PR proteins can improve resistance in plants (Velazhahan and Muthukrishnan 2004). In addition, strong chitinase and  $\beta$ -1,3-glucanase activities were related to high resistance levels in chickpea inoculated with the fungus *Ascochyta rabiei* (Hanselle and Barz 2001).

In this study, higher correlation coefficients obtained with the resistant cultivar confirmed the role of the studied biochemical and physiological factors in the defense against VWO. However, correlation coefficients obtained with the susceptible cultivar deemed also high, which indicates that resistance to *V. dahliae* is often quantitative and not only limited to the studied factors, but involved also other factors that need deeper research studies.

In conclusion, olive plants are able to induce efficient set of defense mechanisms, if harmful pathogens are timely recognized and the resulting signals are appropriately processed. The overall results obtained in the present study indicate that among the biochemical factors, membrane conductivity, polyphenols, lignin, and POX enzyme play central role in root resistance to *V. dahliae*. All the biochemical factors investigated in this study have been subjected to differential significant changes in stems and roots of inoculated olive plants from both cultivars as compared to their corresponding uninoculated plants. This result could reflect the activation of systemic defense response in these cultivars against upon *V. dahliae* infection. Despite the importance of these new findings, deeper research still required to confirm these observations using new anatomical and molecular tools.

**Author contribution statement** The work presented here was carried out in collaboration with all authors: YG designed, accomplished the experiment, interpreted the results and wrote the article manuscript. MB participated in the study design, accomplished the statistical analysis, interpreted the results and revised critically the manuscript. EB participated in all laboratory analysis, especially the physiological analyses and revised critically the manuscript. KH helped in the revision step, and revised the language of the manuscript. RG revised the statistical analysis of q-PCR data and revised critically the manuscript. MAT supervised the experimental work, interpretation of results and revised critically the manuscript.

**Acknowledgements** This study was supported in part by a grant from the PESTOLIVE project in Tunisia to M.A. Triki, and Research Laboratory APREGO, and the research unit of toxicology–environmental microbiology and health to R. Gdoura.

#### Compliance with ethical standards

**Conflict of interest** We have read and understood the journal policy on declaration of interests and we have no conflict of interest to declare.

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