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Tetraclinis articulata essential oil emulsion use as alternative to chemical fungicide to control tomato grey mould disease

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ABSTRACT

Tetraclinis articulata essential oil proved to be effective in controlling tomato grey mould, so we would investigate its effect on some tomato defense mechanisms. The pretreatment of Botrytis cinerea infected tomato plants with TAEO emulsion enhanced the activity of antioxidant enzymes activity SOD, CAT, APX, and GPX, and total polyphenols content and it decreased IC_{50} of free radical-scavenging activity and H_2O_2 content. Results showed amelioration in antioxidant status in TAEO emulsion treated and B. cinerea infected plants indicating that treatment decreased infection in tomato plants. The qRT-PCR analysis of defense genes expression Chitinase SlChi, transcription factors SlWRKY and SlAP2/ERF, Lipoxygenase SlLOX, and Thioredoxin SlTRX showed that they were up-regulated as early as 12 hpi sustained with a second increase at 48 hpi in TAEO emulsion pretreated and infected plants. These results suggest the potential use of TAEO emulsion as natural product to induce tomato antioxidant status and activate defense genes.

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Introduction

Tomato (*Solanum lycopersicum* L.) is an important solanaceous vegetable crop consumed worldwide as fresh and after food industry processing (Rguez et al. 2020). Tomato crop is cultivated in greenhouse or open field conditions with a total world production about 17.46 million tons in 2020. Tomato fruit constitutes an important source of several vitamins like C, B6, and E, minerals potassium), and a variety of natural antioxidants including carotenoids and polyphenols (de Souza Av et al. 2020), which are essential in human dietary.

Botrytis cinerea the causal agent of grey mould disease, is one of the most devastating fungal phytopathogen worldwide (Mouekouba et al. 2013). This fungal disease leads to severe losses in tomato production in the field, in greenhouses as well as in postharvest conditions during storage and transport (Rguez et al. 2018). The lack of tomato varieties resistant to this pathogen implies the development and the use of synthetic fungicides that inhibit the growth or kill the fungal pathogen. Despite their usefulness in agriculture to increase crop production, the use of fungicides represents a potential danger for human health through their harmful residues. In addition, the repetitive and excessive use of these chemicals lead to the loss of effectiveness through the emergence and the proliferation of resistant

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B. cinerea strains (Saito et al. 2019). Therefore, the development of safe alternatives to fungicides is of major importance in modern agriculture systems.

The biological control of plant diseases by microorganisms and plant extracts including essential oils (EOs) and their active compounds has been considered as an efficient and eco-friendly alternative to chemical control methods (Elmsellem et al. 2019). Essential oils are complex organic compounds, synthesized through secondary metabolic pathways of plants playing important roles as signaling molecules in plant defense against biotic aggressors including pests, fungi, bacteria, and viruses (El Ouadi et al. 2017; Bendaif et al. 2018). Several *in vitro* and *in vivo* studies showed that TAEO exhibited an important activity against *B. cinerea* mycelial growth and conidia germination (Rguez et al. 2020). This EO applied on tomato fruit reduced infection caused by *B. cinerea* by 64.01%. The pulverization of TAEO on detached leaflets also reduced *B. cinerea* necrosis to 14.50% in comparison to only infected leaflets 76.00%. The TAEO applied as preventive treatment on tomato plants under greenhouse conditions enhanced plant growth and reduced *B. cinerea* infection to 17.72% in comparison to infected and non-treated plants. Recent studies indicated that *thymus vulgaris* and *Satureja hortensis* EOs effectively reduced *B. cinerea* infections on apple fruit by the activation of pathogenesis-related protein such as PR-5 and PR-8 involved in defense against pathogens (Banani et al. 2018). In addition, the use of *Gaultheria procumbens* EO-induced defense mechanisms in rice plants against *Colletotrichum higginsianum* (Vergnes et al. 2014). Many reaserch work interested to the biological control of phytopathogenic fungal diseases by EO, however this work consists of the first to describe its mechanism of action.

In this context, this work aimed to analyze reactive oxygen species, antioxidant enzyme activity and quantitative expression of some defense genes in tomato plants pretreated by *T. articulata* EO and inoculated with *B. cinerea* in order to explain for the first time defense mechanisms underlying the effectiveness of *T. articulata* EO in controlling *B. cinerea* infection in tomato plants.

Material and methods

Plant material and culture conditions

Tomato seeds (cultivar RioGrande) were planted in autoclaved peat and transplanted to

2 L pots at cotyledon stage according to Rguez et al. (2020). They were grown in a greenhouse under natural temperature and photoperiod and irrigated twice a week with Vadez nutritive solution (Vadez et al. 1996). Plants were grown in greenhouse under natural conditions of temperature and photoperiod. Tomato plants were inoculated by putting 200 µl of *B. cinerea* conidia suspension in wound performed on the steam. Tomato plants were treated with TAEO emulsion at a concentration of 2 g L- in powder milk solution using a manual sprayer. Control plants were sprayed with the fungicide Sp-Vegetaux at 119 a.i. mg $L^{-1.}$ Plants were covered by an air-tight transparent plastic bag to maintain high relative humidity (Rguez et al. 2020).

Essential oil extraction isolation method and emulsion preparation

Tetraclinis articulata essential oil (TAEO) was isolated from plants aerial parts collected at flowering stages (Rguez et al. 2020). Essential oil was extracted by hydrodistillation method and the identity of the plant was confirmed by Prof. Abderrazzak Smaoui and a voucher specimen was deposited under the reference number LN 07002 in the herbarium at the Biotechnological Centre of Borj-Cedria and a sample have been retained for any further reference.

Treatment of tomato plants

The TAEO emulsion was used for foliar pulverization of tomato plants at two leaf stage and after 24 h plants were infected with *B. cinerea* fungal strain TRG (MK854557) (Rguez et al. 2020). The chemical fungicide "Désogerme SP Végétaux" (20 g/L of polyhexamethylene

guanidine and 50 g/L of alkyl dimethyl benzyl ammonium chloride) was used as a control (119 mg/L), after 24 h plants were infected with *B. cinerea* strain TRG. Plants treated separately with powder-milk solution (10 g/L), the TAEO emulsion, the fungicide, and infected with *B. cinerea* were included as controls.

For biochemical and gene expression analyses, tomato leaves were harvested at 0, 12, 24, 48, and 72 h post infection (hpi). Samples were immediately frozen in liquid nitrogen and stored at −80°C until use. The experiment was repeated three times.

Antioxidant enzyme activities

Antioxidant enzyme activities were analyzed in this study. Enzymatic extracts were prepared, and the protein content was determined using the Bradford method. The activity of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) were measured spectrophotometrically. The total phenolic content was determined using the Folin-Ciocalteu reagent, and the free radical-scavenging activity was evaluated by 1,1,-diphenyl-2-picryl-hydrazil (DPPH). Additionally, the concentration of hydrogen peroxide (H_2O_2) was determined at 390 nm. Bradford (1976), Hemeda and Klein (1990), Nakano and Asada (1981), Mau et al. (2001), Hatano et al. (1988), and Velikova et al. (2000) were the references used for these methods.

Identification of phenolic compounds

The HPLC separation of leaf methanolic extracts used a Gemini C18 column (150 × 2.1 mm, i.d. 5 µm) from Phenomenex (USA). A mobile phase of 0.1% formic acid solution and acetonitrile with a flow rate of 0.3 ml min-1 was used with a gradient elution. The Thermo Finnigan Surveyor Plus HPLC apparatus was used with a quaternary pump, Surveyor Plus-auto-sampler, and a vacuum degasser connected to an LCQ Advantage Max ion trap mass spectrometer through an ESI source. The ion trap operated in data-dependent, full scan (500–2000 m/z) and MSn mode, and the optimized ESI source parameters were obtained using a 10 ppm 5-caffeoylquinic acid solution.

Quantitative gene expression analysis

Total RNA extraction was performed according to Deepa et al. (2014). The cDNA was synthesized from 10 µg of total RNA by Turbo-I First Strand cDNA Synthesis Kit (Biomatik, Wilmington, Delaware, United States) as described by the manufacturer. The reverse transcription reactions were performed at 42°C for 60 min in a total volume of 20 µL.

cDNA was diluted (1:10) and used as template for qRT-PCR with specific primers designed for Chitinase (SlChi), APETALA2/ethylene responsive factor (SlAP2/ERF), Lipoxygenase (SlLOX), WRKY-type transcription factor (SlWRKY), and Thioredoxin enzyme (SlTRX) genes. Tomato 18S rRNA gene primer pair was used as endogenous control. Quantitative real-time RT-PCR was done using Maxima SYBR Green/ROX qPCR Master Mix (2×) Kit, and a melting curve analysis was performed. Data were normalized to the housekeeping gene SlEF1α and calculated using the $2^{-\Delta\Delta CT}$ method (Table 1) (Untergasser et al. 2012).

Statistical analysis

Duncan's test was performed using the SPSS (IBM SPSS Statistics version 22.0). Pearson correlation heatmaps were realized by ClustVis (Online web tool, https://biit.cs.ut.ee/clustvis/)

Table 1. The list of genes and their primers used in quantitative real-time RT-PCR assays.

Gene name	Gene id.	Forward (F) and reverse (R) primer sequence
Chitinase	*SlChi* (F)	5′-GCAGGGAGTGCAATAGGTGT-3′
	(R)	5′-CCACAACGCTGTTTTGAATG-3′
WRKY-type transcription factor	*SlWRKY* (F)	5′-GAAGAAGGTTGAGCGGTCTG-3′
	(R)	5′-TGGTTGGTTGATCATGGTTG-3′
APETALA2/ethylene responsive factor (transcription factors)	*SlAP2/ERF* (F)	5′-TCCGATCACATCTCCTCCTC-3′
	(R)	5′-ATTGGGGAGTTGGGTTCATT-3′
Lipoxygenase	*SlLOX* (F)	5′-ACTCCAGTCTTGGTGGATGG-3′
	(R)	5′-ACAGCAGCATGAAGAGCAGA-3′
Thioredoxin enzyme	*SlTRX* (F)	5′-TTTTGGCCTTTTTCACCATC-3′
	(R)	5′-ATCGGTTTACCTGCACTTGG-3′
Tomato *elongation factor* 1 alpha (reference gene)	*SlEF1α** (F)	5′-GGAACTTGAGAAGGAGCCTAAG-3′
	(R)	5′-CAACACCAACAGCAACAGTCT-3′

*The reference housekeeping gene SlEF1α was used for data normalization.

Results

TAEO effect on tomato antioxidant enzymes

The antioxidant enzyme activities varied significantly with treatments and time of analysis (Table 2). Plants treated with TAEO emulsion, fungicide, and infected with *B. cinerea* showed a significant increase in guaiacol peroxidase (GPX) activity compared to non-treated and non-infected plants, with fold increases ranging from 2.90 to 3.50 at 12 hpi. Pretreatment with TAEO emulsion and fungicide increased GPX activity by 1.26 and 1.03-fold, respectively, at 12 hpi. At 72 hpi, pretreatment with TAEO emulsion and fungicide significantly enhanced GPX activity by 3.67 and 3.22-folds compared to only infected plants (Table 2).

Table 2. Kinetic variation of the activities of some antioxidant enzymes in tomato plants treated or not with *Tetraclinis articulata* essential oil at different hours post infection (hpi) with *Botrytis cinerea*. Guaiacol peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX).

Enzymes	Treatment	12 hpi	24 hpi	48 hpi	72 hpi
GPX	T1	$21.55^{eC}\pm0.02$	$22.22^{dB}\pm00.00$	$23.91^{fA}\pm0.00$	$21.31^{fC}\pm0.04$
(μmoles $mn^{-1}mg^{-1}$)	T2	$62.65^{dD}\pm5.04$	$89.64^{aB}\pm2.73$	$80.13^{aA}\pm9.2$	$74.20^{dC}\pm3.16$
	T3	$75.53^{bB}\pm0.98$	$54.64^{cD}\pm3.20$	$62.02^{eC}\pm4.06$	$91.80^{bA}\pm1.22$
	T4	$73.51^{cA}\pm6.54$	$56.27^{cC}\pm0.52$	$70.00^{dB}\pm0.38$	$26.47^{eD}\pm0.02$
	T5	$93.18^{aB}\pm6.29$	$64.85^{bD}\pm1.42$	$73.15^{cC}\pm0.50$	$97.15^{aA}\pm3.97$
	T6	$75.94^{bC}\pm4.65$	$63.01^{bD}\pm1.07$	$90.13^{bA}\pm0.00$	$85.49^{cB}\pm1.12$
SOD	T1	$45.72^{eB}\pm12.08$	$45.07^{eB}\pm5.63$	$49.53^{eA}\pm1.18$	$45.30^{eB}\pm3.45$
(mg^{-1}. g^{-1}protein)	T2	$98.26^{dC}\pm2.38$	$225.41^{cB}\pm2.38$	$29.15^{dA}\pm0.88$	$94.05^{cD}\pm4.87$
	T3	$104.27^{cC}\pm8.16$	$229.20^{cB}\pm5.38$	$320.35^{bA}\pm1.30$	$52.85^{dD}\pm2.49$
	T4	$161.68^{bC}\pm2.58$	$237.01^{bB}\pm12.42$	$299.41^{cA}\pm1.98$	$149.34^{aD}\pm12.45$
	T5	$212.82^{aC}\pm2.28$	$268.41^{aB}\pm8.87$	$364.98^{aA}\pm1.01$	$116.96^{bD}\pm6.97$
	T6	$168.18^{bB}\pm2.63$	$167.36^{dB}\pm0.75$	$328.05^{bA}\pm8.32$	$120.58^{bC}\pm4.58$
CAT	T1	$21.16^{dB}\pm0.00$	$24.35^{eA}\pm1.97$	$24.23^{bA}\pm1.82$	$21.86^{dB}\pm0.47$
(mmoles. min^{-1}. mg^{-1})	T2	$27.49^{bB}\pm0.86$	$38.92^{bA}\pm0.59$	$27.70^{aB}\pm2.19$	$26.38^{cC}\pm0.15$
	T3	$25.78^{cC}\pm1.63$	$35.71^{cA}\pm2.45$	$23.56^{cD}\pm0.82$	$28.17^{aB}\pm2.32$
	T4	$24.95^{cC}\pm0.00$	$33.71^{dA}\pm1.40$	$23.77^{cD}\pm0.81$	$27.23^{bB}\pm1.31$
	T5	$30.70^{aB}\pm1.49$	$53.66^{aA}\pm0.71$	$27.74^{aC}\pm0.78$	$27.36^{bC}\pm0.00$
	T6	$13.72^{eC}\pm1.41$	$17.66^{fA}\pm0.78$	$17.30^{dA}\pm1.53$	$16.18^{eB}\pm0.42$
APX	T1	$9.06^{eC}\pm0.02$	$12.27^{dA}\pm0.09$	$11.98^{dA}\pm0.85$	$10.20^{eB}\pm0.45$
(mmoles. min^{-1}. mg^{-1})	T2	$80.72^{aA}\pm3.21$	$43.27^{aB}\pm1.56$	$21.82^{aD}\pm1.40$	$23.27^{aC}\pm2.60$
	T3	$10.12^{dD}\pm1.32$	$17.74^{cA}\pm1.75$	$15.15^{cB}\pm1.06$	$12.10^{dC}\pm0.12$
	T4	$18.57^{cB}\pm0.00$	$23.80^{bA}\pm0.38$	$17.24^{bC}\pm2.01$	$15.09^{cD}\pm0.08$
	T5	$43.04^{bA}\pm1.79$	$21.14^{bB}\pm2.02$	$21.59^{aB}\pm0.04$	$20.22^{bC}\pm1.06$
	T6	$10.14^{dC}\pm1.02$	$9.90^{eC}\pm0.80$	$16.59^{bA}\pm0.60$	$14.06^{cB}\pm1.41$

T1: Non-infected plants; T2: Essential oil treated plants; T3: Fungicide-treated plants; T4: *Botrytis cinerea* infected plants; T5: Plants treated with EO and infected with *Botrytis cinerea*; T6: Plants treated with fungicide and infected with *Botrytis cinerea*. hpi: Hour post inoculation. Different tiny letters indicate significant variation of means between the different treatments for each time of observation at $p < 0.05$ according to Duncan's multiple range test. Different capital letters indicate significant variation of means between times of observation in the same treatment at $p < 0.05$ according to the Duncan test.

The SOD enzyme activity showed a significant increase at 48 hpi in most treatments. At this time, plants treated with TAEO emulsion, the fungicide, and infected with *B. cinerea* separately exhibited a 6.05, 6.46, and 6.04-fold increase in SOD activity, respectively, compared to non-treated and non-infected plants. Treatment with TAEO emulsion increased SOD activity by 1.16-fold, while fungicide treatment decreased the activity of this enzyme in infected plants by 0.72-fold relative to only infected plants (Table 2).

CAT activity peaked at 24 hpi in all treatments. Plants treated with TAEO, fungicide, and infected with *B. cinerea* separately showed 1.59, 1.46, and 1.38-fold increases in CAT activity. TAEO pretreatment increased CAT activity by 1.50-fold, while fungicide pretreatment decreased it by 0.52-fold compared to only infected plants (Table 2).

APX activity varied with treatment and time (Table 2). At 12 hpi, plants treated with TAEO, the fungicide, and infected with *B. cinerea* showed increased APX activity by 8.90, 1.11, and 2.16, respectively, compared to non-treated and non-infected plants. Pretreatment with TAEO increased APX activity by 2.31, while pretreatment with the fungicide decreased it by 0.54-fold compared to only infected plants.

Comparing the effects of pretreatments with TAEO emulsion and the fungicide on only infected tomato plants over time, at 12 hpi, TAEO emulsion induced the activity of all four enzymes (GPX, SOD, CAT, and APX), while the fungicide maintained the activity of GPX and SOD but decreased the activity of CAT and APX. At 72 hpi, the TAEO emulsion increased the activities of GPX and APX, maintained the activity of CAT, and decreased the activity of SOD, while the fungicide only increased the GPX activity, maintained the APX activity, and decreased the activities of SOD and CAT (Table 2).

TAEO effect on total polyphenol content, free radical-scavenging activity, and hydrogen peroxide content of tomato plants

The study found that the total polyphenol content, antioxidant activity, and free radical content varied significantly with treatment and time (Table 3). At 48 hpi, TAEO emulsion treatment increased polyphenols by 1.6-fold, while fungicide treatment reduced polyphenols by 0.95-fold. TAEO pretreatment increased polyphenols by 1.36-fold, while fungicide pretreatment decreased them by 0.86-fold at 48 hpi. Additionally, TAEO emulsion treated tomato plants showed the maximum IC_{50} of free radical-scavenging activity. Treatment with TAEO and *B. cinerea* decreased IC_{50}, but fungicide maintained the same level of activity. Pretreatment with TAEO and fungicide of infected plants decreased and increased, respectively, the free radical-scavenging activity at 12 hpi.

The H_2O_2 content varied significantly according to the hour post inoculation and treatments (Table 3). In almost all the treatments the maximum of H_2O_2 contents was observed at 12 hpi with a decreasing trend over time until 72 hpi (Table 3). Currently point, treatment with TAEO emulsion, fungicide, and *B. cinerea* infection separately increased H_2O_2 content by 2.18, 3.02 and 6.46 folds, respectively, and in comparison, to non-treated and non-infected plants (Table 3). Pretreatment with TAEO emulsion and fungicide of *B. cinerea* infected plants decreased H_2O_2 content by 0.49 and 0.73-fold in comparison to only infected plants at 12 hpi (Table 3).

The analysis of correlation between the measured parameters of the total polyphenols content, the IC_{50} free radical-scavenging, and the hydrogen peroxide content in tomato leaves showed a positive correlation between the total polyphenols and the total antioxidant activity at all studied time points and a negative correlation between the total polyphenols and IC_{50} of the free-radical scavenging at 12, 24, and 48 hpi (Figure 1).

TAEO effect on phenolic composition

Table 4 shows the identified phenolic compounds in the methanolic leaf extracts of tomato plants, with no differences observed between treatments. Four hydroxycinnamic acid and two quercetin derivatives were present in all treatments.

Table 3. Kinetic variation of total polyphenol content, free radical-scavenging activity and hydrogen peroxide (H_2O_2) content in tomato plants treated or not with *Tetraclinis articulata* essential oil at different hours post infection (hpi) with *Botrytis cinerea*.

	Treatment	12 hpi	24 hpi	48 hpi	72 hpi
Total polyphenols (mg GAE g^{-1} FW)	T1	6.02^{dA}±0.02	5.23^{dB}±0.03	5.23^{dB}±0.56	5.48^{dB}±0.00
	T2	7.32^{bB}±0.07	6.92^{cC}±0.01	8.46^{bA}±0.05	6.05^{cD}±0.06
	T3	5.41^{eA}±0.07	5.08^{eB}±0.45	4.99^{eB}±0.09	5.07^{eB}±0.29
	T4	7.02^{cC}±0.63	7.80^{bB}±0.08	8.15^{cA}±0.02	8.05^{bA}±0.19
	T5	7.98^{aC}±0.23	8.88^{aB}±0.29	11.11^{aA}±0.17	9.02^{aB}±0.05
	T6	6.79^{cB}±0.40	6.63^{dB}±0.46	7.02^{cA}±0.02	4.29^{fC}±0.45
IC_{50} of free radical-scavenging activity (µg mL^{-1})	T1	570.84^{bA}±9.48	527.14^{bB}±9.23	580.47^{aA}±9.05	527.91^{bB}±7.32
	T2	370.66^{cA}±7.33	265.57^{cB}±6.58	222.23^{cC}±2.23	257.42^{eB}±8.38
	T3	581.45^{aB}±9.38	551.28^{aC}±3.80	587.37^{aA}±4.13	598.99^{aA}±1.30
	T4	309.65^{dB}±12.16	230.53^{eC}±7.21	217.20^{dD}±1.01	321.32^{cA}±1.09
	T5	245.12^{eA}±14.00	174.47^{FC}±1.40	121.83^{eD}±2.93	218.64^{fB}±11.53
	T6	368.62^{cA}±21.94	247.52^{dC}±0.80	252.96^{bC}±9.21	306.90^{dB}±11.99
H_2O_2 (µmol g^{-1} FW)	T1	151.53^{fA}±1.42	117.00^{fB}±1.67	112.70^{cC}±1.49	103.46^{cD}±5.81
	T2	330.90^{eB}±11.35	349.53^{aA}±12.95	301.43^{bC}±11.73	129.83^{aD}±3.12
	T3	459.03^{dA}±10.87	268.10^{cC}±4.84	334.60^{aB}±12.34	118.13^{bD}±4.90
	T4	979.63^{aA}±8.22	307.53^{bC}±6.16	328.66^{aB}±12.67	129.10^{aD}±2.28
	T5	483.96^{cA}±14.68	219.06^{eC}±11.28	287.36^{bB}±8.97	117.63^{bD}±7.02
	T6	719.26^{bA}±15.16	262.06^{dC}±8.49	326.56^{aB}±6.32	127.66^{aD}±2.75

T1: Non-infected plants; T2: Essential oil treated plants; T3: Fungicide treated plants; T4: *Botrytis cinerea* infected plants; T5: Plants treated with EO and inoculated with *Botrytis cinerea*; T6: Plants treated with fungicide and infected with *Botrytis cinerea*. Different tiny letters indicate significant variation of means between the different treatments for each time of observation at $p < 0.05$ according to the Duncan test. Different capital letters indicate significant variation of means between times of observation in the same treatment at $p < 0.05$ according to Duncan's multiple range test. hpi: hour post infection, GAE: gallic acid equivalent, DW: dry weight, IC_{50}: the concentration required to induce 50% 1,1-diphenyl-2-picrylhydrazyl (DPPH) inhibition. So, low levels of IC_{50} indicate better scavenging activity of the free radical DPPH in the methanolic extract.

The compound 1 was identified as 5-mono-caffeoilquinic acid (CQA) isomer (MM 354 Da) on the basis of its selectivity and fragmentation in MS^2 which matched that for the 5-CQA standard compound and by comparison to literature (Jaiswal et al. 2011) Using the same approach, the compounds 4 and 5 (MM 516) were identified as 1,5-di-CQA and 3,4-di-CQA, respectively, while compound 2 showed the same UV – Vis spectrum, selectivity, and fragmentation pattern of caffeic acid standard. Compounds 3 and 6 were identified as quercetin derivatives. Indeed, the compound 3 in MS/MS experiments fragmentation gives a base peak at m/z 301 due to the loss of a rhamnosylglucose residue (308 amu) and secondary ions at m/z 463 and 300; considering the loss of the sugar residue (Jaiswal et al. 2011) and the relative abundance of the aglycone, that is more abundant then the radical aglycone ion in 7-O glycosylated compounds. So, it was possible to putatively attribute to this compound the chemical structure of quercetin-7-O-rutinoside (Jaiswal et al. 2011). Following these criteria, the compound 6 was identified as quercetin-7-O-glucoside (Table 4).

TAEO effect on the expression of defense genes

The effect of foliar pulverization with TAEO emulsion or fungicide on the quantitative expression of the five defense-related genes (*SlChi*, *SlWRKY*, *SlAP2/ERF*, *SlLOX*, and *SlTRX*) prior to infection or not of tomato plants with *B. cinerea* was evaluated at different time points (Figure 2). The expression of the studied genes of the different treatments was compared to non-treated and non-infected plants.

The treatment of tomato plants only with the fungicide did not induce a substantial increase in *SlChi* gene expression over the time (Figure 2A). The treatment of plants with TAEO emulsion only induced *SlChi* gene expression at 24 hpi by 3.5-folds. The infection of tomato plants with *B. cinerea* induced *SlChi* gene expression at 24 hpi and 48 hpi by 4 and 3.5-folds, respectively (Figure 2A). While a slight increase in *SlChi* gene expression was observed in fungicide pretreated and *B. cinerea* infected plants at 24 hpi and 48 hpi by almost 2-folds, the pretreatment with TAEO emulsion of

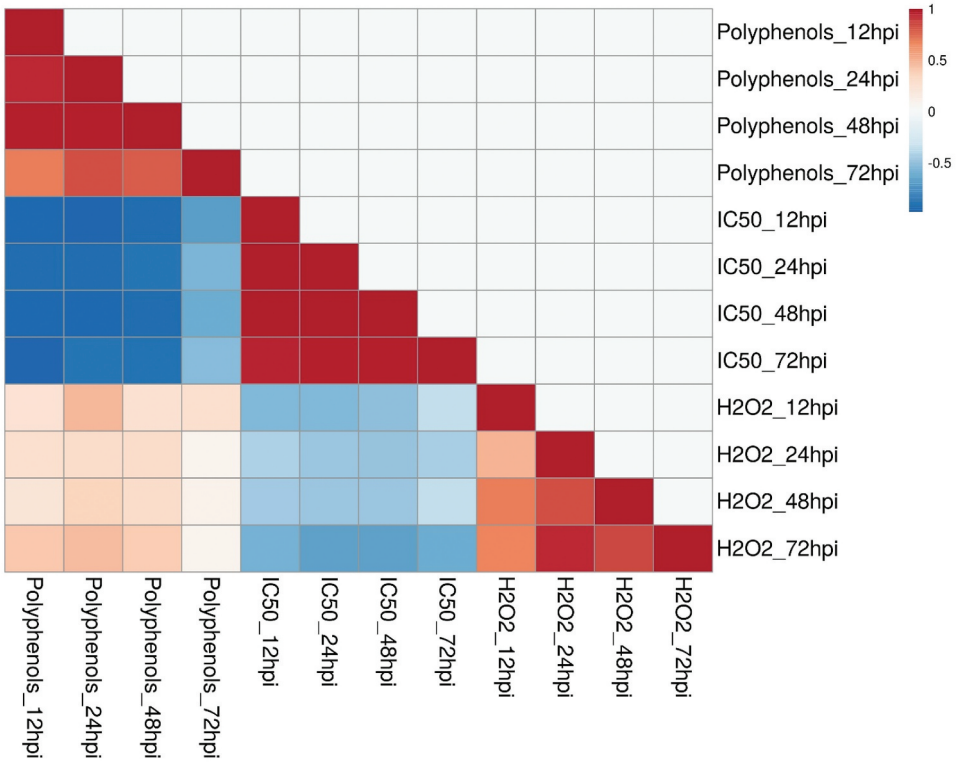

Figure 1. Heatmap of the kinetic variation of total polyphenol content (Polyphenols), IC_{50} of free radical-scavenging activity (IC50), hydrogen peroxide (H2O2) content and the activities of four antioxidant enzymes Guaiacol peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) in tomato plants treated or not with *Tetraclinis articulata* essential oil measured at different hours post infection (hpi) with *Botrytis cinerea*.

Table 4. HPLC-DAD-ESI/MS^n (negative ionization mode) profiling of phenolic compounds extracted from leaves of tomato plants. The MS^2 and MS^3 of phenolic compounds are presented.

Compound n°	Precursor Ion *(m/z)*	HPLC-ESI/MS^n *m/z* (% of base peak)	Compound identity (Jaiswal et al. 2011)
1*	353	MS^2 [353]: 191(100), 179(5), 135(5) MS^3[191]: 172(3), 127(25), 85(100)	5-caffeoylquinic acid
2*	179	MS^2 [179]: 135(100)	caffeic acid
3	609	MS^2[609]: 463(65), 301(100), 300(45)	quercetin-7-*O*-rutinoside
4	515	MS^2 [515]: 353(100), 335(50), 203(10), 191(35) MS^3[353]: 191(100), 179(150)	1,5-di-caffeoylquinic acid
5	515	MS^2[515]: 353(100), 299(20), 255(10), 203(5) MS^3[335]: 191(30), 173(100)	3,4-di-caffeoylquinic acid
6	463	MS^2[463]: 301(100), 300(60)	quercetin-7-*O*-glucoside

The compounds are mentioned according to their order of elution. * Compounds compared to standard.

B. cinerea infected plants induced by 22 and 6.5-folds the expression of this gene at 12 hpi and 48 hpi, respectively.

The treatment of tomato plants with the fungicide only induced *SlWRKY* gene expression at 12 hpi and 48 hpi by almost 2-folds (Figure 2B). The treatment with TAEO emulsion only induced *SlWRKY* gene expression at 48 hpi and 72 hpi by 2 and 10-folds, respectively. The infection of tomato plants with *B. cinerea* induced *SlWRKY* gene expression at 48 hpi and 72 hpi by 24- and 3-folds, respectively. The *SlWRKY* gene expression was induced in fungicide pretreated and *B. cinerea* infected plants at later time 48 hpi and 72 hpi by 2.5 and 9-folds, respectively. The

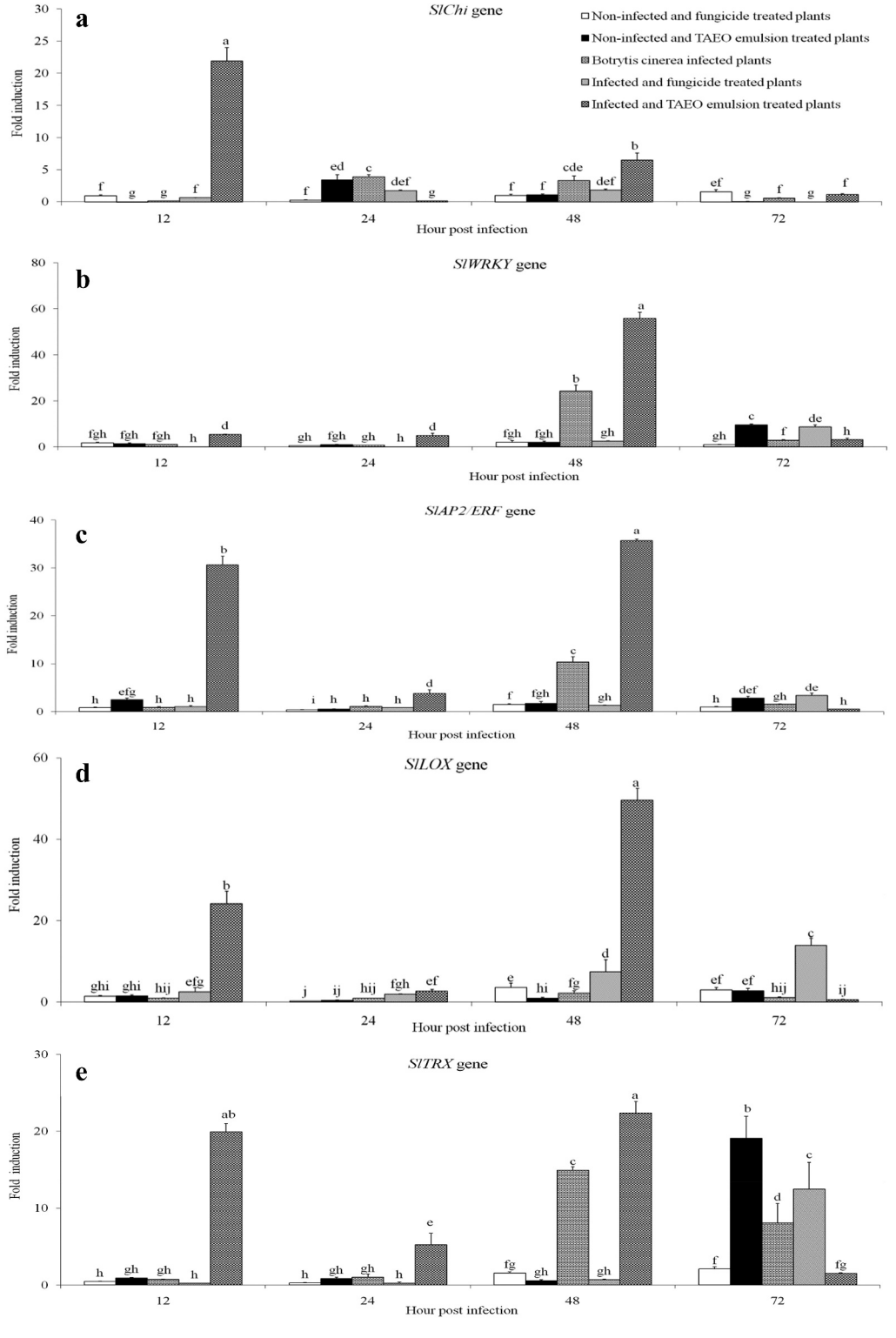

Figure 2. Kinetic of quantitative expression of defense-related genes Chitinase *SlChi* (a), the transcription factors *SlWRKY* (b) and *SlAP2/ERF* (c), Lipoxygenase *SlLOX* (d), and Thioredoxin *SlTRX* (e) in tomato plants treated or not with *Tetraclinis articulata* essential oil (TAEO) emulsion or a fungicide prior to infection or not with *Botrytis cinerea*. Data represent mean values of three independent amplifications and the error bars indicate standard deviation. Bars labeled with different letters indicate significant difference between treatments at $p < 0.05$ according to Duncan's multiple range test.

pretreatment with TAEO emulsion of infected plants induced *SlWRKY* gene expression by 5.5 and 56-folds at 12 hpi and 48 hpi, respectively.

The treatment with the fungicide only induced a slight increase in *SlAP2/ERF* gene expression at 48 hpi by 1.5-folds (Figure 2C). The treatment with TAEO emulsion only induced *SlAP2/ERF* gene expression at 12 hpi, 48 hpi, and 72 hpi by 2.5, 2, and 3-folds, respectively. The infection with *B. cinerea* induced *SlAP2/ERF* gene expression at 48 hpi and 72 hpi by 10 and 1.5-folds, respectively. The *SlAP2/ERF* gene expression was induced in fungicide pretreated and infected plants at later time 72 hpi by 3.5-folds. The pretreatment with TAEO emulsion of infected plants induced *SlAP2/ERF* gene expression by 31, 4, and 36-folds at 12 hpi, 24 hpi, and 48 hpi, respectively.

The treatment with the fungicide only induced an increase in *SlLOX* gene expression at 12 hpi, 48 hpi, and 72 hpi by 1.5, 3.5, and 3-folds, respectively (Figure 2D). The treatment with TAEO emulsion only induced *SlLOX* gene expression at 12 hpi, and 72 hpi by 1.5, and 3-folds, respectively. The infection-induced *SlLOX* gene expression at 48 hpi by 2.5-folds. The *SlLOX* gene expression was induced in fungicide pretreated and infected plants at all studied times with a maximum at 72 hpi by 14-folds. The pretreatment with TAEO emulsion of infected plants induced *SlLOX* gene expression by 24, 3, and 50-folds at 12 hpi, 24 hpi, and 48 hpi, respectively.

The treatment with the fungicide only induced an increase in *SlTRX* gene expression at 48 hpi and 72 hpi by almost 2-folds (Figure 2E). The treatment with TAEO emulsion only induced *SlTRX* gene expression at 72 hpi by 19-folds. The infection with *B. cinerea* induced *SlTRX* gene expression at 48 hpi and 72 hpi by 15- and 8-folds, respectively. The *SlTRX* gene expression was induced in fungicide pretreated and infected plants at 72 hpi by 12.5-folds. The pretreatment with TAEO emulsion of infected plants induced *SlTRX* gene expression by 20, 5.5, and 22-folds at 12 hpi, 24 hpi, and 48 hpi, respectively.

Overall, the results showed a transient or later induction of the five defense genes in solely *B. cinerea* infected plants, while TAEO emulsion pretreatment and infection with B. cinerea induced a great and early expression. Fungicide pretreatment induced a less extent and later expression of the five genes.

Discussion

Plant essential oils have been shown to be effective against agricultural pests and some fungal diseases. The essential oils extracted from *Cupressus sempervirens* and *Tetraclinis articulata* have demonstrated antifungal activities against *B. cinerea* (Rguez et al. 2018, 2020). While the mechanisms of action are still being studied, this report is the first to highlight the effect of foliar pulverization of TAEO on tomato defense against *B. cinerea*, with a focus on the antioxidant enzymatic system, synthesis of phenolic compounds and expression of tomato defense genes.

The pretreatments with TAEO emulsion of *B. cinerea* infected plants revealed an induction of the activity of the four studied enzymes at early infection, whereas the pretreatment with the fungicide maintained the activity of GPX and SOD and decreased of the activity of CAT and APX. APX and CAT constitute the main H_2O_2 scavenging enzyme systems in plants (Vergnes et al. 2014). Indeed, the early and important increase in the activity of these enzymes in TAEO emulsion pretreated and *B. cinerea* infected plants was probably responsible for the decrease of H_2O_2 levels of about 50% in tomato plants. In agreement with this result, H_2O_2 scavenging mechanisms has been shown as components of *Medicago truncatula* partial resistance to the oomycete *Aphanomyces euteiches*, and H_2O_2 levels were negatively correlated to APX, CAT, and lignin production in the root of the partially resistant ecotype in comparison to the sensitive one (Djébali et al. 2011; El Moussaoui et al. 2019).

The accumulation of ROS (Reactive oxygenate species) including H_2O_2 in plants tissues subjected to disease attacks was reported in several studies which are strongly oxidizing species that can react with all types of biomolecules and cause cells damages and enhance pathogens colonization of

plant tissues (Maliki et al. 2021). This study found that polyphenols play an important role in ROS scavenging mechanism. There was a positive correlation between total polyphenol and total antioxidant activity, and a negative correlation between total polyphenol and IC_{50} of free-radical scavenging in tomato leaves. The use of TAEO emulsion increased polyphenol contents and total antioxidant, and decreased IC_{50} free-radical scavenging activity in infected tomato plants at all studied times, while the fungicide pretreatment had the opposite effect. No differences were observed in the phenolic compounds extracted from tomato leaves among the different treatments. Six previously reported compounds were identified using HPLC-DAD-ESI/MSn analysis (Silva et al. 2014). TAEO emulsion treatment did not alter the polyphenols composition of tomato plants but increased their accumulation and antioxidant status. This is similar to the observed accumulation of polyphenols in infected coffee plants treated with *Cymbopogon citratus* EO (Pereira et al. 2012). Phenolic compounds not only scavenge ROS but also reinforce cell walls and increase plant resistance to pathogen penetration, enzymatic degradation of plant tissues, and diffusion of fungal toxins (Silva et al. 2014). TAEO emulsion treatment increased phenolic compounds in tomato plants, which may reduce *B. cinerea* penetration and colonization.

In this study, the expression of five tomato genes (SlChi, SlWRKY, SlAP2/ERF, SlLOX, and SlTRX) involved in biotic stress response were analyzed in *B. cinerea* infected tomato plants pretreated with TAEO emulsion or fungicide. The solely infected plants showed an transient increase in the expression of some genes, while the pre-treatment with TAEO emulsion induced an early and sustained expression of all five genes, whereas the fungicide treatment induced expression to a lesser extent and at a later time. The activity of a *Chitinase* gene was induced at 7 dpi in tomato plants infected with *Clavibacter michiganensis* (Baysal et al. 2003) which degraded fungal cell-wall contributing to plant resistance to disease infection (Raghav 2014). The over expression of *ERF1* gene in *Arabidopsis thaliana* was sufficient to confer resistance against *B. cinerea* (Berrocal-lobo et al. 2002).

The *WRKYs* were involved as regulators in defense responses and can interact directly with pathogen-associated molecular patterns or effector proteins to activate or repress plant defense (Bakshi and Oelmüller 2014). Also, the analysis of *WRKY* genes confirmed that they are involved as effectors to activate local and systemic acquired resistance response by production of ROS and hormonal pathways (Bai et al. 2018). Lipoxygenase (LOX) is involved in the generation of oxilipins derived from lipids which are implicated in jasmonic acid biosynthesis that regulates the expression of plant defense genes and resistance to pathogen attacks (Hu et al. 2015). The thioredoxin gene (*TRX*) has a fundamental role in ROS scavenging system during normal development and pathogens infection (Liu and Han 2010). In fact, ROS such as H_2O_2 can be indirectly scavenged by *TRX* pathway (Wang et al. 2013; Mata-Pérez and Spoel 2019) and consequently reduce oxidative damage of cells.

Conclusion

In conclusion, it was shown that pretreatment of non-infected and *B. cinerea* infected tomato plants with TAEO emulsion increased the antioxidant enzymes activities, total polyphenols contents, and improved the overall antioxidant status of plants. In addition, the pretreatment with TAEO emulsion was more effective than fungicide in inducing plant antioxidant defense system. A quite similar antioxidant status in plants treated with TAEO emulsion or infected with *B. cinerea* was observed, which likely indicate that plant treatment with the essential oil mimic an infection condition leading to a priming status in the plants which may explain the better antioxidant response of *B. cinerea* infected plants when pretreated with TAEO emulsion. The early and great activation of the expression of the defense genes *SlChi, SlWRKY*, *SlAP2/ERF*, *SlLOX*, and *SlTRX* by TAEO emulsion in comparison to the synthetic fungicide "Désogerme SP Végétaux" likely indicates its potential as a broad spectrum biofungicide which enhance plant defenses against several pathogens of tomato offering an alternative to the use of chemical fungicides in conventional and biological agriculture systems.

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Abbreviation list

APX	Ascorbate peroxydase
B. cinerea	*Botrytis cinerea*
CAT	Catalase
CHI	Chitinase
EO	Essential oil
ERF	Ethylene response factor
FG	Fongicide
FM	Fresh matter
GAE	Equivalent acide gallique
GC-MS	Gas chromatography coupled with mass spectrometry
GPOX	Guaïacol peroxydase
HPI	Hour after inoculation
IC_{50}	Inhibition concentration at 50%
LOX	Lipoxygénases
MW	Molecular wight
PCR	Polymerase chain reaction
ROS	Reactive oxygenated species
RP-HPLC-DAD-ESI-MSn	Chromatographie liquide en phase inverse couplée à la spectrométrie de masse par électro-nébulisation
SOD	Superoxyde dismutase
T. articulata	*Tetraclinis articulata*
TAEO	*Tetraclinis articulata* essential oil
TRX	Thioredoxine

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