Environmental and Experimental Botany 208 (2023) 105249

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Significance of phenylpropanoid pathways in the response of two pomegranate cultivars to salinity and ozone stress

Antonella Calzone [a], Mariagrazia Tonelli [a], Lorenzo Cotrozzi [a,b], Giacomo Lorenzini [a,b], Cristina Nali [a,b,*], Elisa Pellegrini [a,b]

[a] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy
[b] Nutrafood Research Center, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy

ARTICLE INFO

Keywords:
Air pollution
Punica granatum
Salt stress
Secondary metabolites
Sequential stress
Stress-tolerance

ABSTRACT

Secondary metabolites play a pivotal role in defense mechanisms, especially in plant-environment interactions. However, there are still difficulties in ascertaining the metabolic effect of their production due to individual and/or combined stress, given the variety of simultaneous and interconnected effects of multiple stresses to metabolic processes in plants. Here, two pomegranate cultivars (Wonderful and Parfianka) were subjected to moderate levels of salt (i.e., 100 mM NaCl for 35 consecutive days) and sequentially exposed to a realistic O_3 concentration (i.e., 100 ppb for 5 h) to evaluate how NaCl treatment and/or O_3 fumigation alter the leaf polyphenolic composition, and what is the role of metabolites and enzymes involved in the phenylpropanoid pathway during sequential abiotic stresses. Wonderful leaves responded to NaCl or O_3 treatments through the production/accumulation of cinnamic acid derivatives (more than 3-fold higher than controls) and by exhibiting chemical composition plasticity against oxidative stress. The unchanged concentrations of benzoic acid derivatives indicate that treated Wonderful leaves could diversify the components of polyphenolic compounds and induce the biosynthesis of the components available (e.g., ellagitannins and punicalagin). During sequential abiotic stresses, Wonderful leaves needed few cellular adjustments to maintain oxidative stress under control (especially during the recovery time). On the other hand, Parfianka leaves had phenolics/polyphenolics amount (at constitutive level) suitable to face out the environmental changes (occurring singly or sequentially) so that no rearrangement of metabolites and enzymes involved in the phenylpropanoid pathway was required. Consequently, it is possible to conclude that Parfianka is more salt- and O_3-tolerant than Wonderful.

1. Introduction

Due to their sessile entities, plants have evolved various mechanisms at different levels for accommodating changes arising in their fluctuating growth conditions to cope with adverse environments (Chen et al., 2022). In particular, plants make use of constitutive and induced defense systems by orchestrating the production/consumption of antioxidants and phytohormones to regulate their basal metabolism, avoid or tolerate stress effects, and ensure survival (Isah, 2019). Secondary metabolites play a pivotal role in defense mechanisms, especially in plant-environment interactions (Srivastava et al., 2021). However, there are still difficulties in ascertaining the metabolic effects of their production under individual and/or combined stress, given the variety of simultaneous and interconnected effects of multiple stresses to metabolic processes in plants (Selmar and Kleinwächter, 2013). Plant responses to single-stress factors are already subject to complex regulatory mechanisms, which can be highly influenced and further intricated by environmental conditions and additional stress factors commonly co-occurring in the field (Sukuzi et al., 2014). How plants regulate and prioritize their adaptive response when subjected to multiple stresses is largely unknown. Evidence suggests that under combined stresses plants exhibit tailored physiological, biochemical and molecular responses, in addition to several cross responses as part of their stress tolerance strategy (Ramegowda and Senthil-Kumar, 2015).

Among the major abiotic stress factors, salinity may be considered a multi-component stress due to the involvement of several metabolic pathways and cellular adaptation (Cabot et al., 2014). Salinity usually occurs simultaneously with other stresses, including ozone (O_3) insult, which represents the major air pollutant in the Mediterranean basin, negatively affecting many biological activities in living organisms

* Corresponding author at: Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, I-56124 Pisa, Italy.
E-mail address: cristina.nali@unipi.it (C. Nali).

https://doi.org/10.1016/j.envexpbot.2023.105249
Received 2 August 2022; Received in revised form 14 January 2023; Accepted 31 January 2023
Available online 1 February 2023

(Sillmann et al., 2021). Plants can respond to salinity or long- and short-term O_3 exposure by synthesizing and/or accumulating different classes/components of secondary metabolites, which are more species specific than primary metabolites and are associated with plant protection due to their multiple functions [e.g., reactive oxygen species (ROS) scavengers, structural polymers (i.e., lignin; Richet et al., 2012), attractants and UV screeners (i.e., flavonoids), signalling (i.e., salicylic acid), and defense response (e.g., phytoalexins); Assaf et al. (2022)]. Recent experimental works studied the association of phenolic compounds and antioxidant activity in plants subjected to salt or O_3 stress, reporting that elevated levels of polyphenolics, flavonoids and/or anthocyanins could be involved in stress tolerance (Ashraf and Ali, 2010; Jolivet et al., 2016; Abdel-Farid et al., 2020; Sarri et al., 2021). These compounds were also proposed as stress sensors (Assaf et al., 2022). Some studies also focused on the combined effects of salinity and O_3, where stressors were supplied simultaneously and for a long time (Guidi et al., 2017; Natali et al., 2018). However, there is little available information on how salinity and O_3 (applied singularly, simultaneously or sequentially) influence polyphenolic composition at quanti-qualitative level. Plant species can respond to the environmental stimuli through diversifying the components and inducing the biosynthesis of the components available (Kulak et al., 2020). Their success depends not only upon the enormous numbers of different metabolites synthesized by different taxa, but also on the multifunctionality of each "specialized metabolite" depending on the type and the intensity of environmental inputs, cultivar/provenance and the plant developmental stage (Cotrozzi et al., 2018; Calzone et al., 2021a, 2021b). Consequently, analysis of cellular and metabolic rearrangements provides complementing evidence to describe the role(s) of secondary metabolites in the adaptation/acclimation of plants to harsh environmental conditions, such as the complex interactions between salinity and O_3.

A plant species that has shown a good tolerance to abiotic stresses is *Punica granatum* L. (pomegranate, *Punicaceae* family). Native to the Middle East and Mediterranean areas, and now naturalized in many other regions worldwide, it is still evaluated as a minor crop, but the demand in pomegranate fruits is rapidly increasing, especially because of their large content of bioactive compounds, most of which are phenylpropanoids. Similarly to other plants (e.g., wheat, rice, bean, maize; Feng et al., 2016; Fatima et al., 2019: Farroq et al., 2021), the degree of salt- and O_3-tolerance in pomegranate is a cultivar-dependent feature. Indeed, in previous papers focused on the physiological, biochemical and molecular responses of the widely grown cultivars Wonderful and Parfianka to salinity and O_3 (applied singularly), we reported a differential salt and O_3 tolerance, this being moderate for Wonderful and elevated for Parfianka (Calzone et al., 2020a, 2021a; b). Thus, there are still many open questions about whether and how the antioxidant strategy adopted by pomegranate cultivars under salinity may affect their potential capability to avoid O_3 stress and/or tolerate O_3-induced effects (e.g., by altering polyphenolic composition at quanti-qualitative level).

The aim of the present study was to characterize the role of metabolites and enzymes involved in the phenylpropanoid pathway in Wonderful and Parfianka plants subjected to moderate levels of salt (i.e., 100 mM NaCl for 35 consecutive days) and sequentially exposed to realistic O_3 concentrations (i.e., 100 ppb for 5 h). Specifically, we hypothesized the following questions: (i) How much NaCl treatment and/or O_3 fumigation alter polyphenolic composition of Wonderful and Parfianka leaves?, and (ii) Are metabolic responses markedly stress- and cultivar-specific? We postulated that differences in cellular and metabolic rearrangement of phenylpropanoids and stress-tolerance between cultivars could occur under singular and sequential stress conditions.

2. Materials and methods

2.1. Plant material and experimental design

On April 2019, three-year-old pomegranate plants of commercial cultivars Wonderful and Parfianka were selected for uniformity of height (ca. 1 m), transplanted into 5-L plastic pots containing sandy soil, and maintained well-watered under standard agronomic conditions. After one month (i.e., May 2019) and one week of gradual salt acclimation (Calzone et al., 2020a), 48 plants *per* cultivar were subjected to two salinity treatments for 35 days by daily applications of 200 mL of 0 (EC = 0.05 mS/cm; controls) and 100 mM NaCl solutions (EC = 8.36 mS/cm). At the end of the NaCl treatment, plants were maintained well-watered with deionized water, and transferred into four greenhouse fumigation chambers and exposed to O_3 (100 ppb for 5 h from 10:00–15:00) or ventilated charcoal-filtered air (<5 ppb of O_3; two chambers *per* O_3 treatment). The O_3 exposure was performed with a Fisher 500 air-cooled O_3 generator (Fisher America Inc., Houston, TX, USA), according to Cotrozzi et al. (2018), and all plants were kept under charcoal-filtered air under after the O_3 exposure.

At 5 and 24 h from the end of NaCl treatment (i.e., re-watering period) or from the beginning of O_3 exposure (FBE; i.e., at the end of O_3 exposure and at recovery, respectively), two leaf samples (each from younger and older mature leaves) were collected *per* three plants *per* cultivar/salt/O_3 treatment combination, instantly frozen in liquid nitrogen, and stored at −80 °C until biochemical analyses.

2.2. Determination of phenylpropanoid contents

Products of phenylpropanoid pathway were assessed according to Jakopic et al. (2011), with minor modifications. Leaf raw material (30 mg fresh weight) was homogenized in 500 μL of 100% methanol and incubated in a water bath for 30 min at room temperature. After centrifugation (12,000 *g* for 10 min at room temperature), the supernatant was filtered through 0.2 μm filters Minisart® SRT 15. High Performance Liquid Chromatography (HPLC; P680 Pump, UVD 170 U UV–VIS detector, Dionex, Sunnyvale, CA, USA) separation was performed with a reverse-phase Acclaim 120 C18 column (5 μm particle size, 4.6 mm internal diameter × 150 mm length) mounted in a Dionex TCC-100 column oven (Thermo Scientific, Waltham, MA, USA) at 30 °C. Setting the flow rate at 1 mL min^{-1}, phenylpropanoids were eluted as follows: 100% solvent A [water/methanol/acetic acid, 75:20:5 (v/v/v)] for 1 min, a 30 min linear gradient to 100% solvent B (water/-methanol/acetic acid, 50:45:5 (v/v/v)], a 5 min linear gradient to 100% solvent A, and finally 5 min 100% solvent A. Phenylpropanoid compounds were detected at 254, 280 and 348 nm. Compound concentrations were quantified using equations developed linearly relating peak areas with known amounts of pure standards injected into the HPLC system. Chromatographic data were processed and recorded by Chromeleon Chromatography Management System software, version 6.60–2004 (Thermo Scientific). Phenylpropanoid metabolites are also grouped on the basis of their chemical structures and presented as cinnamic acid derivatives (total amount of caffeic, ferulic and trans-cinnamic acids), benzoic acid derivatives (total amount of gallic and ellagic acids), and ellagitannins [total amount of punicalin (α + β) and punicalagin (α + β)].

2.3. Determination of enzymes involved in the phenylpropanoid metabolism

The activity of shikimate dehydrogenase (SKDH; EC 1.1.1.25) was determined according to Diaz et al. (1994), with minor modifications. Leaf raw material (50 mg fresh weight) was extracted with 1 mL potassium phosphate buffer (0.1 M; pH 7.4) containing dithiotreitol (DTT; 0.5 mM), L-cysteine (2 mM), EDTA (2 mM) and 2-mercaptoethanol (8 mM). After centrifugation (19,000 *g* for 20 min at 4 °C), the supernatant

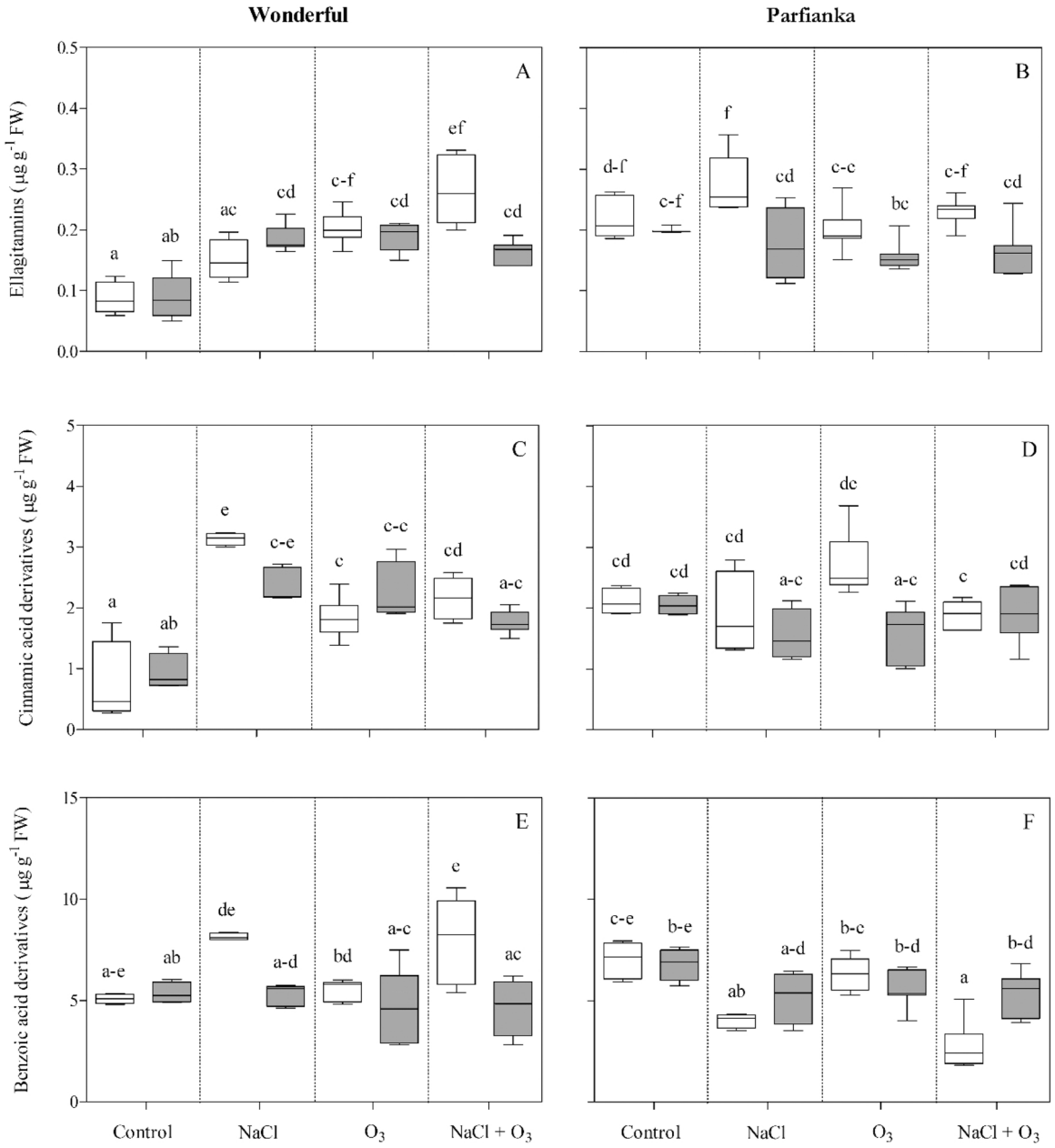

Fig. 1. Box and whiskers representation of the content of ellagitannins (A, B), cinnamic acid derivatives (C, D) and benzoic acid derivatives (E, F) in Wonderful and Parfianka leaves subjected to salt treatment (NaCl, 100 mM for 35 consecutive days) and ozone fumigation (O_3, 100 ppb for 5 h) applied alone or sequentially (NaCl + O_3). Measurements were carried out at 5 and 24 h (white and grey coloured bars, respectively) after the re-watering and/or from the beginning of O_3 exposure. For each boxplot, median, 25th-75th percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. Different letters indicate significant differences among experimental groups ($P \leq 0.05$, Tukey's post-hoc test, N = 6). Abbreviation: FW, fresh weight.

was directly used in the enzyme assays. The reaction mixture containing 50 µL of enzymatic extract, 100 µL of shikimic acid (40 mM) and 100 µL of $NADP^+$ (20 mM) in 750 µL of Tris-HCl (0.1 M; pH 9) was incubated at 25 °C. The reduction of NADP (due to the oxidation of shikimic acid to dehydroshikimic acid) was followed at 340 nm for 5 min by using a spectrophotometer (UV-1900, Shimadzu, Kyoto, Japan).

The activity of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) was determined in terms of trans-cinnamic acid production according to Mitchell et al. (1994), with minor modifications. Leaf raw material (50 mg fresh weight) was extracted with 1 mL of potassium phosphate buffer (0.1 M; pH 8) containing EDTA (2 mM), 2-mercaptoethanol (1.4 mM) and 1% PVPP (w/v). Particulate material was removed by centrifugation (19,000 *g* for 8 min at 4 °C), and the supernatants were used directly in the enzyme assays. The reaction mixture containing 100 µL of enzymatic extract, 900 µL of L-phenylalanine (100 mM in Tris-HCl 100 mM, pH 8) was incubated at 37 °C. After 2 h, 50 µL of HCl (6 N) were added to stop the reaction. Absorbance of the final product was measured at 290 nm with the same spectrophotometer reported above. The activity of PAL was determined using the molar extinction coefficient for trans-cinnamic acid (9.63 mM^{-1} cm^{-1}).

The activity of cinnamyl alcohol dehydrogenase (CAD; 1.1.1.195) was determined according to Mitchell et al. (1994), with minor modifications. Leaf raw material (50 mg fresh weight) was extracted with 1 mL of monobasic sodium phosphate buffer (0.1 M; pH 7.3) containing DTT (100 mM) and 2-mercaptoethanol (40 mM). Particulate material was removed by centrifugation (19,000 *g* for 8 min at 4 °C), and the supernatants were used directly in the enzyme assays. The reaction mixture containing 100 µL of enzymatic extract, 400 µL of coniferyl

Table 1
Content of punicalagin and of caffeic, trans-cinnamic and gallic acids (μg g^{-1} fresh weight) in Wonderful and Parfianka control leaves or subjected to salt (NaCl, 100 mM for 35 consecutive days) treatment and ozone (O_3, 100 ppb for 5 h) applied alone or sequentially (NaCl + O_3). Measurements were carried out at 5 and 24 h after the re-watering and/or from the beginning of O_3 exposure. All data are reported as mean ± standard deviation (N = 6). Different letters indicate significant differences among treatments according to Tukey's post-hoc test ($P \leq 0.05$).

	Punicalagin	Caffeic acid	Trans-cinnamic acid	Gallic acid
Wonderful				
5 h				
Control	0.077 ± 0.024a	0.135 ± 0.090ab	0549 ± 0314a	2.516 ± 1.400ab
NaCl	0.134 ± 0.034b-e	0.279 ± 0.050a-d	0794 ± 0294a-d	3.760 ± 2.544ab
O_3	0.180 ± 0.024d-g	0.142 ± 0.013ab	1070 ± 0382b-e	4.345 ± 0.044bc
NaCl + O_3	0.234 ± 0.045fg	0.253 ± 0.055b-d	1254 ± 0197de	6.981 ± 2.006c
24 h				
Control	0.062 ± 0.029ab	0.100 ± 0.015a	0636 ± 0269ab	3.679 ± 1.700ab
NaCl	0.151 ± 0.027c-e	0.825 ± 0.122d	1279 ± 0159c-e	4.431 ± 0.431bc
O_3	0.154 ± 0.019c-e	0.209 ± 0.072b-d	1399 ± 0232e	3.717 ± 1.850bc
NaCl + O_3	0.134 ± 0.018c-d	0.194 ± 0.088a-d	1026 ± 0187a-e	3.998 ± 1.312bc
Parfianka				
5 h				
Control	0.168 ± 0.010c-f	0.205 ± 0.043a-d	1061 ± 0117a-e	5.266 ± 0.758bc
NaCl	0.246 ± 0.044g	0.172 ± 0.053a-d	0893 ± 0235a-e	3.498 ± 1.803a-c
O_3	0.175 ± 0.033c-f	0.276 ± 0.057cd	1210 ± 0291c-e	4.869 ± 0.641bc
NaCl + O_3	0.195 ± 0.032e-g	0.181 ± 0.048a-d	0825 ± 0100a-d	2.256 ± 2.457a
24 h				
Control	0.111 ± 0.052a-c	0.165 ± 0.097a-d	0733 ± 0356a-c	5.871 ± 0.335a-c
NaCl	0.164 ± 0.055c-f	0.140 ± 0.057a-d	0713 ± 0230a-c	3.503 ± 1.284a-c
O_3	0.126 ± 0.025b-d	0.164 ± 0.040a-d	1039 ± 0217b-e	4.562 ± 0.843bc
NaCl + O_3	0.135 ± 0.037b-e	0.155 ± 0.086a-c	1103 ± 0294b-e	3.931 ± 1.252a-c

alcohol (0.5 mM) and 50 μL of $NADP^+$ was incubated at 30 °C. The formation of coniferaldehyde was monitored at 400 nm for 30 min at 30 °C by using the same spectrophotometer reported above.

Protein concentrations were determined with the same spectrophotometer reported above using bovine serum albumin as standard (Bradford, 1976).

2.4. Statistical analysis

Data distribution was preliminary tested by the Shapiro-Wilk test. The effects of cultivar, NaCl, O_3, time and their interactions on leaf parameters were mostly investigated by using a four-way analysis of variance (ANOVA). However, as the values of all tested enzymes, catechins and ferulic acid were not normally distributed, a Kruskall-Wallis test was used to determine significant differences of these parameters among experimental groups. The Tukey's honestly significant difference (HSD) and Dunn's multiple comparison post-hoc tests were used for normally and not normally distributed parameters, respectively. Statistically significant effects were considered for $P \leq 0.05$. Statistical analyses were run in JMP 13.2.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Phenylpropanoid profiles

Ten phenolic compounds were identified in *P. granatum* leaves: five of these compounds were simple phenolic acids (i.e., non-flavonoid phenolic compounds), which can be further divided into two main types, cinnamic and benzoic acid derivatives. Specifically, three hydroxycinnamic acids (caffeic, ferulic and trans-cinnamic acids), and two hydroxybenzoic acids (ellagic and gallic acids) were identified. The five remaining compounds belonged to flavones (apigenin), flavonols (quercetin), flavanols (catechins) and ellagitannins (punicalin α + β and punicalagin α + β). A significant "cultivar × NaCl × O_3 × time" interaction was reported for caffeic, trans-cinnamic and gallic acids, and punicalagin, as well as for the fractions of cinnamic and benzoic acid derivatives, and ellagitannins. Kruskall-Wallis test also showed significant differences for PAL and CAD activities ($P \leq 0.01$), but Dunn's multiple comparison test showed significant difference among means only in terms of CAD activity (Table 2).

Significant differences were observed in the phenylpropanoid profiles between controls of the two cultivars at the beginning of the experiment. Specifically, Parfianka leaves exhibited higher concentrations than Wonderful ones, especially in terms of ellagitannins (2-fold; Fig. 1A-B) and ferulic, gallic and ellagic acids (more than 2-fold, Tables 1 and 2). In both untreated cultivars, highest concentrations were detected for gallic acid, which reached around 70% of the total amount of phenolic acids. No visible symptoms due to NaCl treatment and/or O_3 exposure were observed throughout the whole experiment.

3.2. Effects of NaCl treatment

In Wonderful leaves, NaCl treatment induced a marked accumulation of ellagitannins after 24 h of re-watering (2-fold higher than untreated ones) and a concomitant increase of punicalagin levels (about 2-fold higher; Tables 1 and 2). No significant differences were observed after 5 h of re-watering (Fig. 1 A). A production of cinnamic acid derivatives was observed at the end of NaCl treatment, reaching a maximum after 5 h of re-watering (Fig. 1 C). A concomitant rise of trans-cinnamic acid was observed (Table 2). No significant differences were reported after the end of NaCl treatment in terms of benzoic acid derivatives (Fig. 1E). PAL and SKDH activities revealed no significant differences between salt-treated and untreated plants after the end of NaCl treatment (independently of sampling times; Fig. 2A and Table S1). Conversely, the activity of CAD slightly decreased after 5 h of re-watering (−45%) and strongly increased at 24 h after the end of NaCl treatment (about 2-fold higher; Fig. 2 C).

In Parfianka leaves, NaCl treatment did not alter the content of ellagitannin and cinnamic acid derivatives (Fig. 1B-D). A decrease of benzoic acid derivatives level was observed after 5 h of re-watering (−25% compared to untreated ones; Fig. 1 F), even if no significant differences were observed in terms of individual benzoic acids (i.e., gallic and ellagic acids; Tables 1 and 2). A production of punicalagin was reported at 5 h (+14%; Tables 1 and 2). PAL and SKDH activities revealed no significant differences between salt-treated and untreated plants throughout the whole experiment (Fig. 2B and Table S1). Conversely, the activity of CAD significantly increased at 5 h (+65%; Fig. 2D).

Although the interaction "cultivar × NaCl × O_3 × time" was not significant for punicalin and ellagic acid, statistical differences were observed for the interactions "cultivar × NaCl × O_3" [Parfianka leaves responded to both treatments by reducing the content of punicalin (−15% compared with controls), whereas no significant differences were observed in Wonderful] and "NaCl × O_3" [NaCl treatment stimulated the synthesis of ellagic acid (+79%)] (Table S1).

Table 2
Content of punicalin, ferulic acid, ellagic acid, catechins, quercitin and apigenin (μg g^{-1} fresh weight, FW) in Wonderful and Parfianka leaves subjected to salt (NaCl, 100 mM for 35 consecutive days) treatment and ozone (O_3, 100 ppb for 5 h) applied alone or sequentially (NaCl + O_3). Measurements were carried out at 5 and 24 h after the re-watering and/or from the beginning of O_3 exposure. All data are reported as mean ± standard deviation (N = 6).

	Punicalin	Ferulic acid	Ellagic acid	Catechins	Quercitin	Apigenin
Wonderful						
5 h						
Ctrl	0.011 ± 0.002	0.199 ± 0.160	0.404 ± 0.450	0.236 ± 0.182	0.136 ± 0.088	0.143 ± 0.089
NaCl	0.023 ± 0.001	0.973 ± 0.949	1.170 ± 1.082	0.378 ± 0.257	0.241 ± 0.074	0.227 ± 0.072
O_3	0.022 ± 0.003	0.611 ± 0.136	1.070 ± 0.143	0.396 ± 0.046	0.193 ± 0.126	0.248 ± 0.015
NaCl + O_3	0.030 ± 0.016	0.654 ± 0.220	1.044 ± 0.246	0.373 ± 0.103	0.290 ± 0.051	0.126 ± 0.085
24 h						
Ctrl	0.028 ± 0.009	0.219 ± 0.040	0.636 ± 0.269	0.173 ± 0.101	0.115 ± 0.039	0.202 ± 0.079
NaCl	0.034 ± 0.010	0.783 ± 0.270	1.279 ± 0.159	0.457 ± 0.127	0.248 ± 0.108	0.224 ± 0.118
O_3	0.035 ± 0.017	0.726 ± 0.297	1.399 ± 0.232	0.363 ± 0.093	0.247 ± 0.034	0.268 ± 0.076
NaCl + O_3	0.027 ± 0.006	0.537 ± 0.159	0.888 ± 0.333	0.366 ± 0.076	0.248 ± 0.049	0.239 ± 0.042
Parfianka						
5 h						
Ctrl	0.045 ± 0.022	0.825 ± 0.315	1.521 ± 0.244	0.498 ± 0.122	2.091 ± 0.475	6.788 ± 1.001
NaCl	0.031 ± 0.020	0.940 ± 0.437	1.584 ± 0.605	0.342 ± 0.145	2.778 ± 0.771	6.401 ± 0.974
O_3	0.025 ± 0.009	1.292 ± 0.423	1.532 ± 0.334	0.358 ± 0.078	2.006 ± 0.725	5.082 ± 2.408
NaCl + O_3	0.024 ± 0.008	0.862 ± 0.146	1.319 ± 0.337	0.312 ± 0.108	1.868 ± 0.294	3.575 ± 2.794
24 h						
Ctrl	0.030 ± 0.014	0.838 ± 0.151	0.870 ± 0.463	0.410 ± 0.270	1.736 ± 0.604	6.741 ± 0.798
NaCl	0.041 ± 0.017	0.712 ± 0.168	1.681 ± 0.082	0.160 ± 0.148	1.623 ± 0.561	5.647 ± 1.061
O_3	0.034 ± 0.011	0.419 ± 0.305	1.085 ± 0.218	0.337 ± 0.100	1.565 ± 0.455	5.183 ± 1.366
NaCl + O_3	0.031 ± 0.007	0.628 ± 0.261	1.169 ± 0.351	0.380 ± 0.110	1.886 ± 0.641	5.100 ± 1.603

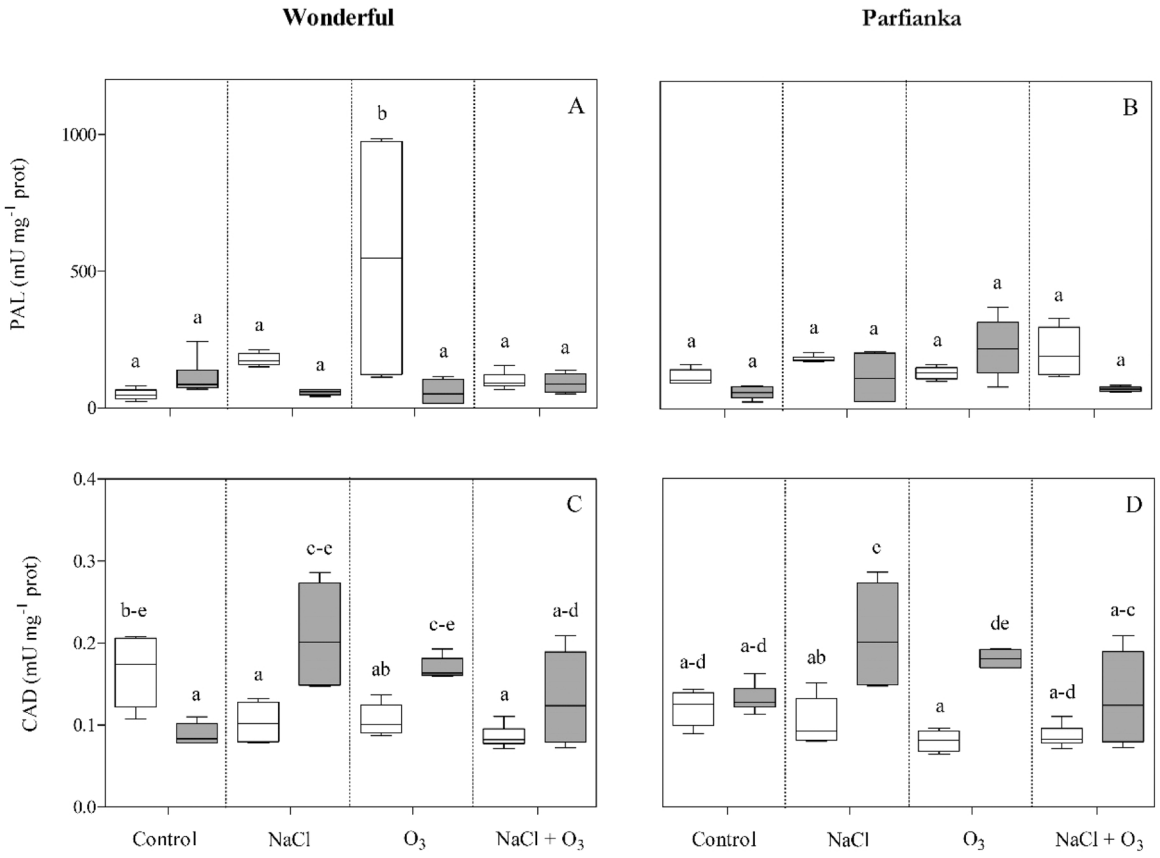

Fig. 2. Box and whiskers representation of the activity of phenylalanine ammonia-lyase (PAL; A, B) and cinnamyl alcohol dehydrogenase (CAD; C, D) in Wonderful (left) and Parfianka (right) control leaves or subjected to salt treatment (NaCl, 100 mM for 35 consecutive days) and ozone fumigation (O_3, 100 ppb for 5 h) applied alone or sequentially (NaCl + O_3). Measurements were carried out at 5 and 24 h (white and grey coloured bars, respectively) after the re-watering and/or from the beginning of O_3 exposure. For each boxplot, median, 25th-75th percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported. Different letters indicate significant differences among experimental groups ($P \leq 0.05$, Dunn's multiple comparison post-hoc test, N = 6).

3.3. Effects of ozone fumigation

In Wonderful leaves, O_3 fumigation induced a marked accumulation of ellagitannins at 5 and 24 h FBE (2-fold higher than untreated ones; Fig. 1 A), and a concomitant increase of punicalagin levels was observed (about 2-fold higher; Tables 1 and 2). A production of cinnamic acid

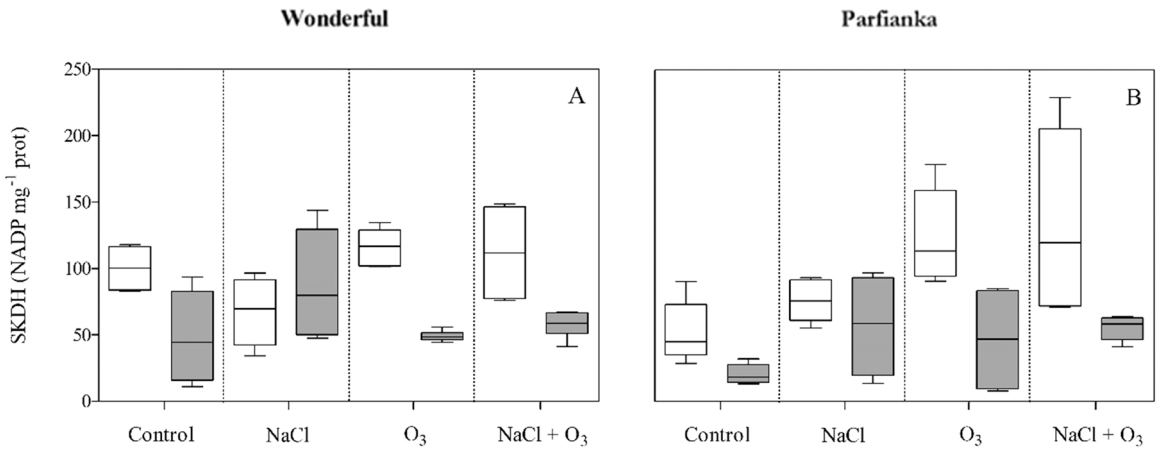

Fig. 3. Box and whiskers representation of the shikimate dehydrogenase (SKDH) activity in Wonderful (A) and Parfianka (B) control leaves or subjected to salt (NaCl, 100 mM for 35 consecutive days) treatment and ozone (O_3, 100 ppb for 5 h) applied alone or sequentially (NaCl + O_3). Measurements were carried out at 5 and 24 h (white and grey coloured bars, respectively) after the re-watering and/or from the beginning of O_3 exposure. For each boxplot, median, 25th-75th percentiles (boxes), non-outlier minimum and maximum (whiskers) are reported (N = 6).

derivatives (trans-cinnamic and ferulic acids; Tables 1 and 2) was recorded throughout the whole experiment (Fig. 1 C). No significant differences were observed in terms of benzoic acid derivatives (Fig. 1E) and individual benzoic acids (i.e., gallic and ellagic acids; Table 2). SKDH activity revealed no significant differences between O_3-treated and untreated plants throughout the whole experiment (Fig. 3). Conversely, the activities of PAL and CAD markedly increased at 5 and 24 h (9-fold higher and +20%, respectively; Fig. 2A-C).

In Parfianka leaves, O_3 fumigation did not alter the metabolites/enzymes involved in the phenylpropanoid pathway (Figs. 1 and 2, Tables 1 and 2).

Although the interaction "cultivar × NaCl × O_3 × time" was not significant for apigenin and no significant differences were reported for catechins and SKDH activity, some differences were observed if "time" was not considered: Wonderful and Parfianka leaves responded to treatments by increasing (3-fold higher) and reducing the content of catechin (−45% compared with controls), respectively, O_3 fumigation stimulated the synthesis of apigenin (+31%) and Parfianka leaves response to O_3 treatment by increasing the activity of SKDH (about 2-fold higher; Fig. 3).

3.4. Effects of NaCl and ozone sequential treatment

In Wonderful leaves, double-stress treatments induced a marked accumulation of ellagitannins over time (more than 2-fold higher than untreated ones; Fig. 1A), as wells as a concomitant increase of punicalagin levels (about 2-fold higher; Table 2). A production of cinnamic acid derivatives was recorded at 5 h (about 2-fold higher; Fig. 1C). A concomitant rise of trans-cinnamic acid was observed (more that 2-fold higher; Table 2). No significant differences were observed in terms of ellagitannins and cinnamic acid derivatives in Wonderful leaves during the recovery time. An increase of gallic acid level was observed at 5 h FBE (about 2-fold higher; Table 2), even if no significant differences were observed in terms of benzoic acid derivatives (Fig. 1E). PAL and SKDH activities revealed no significant differences between NaCl + O_3-treated and untreated plants (controls) at 5 and 24 h FBE (Fig. 2A and S2). Conversely, the activity of CAD significantly decreased at 5 h (−48%; Fig. 2C).

In Parfianka leaves, no differences were observed under sequential stress in terms of ellagitannins and cinnamic acid derivatives. A significant reduction of benzoic acid derivatives and gallic acid levels was documented at 5 h (−60 and −57%; Fig. 1F and Table 2).

Although the interaction "cultivar × NaCl × O_3 × time" was not significant for punicalin, and quercetin, statistical differences were observed for the interaction "cultivar × NaCl × O_3" [Parfianka leaves responded to treatments by reducing the content of punicalin (−33% compared with controls, independently to the sampling time)] and "NaCl × O_3" [both treatments stimulated the synthesis of quercetin (+34%), regardless of cultivar] (Table S1).

4. Discussion

It is known that the degree of cellular oxidative damage in plants subjected to stressful conditions is regulated by their ability to produce antioxidant agents. Therefore, stress tolerance seems to be favoured by the increase in plant antioxidant levels to detoxify the ROS produced under these conditions (Noctor and Foyer, 1998). However, there is little available information about whether single or multiple stresses influence polyphenolic composition at quanti-qualitative level.

4.1. NaCl treatment

The effect of NaCl treatment on leaf concentrations of measured secondary metabolites varied between cultivars. Wonderful leaves coped with NaCl through the production/accumulation of cinnamic acid derivatives and by exhibiting chemical composition plasticity against salinity (Sarri et al., 2021). Conversely, the unchanged concentrations of benzoic acid derivatives indicate that salt-treated Wonderful leaves could diversify the components of polyphenolic compounds and induce the biosynthesis of the available components (Kulak et al., 2020). Gallic acid (the major component of pomegranate leaves) together with ellagic acid is a substituent of polyphenolic biomolecules (called hydrolysable tannins; Šamec et al., 2021). These compounds are hydrolysed by weak acids and can be divided into gallotannins (which provide sugar and gallic acid), and ellagitannins (which yield ellagic acid after hydrolysis). The observed increase of individual polyphenolics (e.g., punicalagin), and the concomitant changes in ellagitannins content suggest that these metabolites may enhance antioxidant activities in salt-treated Wonderful leaves by detoxifying the ROS produced under these conditions (Calzone et al., 2020b). It is worth to pointing out that NaCl treatment significantly improved the levels of phenolic acids/polyphenolics during the recovery time (as reported above), and concomitantly increased the activity of CAD (enzyme involved in the last steps of monolignols synthesis pathway, which play a pivotal role in lignin synthesis; Mitchell et al., 1994). All the information collected tends to underline that Wonderful leaves react to NaCl by enhancing the synthesis of cinnamyl alcohols, and the degree of lignification to provide mechanical support to cell walls (Oliveira et al., 2020). In fact, besides their antioxidant

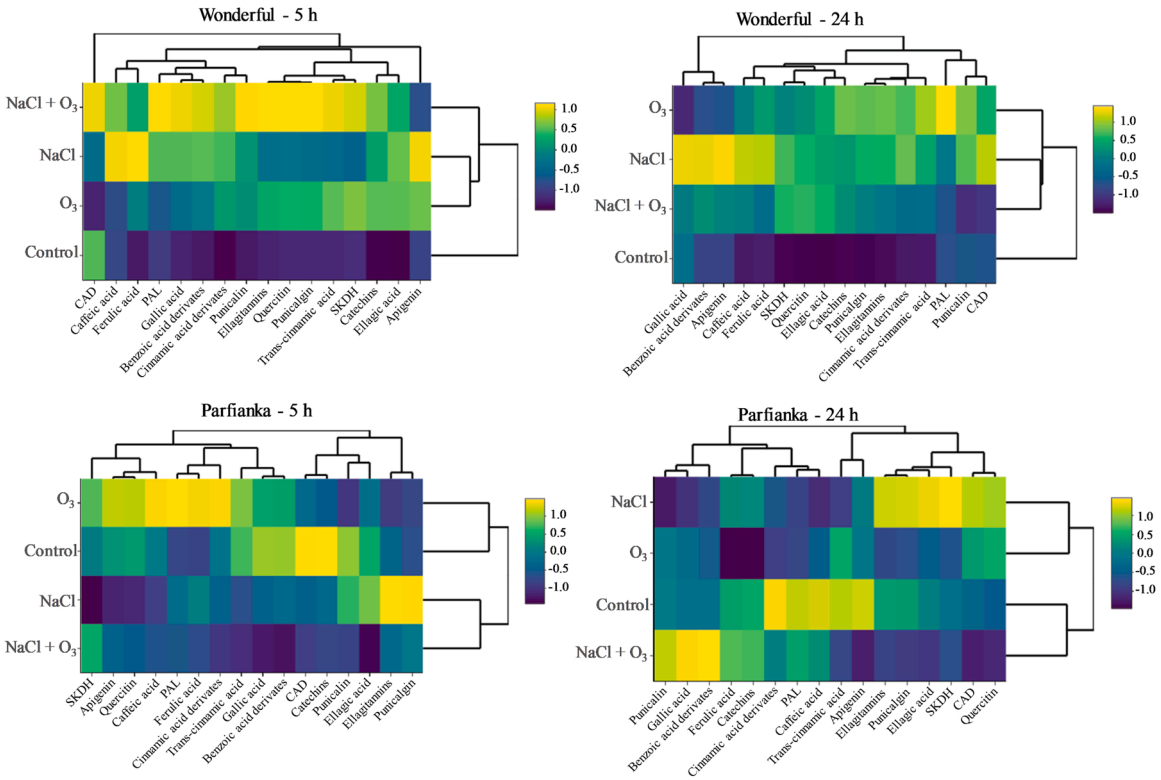

Fig. 4. Heat maps of enzymatic and non-enzymatic antioxidant compounds in Wonderful and Parfianka leaves subjected to salt (NaCl, 100 mM for 35 consecutive days) treatment and ozone (O_3, 100 ppb for 5 h) applied alone or sequentially (NaCl + O_3). Enzymatic activities and phytochemicals intensities (at 5 and 24 h after the re-watering and/or from the beginning of O_3 exposure) were log_{10} transformed and are displayed as colours ranging from dark blue to yellow. Abbreviations: CAD, cinnamyl alcohol dehydrogenase; PAL, phenylalanine ammonia-lyase; SKDH, shikimate dehydrogenase.

capacity, a strong correlation was observed in root lignification and cell wall solidification in vascular and xylem tissues subjected to salt stress (Chun et al., 2019). This could be beneficial to provide protection against further oxidative injury, thus they have a positive role in salt tolerance (Gupta and De, 2017). However, sufficient evidence is still lacking, so further studies are needed to investigate this possibility.

In Parfianka leaves, NaCl treatment did not induce a rearrangement of metabolites and enzymes involved in the phenylpropanoid pathway. Few quanti-qualitative changes in phenolic/polyphenolic contents were reported over time suggesting that NaCl treatment induced only structural changes in terms of gallic and ellagic acids. It is possible to speculate that these compounds may work as primary oxidants (as free-radical scavengers) or "secondary" antioxidants (e.g., indirect pathway; Šamec et al., 2021) in order to alleviate the excess of excitation pressure or provide antioxidative protection to chloroplasts, respectively (Natali et al., 2018).

4.2. Ozone fumigation

Likewise to salt treatment, the effect of O_3 fumigation on leaf concentrations of measured secondary metabolites varied between cultivars. Wonderful leaves responded to O_3 through the production/accumulation of cinnamic acid derivatives by exhibiting chemical composition plasticity against oxidative stress. The significant induction of trans-cinnamic acid observed at the end of O_3 treatment and even more at recovery suggested that the leaves previously experiencing a single pulse of O_3 activated distinct pathways with specific functions likely to cope with potential future stress episodes (Zhao et al., 2005). Conversely, the unchanged concentrations of benzoic acid derivatives indicate that O_3-treated Wonderful leaves can diversify the components of polyphenolic compounds by inducing the biosynthesis of the components available to regulate the cellular redox state (Kulak et al., 2020), as previously reported by Calzone et al. (2021b). In addition, O_3 induced an activation of some enzymes involved in phenolic metabolism indicating the role of the specialized metabolism in plant defense under O_3 pressure, as well as the possible fine regulation of this mechanism through time (Cotrozzi et al., 2018; Döring et al., 2020).

In Parfianka leaves, O_3 fumigation did not give rise to the same effects induced in Wonderful ones (e.g., partial rearrangement of phenylpropanoid pathways) by documenting that the constitutive biochemical features of Parfianka cultivar were enough to explain its superior ability to counteract short-term O_3 treatment.

4.3. Salt and ozone sequential treatment

Likewise to single stress, the effect of salt and O_3 sequential treatment on leaf concentrations of measured secondary metabolites varied between cultivars. Wonderful leaves responded to multiple stresses through the production/accumulation of cinnamic acid derivatives by exhibiting chemical composition plasticity against NaCl and O_3 treatments. Interestingly, accumulations of cinnamic acid derivatives disappeared at recovery time suggesting that Wonderful leaves finally lost their ability or interest in investing in this response strategy (Marchica et al., 2021). The unchanged concentrations of benzoic acid derivatives throughout the whole experiment indicate that multiple-stressed Wonderful leaves could diversify the components of polyphenolic compounds in order to regulate ROS levels (Kulak et al., 2020). It is worth pointing out that the partial rearrangement of phenylpropanoids was accompanied by a decrease of CAD activity. All the information collected tends to underline that Wonderful leaves react to salt and O_3 sequential treatment by reducing the turnover of phenolic acids and polyphenolics (e.g., lignins) at the end of O_3 fumigation (Arab et al., 2022). In addition, when stresses were individually applied, Wonderful leaves showed variations of metabolites involved in the

phenylpropanoid pathways both at the end of O_3-fumigation and recovery. Conversely, when applied sequentially, most of the arrangements/responses were only observed at the end of O_3-fumigation. Coolen et al. (2016), studying *Arabidopsis thaliana* plants response to abiotic sequential stress, hypothesized that the first stress may influence the timing of the response to the second ones.

In Parfianka leaves, NaCl and O_3 sequential treatment did not induce a rearrangement of phenylpropanoid pathways. We only observed a consumption of benzoic acid derivatives at the end of O_3 treatment. No other quanti-qualitative changes in phenolic/polyphenolic contents were reported over time suggesting that multiple stresses could induce transient structural changes in terms of gallic acid. The decrease of the main component of Parfianka leaves could be related to its consumption by the cell to counteract the accumulation of ROS, thus representing an important defense mechanism against the increased oxidative metabolism induced by NaCl and O_3 sequential treatments (Pellegrini et al., 2019).

5. Conclusions

Significant differences of secondary metabolites (in terms of classes/types/components) were detected between the constitutive levels of Wonderful and Parfianka untreated plants. This feature could be crucial in sustaining cultivar response under single- or double-stress conditions. When NaCl and O_3 were applied sequentially, they induced different responses in the two pomegranate cultivars than when they were applied individually (Fig. 4). This divergence was due to a different perception of the stress, i.e., as a new one and not as a combination of two different stress factors. Although oxidative stress was not estimated in plants subjected to the sequential stress, the observed transient rearrangements of phenylpropanoids could be attributed to a negative effect induced by the combination of the two stresses. Under sequential double-stress conditions, Wonderful leaves needed less adjustments (with respect to single-stress ones) in the antioxidant system components to maintain oxidative stress under control (especially at recovery). On the other hand, Parfianka leaves had phenolics/polyphenolics amount (at constitutive level) suitable to face out the environmental changes (single or sequentially), so no rearrangement of phenylpropanoids was required. According to our results, Parkianka leaves seem to be more salt- and O_3-tolerant than Wonderful.

CRediT authorship contribution statement

Antonella Calzone: Formal analysis, Investigation, Data curation, Writing – original draft. **Mariagrazia Tonelli:** Formal analysis, Investigation. **Lorenzo Cotrozzi:** Methodology, Writing – review & editing, Supervision. **Giacomo Lorenzini:** Writing – review & editing, Supervision. **Cristina Nali:** Conceptualization, Writing – review & editing, Supervision, Resources. **Elisa Pellegrini:** Conceptualization, Writing – original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Authors gratefully acknowledge Rebecca Detti and Alberto Contarin for helping with data organization, Samuele Risoli for drawing the heat maps and Andrea Parrini for supervising experimental facilities.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2023.105249.

References

Abdel-Farid, I.B., Marghany, M.R., Rowezek, M.M., Sheded, M.G., 2020. Effect of salinity stress on growth and metabolomic profiling of *Cucumis sativus* and *Solanum lycopersicum*. Plants 9, 1626.

Arab, L., Hoshika, Y., Müller, H., Cotrozzi, L., Nali, C., Tonelli, M., et al., 2022. Chronic ozone exposure preferentially modifies root rather than foliar metabolism of date palm (*Phoenix dactylifera*) saplings. Sci. Total Environ. 806, 150563.

Ashraf, M., Ali, Q., 2010. Response of two genetically diverse wheat cultivars to salt stress at different growth stages: leaf lipid peroxidation and phenolic contents. Pak. J. Bot. 42, 559–565.

Assaf, M., Korkmaz, A., Karaman, Ş., Kulak, M., 2022. Effect of plant growth regulators and salt stress on secondary metabolite composition in Lamiaceae species. S. Afr. J. Bot. 144, 480–493.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cabot, C., Sibole, J.V., Barceló, J., Poschenrieder, C., 2014. Lessons from crop plants struggling with salinity. Plant Sci. 266, 2–13.

Calzone, A., Cotrozzi, L., Pellegrini, E., Guidi, L., Lorenzini, G., Nali, C., 2020a. Differential response strategies of pomegranate cultivars lead to similar tolerance to increasing salt concentrations. Sci. Hortic. 271, 109441.

Calzone, A., Cotrozzi, L., Remorini, D., Lorenzini, G., Nali, C., Pellegrini, E., 2020b. Oxidative stress assessment by a spectroscopic approach in pomegranate plants under a gradient of ozone concentrations. Environ. Exp. Bot. 182, 104309.

Calzone, A., Cotrozzi, L., Lorenzini, G., Nali, C., Pellegrini, E., 2021a. Hyperspectral detection and monitoring of salt stress in pomegranate cultivars. Agronomy 11, 1038.

Calzone, A., Cotrozzi, L., Pellegrini, E., Lorenzini, G., Nali, C., Maathius, F., 2021b. Can the transcriptional regulation of *NHX1*, *SOS1* and *HKT1* genes handle the response of two pomegranate cultivars to moderate salt stress. Sci. Hortic. 288, 110309.

Chen, D., Mubeen, B., Hasnain, A., Rizwan, M., Adrees, M., Naqci, S.A.H., et al., 2022. Role of promising secondary metabolites to confer resistance against environmental stresses in crop plants: current scenario and future perspectives. Front. Plant Sci. 13, 881032.

Chun, H.J., Baek, D., Cho, H.J., Lee, S.H., Jin, B.J., Yun, D.-J., et al., 2019. Lignin biosynthesis genes play critical roles in the adaptation of *Arabidopsis* plants to high-salt stress. Plant Signal. Behav. 14, 8.

Coolen, S., Proietti, S., Hickman, R., Olivas, N.H.D., Huang, N., Verk, P., et al., 2016. Transcriptome dynamics of *Arabidopsis* during sequential biotic and abiotic stresses. Plant J. 86, 249–267.

Cotrozzi, L., Campanella, A., Pellegrini, E., Lorenzini, G., Nali, C., Paoletti, E., 2018. Phenylpropanoids are key players in the antioxidant defense to ozone of European ash, *Fraxinus excelsior*. Environ. Sci. Pollut. Res. 25, 8137–8147.

Diaz, J., Bernal, A., Merino, F., 1994. Changes in shikimate dehydrogenase activity during the development of pepper plants (*Capsicum annuum* L.). Ontogenez 25, 63–70.

Döring, A., Cotrozzi, L., Lorenzini, G., Nali, C., Petersen, M., Pellegrini, E., 2020. Deciphering the role of low molecular weight antioxidants in the sensitivity of *Melissa officinalis* L. to realistic ozone concentrations. Ind. Crop Prod. 150, 112369.

Farroq, M., Park, J.-R., Jang, Y.-H., Kim, E.G., Kim, K.-M., 2021. Rice cultivars under salt stress show differential expression of genes related to the regulation of Na^+/K^+ balance. Front. Plant Sci. 12, 680131.

Fatima, A., Singh, A.A., Mukherjee, A., Dolker, T., Agrawal, M., Agrawal, S.B., 2019. Assessment of ozone sensitivity in three wheat cultivars using ethylenediurea. Plants 8, 80.

Feng, Z., Wang, L., Pleijel, H., Zhu, J., Kobayashi, K., 2016. Differential effects of ozone on photosynthesis of winter wheat among cultivars depend on antioxidative enzymes rather than stomatal conductance. Sci. Total Environ. 572, 404–411.

Guidi, L., Remorini, D., Cotrozzi, L., Giordani, T., Lorenzini, G., Massai, R., et al., 2017. The harsh life of an urban tree: the effect of a single pulse of ozone in salt-stressed *Quercus ilex* saplings. Tree Physiol. 37, 246–260.

Gupta, P., De, B., 2017. Differential responses of cell wall bound phenolic compounds in sensitive and tolerant varieties of rice in response to salinity. Plant Signal. Behav. 12, e1379643.

Isah, T., 2019. Stress and defense responses in plant secondary metabolites production. Biol. Res. 52, 39.

Jakopic, J., Petkovsek, M.M., Likozar, A., Solar, A., Stampar, F., Veberic, R., 2011. HPLC-MS identification of phenols in hazelnut (*Corylus avellana* L.) kernels. Food Chem. 124, 1100–1106.

Jolivet, Y., Bagard, M., Cabané, M., Vaultier, M.V., Gandin, A., Afif, D., et al., 2016. Deciphering the ozone-induced changes in cellular processes: a prerequisite for ozone risk assessment at the tree and forest levels. Ann. Sci. 73, 923–943.

Kulak, M., Gul, F., Sekeroglu, N., 2020. Changes in growth parameter and essential oil composition of sage (*Salvia officinalis* L.) leaves in response to various salt stresses. Ind. Crops Prod. 145, 112078.

Marchica, A., Ascrizzi, R., Flamini, G., Cotrozzi, L., Tonelli, M., Lorenzini, G., et al., 2021. Ozone as eustress for enhancing secondary metabolites and bioactive properties in *Salvia officinalis*. Ind. Crops Prod. 170, 113730.

Mitchell, H.J., Hall, J., Barber, M.S., 1994. Elicitor-induced cinnamyl alcohol dehydrogenase activity in lignifying wheat (*Triticum aestivum* L.) leaves. Plant Physiol. 104, 551–556.
Natali, L., Vangelisti, A., Guidi, L., Remorini, D., Cotrozzi, L., Lorenzini, G., et al., 2018. How *Quercus ilex* L. saplings face combined salt and ozone stress: a transcriptome analysis. BMC Genom. 19, 872.
Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Biol. 49, 249–279.
Oliveira, D.M., Mota, T.R., Salatta, F.V., Sinzker, R.C., Končitíková, R., Kopečný, D., et al., 2020. Cell wall remodeling under salt stress: insights into changes in polysaccharides, feruloylation, lignification, and phenolic metabolism in maize. Plant Cell Environ. 43, 2172–2191.
Pellegrini, E., Hoshika, Y., Dusart, N., Cotrozzi, L., Gérard, J., Nali, C., et al., 2019. Antioxidative responses of three oak species under ozone and water stress conditions. Sci. Total Environ. 647, 390–399.
Ramegowda, V., Senthil-Kumar, M., 2015. The interactive effects of simultaneous biotic and abiotic stresses on plants: mechanistic understanding from drought and pathogen combination. J. Plant Physiol. 176, 47–54.
Richet, N., Tozo, K., Afif, D., Banvoy, J., Lingay, S., Dizengremel, P., Cabané, M., 2012. The response to daylight or continuous ozone of phenylpropanoid and lignin biosynthesis pathways in poplar differs between leaves and wood. Planta 236, 727–737.
Šamec, D., Karalija, E., Šola, I., Vujčić, B., Salopek-Sondi, B., 2021. The role of polyphenols in abiotic stress response: the influence of molecular structure. Plants 10, 118.
Sarri, E., Termentzi, A., Abraham, E.M., Papadopoulos, G.K., Baira, E., Machera, K., et al., 2021. Salinity stress alters the secondary metabolic profile of *M. sativa*, *M. arborea* and their hybrid (*Alborea*). Int. J. Mol. Sci. 22, 4882.
Selmar, D., Kleinwächter, M., 2013. Stress enhances the synthesis of secondary plant products: the impact of stress-related over-reduction on the accumulation of natural products. Plant Cell Physiol. 54, 817–826.
Sillmann, J., Aunan, K., Emberson, L., Büker, P., Oort, B.V., O'Neil, C., et al., 2021. Combined impacts of climate and air pollution on human health and agricultural productivity. Environ. Res. Lett. 16, 093004.
Srivastava, A.K., Mishra, P., Mishra, A.K., 2021. Effect of climate change on plant secondary metabolism: an ecological perspective. In: Srivastava, A.K., Kumar, V. (Eds.), in Evolutionary Diversity as a Source for Anticancer Molecules. (Cambridge, MA: Academic Press), pp. 47–76.
Sukuzi, N., Rivero, R., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress combinations. New Phytol. 203, 32–43.
Zhao, J., Davis, L.C., Verpporte, R., 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. 23, 283–333.