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Effects of Increasing Ozone Levels on Leaf Biochemistry and Flower Development in Petunia Varieties with Different Floral Pigmentation

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Abstract: In this work, we assessed the effects of increasing ozone (O_3) on four petunia varieties with different floral pigmentation (pink, red, rose-red, and white). Plants were exposed, in open-top chambers located in China, to three O_3 concentrations, i.e., (i) ambient air (AA), (ii) AA + 60 ppb O_3 (AA + 60), and (iii) AA + 120 ppb O_3 (AA + 120), for 85 days (9 h day^{-1}). Flower diameter and duration were assessed, together with leaf chlorophyll and flavonoid contents. White petunia showed a reduced flower diameter and longevity under AA + 60 (−7 and −6%, respectively, in comparison to AA), whereas pink and red petunias only showed this under AA + 120 (−8 and −7%, on average, respectively). Chlorophyll loss occurred in all varieties under AA + 60 (−30%, on average), and at AA + 120 in white and red petunias (−54%, on average). The total flavonoid content in the pink and white varieties increased only under AA + 120 (around +85%), while it grew at both AA + 60 and AA + 120 (+92% and two-fold higher, respectively) in the red variety. Increasing O_3 concentrations did not affect particularly the red-rose variety. The white variety showed the strongest correlations among flower and leaf properties, confirming a variety-related O_3 response, as well as demonstrating that it had the highest O_3 sensitivity.

Keywords: flavonoids; flower diameter and longevity; ornamental plants; *Petunia hybrida*; photosynthetic pigments; HPLC; chlorophyll; ozone pollution

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1. Introduction

Tropospheric ozone (O_3) is a harmful secondary pollutant and the third most important greenhouse gas [1]. A series of clean air policies adopted in China since 2013 in order to reduce environmental pollution (e.g., the "Air Pollution Prevention and Control Action Plan" and the "Three-Year Action Plan for Winning the Blue Sky Defense Battle" [2]) have been effective in reducing particulate matter of less than 2.5 µm in diameter (PM 2.5 [3]), but photochemical pollution remains a serious issue, with O_3 trends continuing to increase. For example, daily maximum 8 h average O_3 concentrations have been measured at around and over 150 µg m^{-3} in many areas of China (e.g., Beijing–Tianjin–Hebei and the Yangtze River Delta area) in the last three years [4–6], abundantly overcoming the threshold established at 100 µg m^{-3} by the World Health Organization [7]. Here, continuous and extensive emissions of O_3 precursors (mostly of anthropogenic origin), together with favorable meteorology for O_3 formation (i.e., frequent heat waves and elevated solar radiation), lead

to persistent O_3 pollution events [8], challenging not only human and animal health [9,10], but also vegetation [11,12] and crops [13].

Ozone enters plants through open stomata on leaf surfaces, arrives at the substomatal cavity, reacts with cell wall and plasma membrane constituents, and generates reactive oxygen species (ROS) in the apoplast (i.e., oxidative burst [14]), which commonly have a detrimental impact on plant metabolism. The deleterious effects of O_3 usually include decreases in chlorophyll content and photosynthesis, with consequent reductions in assimilate availability (and thus biomass) and alterations in their partitioning [15,16]. On the other hand, plants can develop a set of morphological, physiological, and biochemical responses (e.g., the activation of enzymatic and non-enzymatic antioxidants [17]) to the increasing O_3 concentrations in order to not be weeded out [18]. These adjustments differ depending on several factors, including O_3 concentrations, exposure conditions (e.g., a controlled or field environment), the timings of exposure, and plant species and varieties [16], so their evaluation can provide decisive information for the selection of O_3-tolerant plant material [19]. This is crucial not only for crop species, but also for ornamental plants used in public and private gardens or urban green spaces.

Ornamental plants provide several ecosystem services to the urban environment because of their esthetic and social values [20] but also their positive effects in terms of improving air quality [21], supporting biodiversity [22,23], favoring environmental conservation [24], capturing storm water runoff [25], and sequestering carbon dioxide [26]. However, the benefits provided by ornamental plants may be weakened by alterations in plant metabolism due to increasing O_3 [27]. This phenomenon has been highlighted by a large number of studies (e.g., [28]), including those that we performed to assess the suitability of ornamental plant species (*Cotinus coggygria*, *Rosa chinensis*, and *Tagetes herecta*) largely used in gardens and urban green spaces in China subjected to increasing O_3 concentrations (using open-top chambers (OTCs) [27,29]). However, although the detrimental effects of elevated O_3 have been largely investigated at the leaf level [30,31], the effects of this pollutant on flowers have been less investigated (and most investigations have focused on plant–insect interactions [32]), even though it is known that O_3 can alter the flowering phenology, either accelerating [33,34] or delaying it [35,36], and can severely reduce the number and the final biomass of flowers, as well [34,36].

The garden petunia, *Petunia hybrida* Vilm. (Solanaceae), with its diversity of flower colors and morphology, is the world's most popular bedding plant, with a long history as a model species for scientific research [37,38], especially regarding interactions with microbes, herbivores, and pollinators [39]. Since the middle of the last century, petunia plants have been shown to be sensitive to O_3 [40] and have shown different degrees of O_3 sensitivity according to variety and leaf age [41]. Here, this species was selected to elucidate the potential involvement of flower pigmentation in the sensitivity/responses of ornamental plants to O_3 challenge.

Specifically, we maintained the same experimental conditions as those in our previous studies, with the aim of elucidating the effects of elevated O_3 on the leaf physiology and biochemistry of ornamental plants suitable for gardens and urban green spaces [27,29]; here, four petunia varieties characterized by different flower colors (pink, red, rose-red, and white) were exposed to increasing O_3 concentrations (using the same OTCs reported above). Specifically, the present study aimed to (i) understand whether petunia varieties with various floral pigmentation show different responses to increasing O_3 concentrations (at both the flower and leaf levels) and (ii) assess a potential relationship between flower development (morphology and duration) and leaf biochemical responses. Considering the importance of chlorophyll, which plays a crucial role in the photosynthetic process by absorbing light energy and converting it into chemical energy [42], and flavonoids, which are secondary metabolites involved in cell growth regulation, insect pollinator attraction, and plant protection against biotic and abiotic stresses [43], we hypothesized different responses among the varieties and postulated that the chlorophyll and flavonoid contents

(Chl_{TOT} and $Flav_{TOT}$) could influence the flower diameter (F_D) and longevity (F_L) under increasing O_3 stress conditions.

2. Materials and Methods

2.1. Chemicals and Reagents

Ozone was generated from pure oxygen by an O_3 generator (HY003, Chuangcheng Co., Jinan, China) using a high-voltage discharge method [44] and then mixed with AA to achieve the target concentration. Chlorophyll total determination was carried out by using 96% ethanol (Fisher Scientific, Thermo Fischer Scientific Inc., Wilmington, DE, USA). Flavonoid total determination was carried out using high-performance liquid chromatography (HPLC)-grade acetonitrile, HPLC-grade methanol, HPLC-grade trifluoroacetic acid, and ≥96% formic acid (Fisher Scientific, Thermo Fischer Scientific Inc., Wilmington, DE, USA), rutin standard (Sinopharm Chemical Reagent Co. Ltd., Beijing, China).

2.2. Plant Material

The present study was carried out from August to October 2019 in Zhangtou Village (40°12′ N, 116°8′ E), Changping District, Beijing, China. The site is in the temperate monsoon climate zone, with an average annual temperature of 12.1 °C and an annual rainfall of 542 mm, concentrated from June to August [45]. Seedlings of *P. hybrida* equally distributed among the pink (Spreading Petunia Easy Wave® Pink, #PAS3189; Figure S1), red (Spreading Petunia Easy Wave® Red, #PAS1016994), rose-red (Spreading Petunia Easy Wave® Red Velour, #PAS933560), and white varieties (Spreading Petunia Easy Wave® White, #PAS760712) (i.e., 45 plants per variety), purchased from a nursery (Beijing Jindetai Landscape Engineering Co., Ltd., Beijing, China), were transplanted into plastic pots (17.0 cm in height and 22.0 cm in diameter, filled with a mixture of turfy soil, vermiculite, and perlite (97:1:2)), at the stage when no plants had buds and the average height of plants was around 7 cm.

2.3. Experimental Design

Forty-five pots per treatment, each containing one individual seedling, were placed in three octagonal open-top chambers (OTCs, 2.8 m in height and 4.0 m in diameter, with aluminium alloy frame and embedded stalinite, i.e., 5 plants per variety per OTC), and exposed to three O_3 concentrations, i.e., (i) ambient air (AA), (ii) AA with a targeted O_3 addition of 60 ppb (AA + 60), and (iii) AA with a targeted O_3 addition of 120 ppb (AA + 120; for O_3, 1 ppb = 1.96 μg m^{-3}, at 25 °C and 101.325 kPa), for 85 consecutive days (9 h day^{-1}, from 8:30 am to 5:30 pm), with the exception of rainy days. The O_3 levels were recorded at the end of the exposure in terms of accumulated exposure over a threshold of 40 ppb (AOT40). To alleviate the OTC effects, plants were rotated within the OTC every 5–7 days, while every 10–15 days, plants were reassigned among the OTCs, turning the O_3 concentration in each OTC.

Total biomass and flower evaluations were carried out at the end of exposure. At the same time, the youngest mature and fully expanded leaf from each plant was collected in liquid nitrogen, ground, and stored at −80 °C until biochemical analyses were conducted. A summary of the experimental activity is given in Figure S2.

2.4. Evaluation of Symptoms, Flower Diameter and Longevity, and Total Biomass

Symptoms were naked-eye-evaluated by two independent evaluators using fully expanded leaves from each plant.

A flower from each plant was selected and evaluated in terms of F_D, i.e., an averaged measure of orthogonal diameters, and longevity, i.e., the number of days from the first opening to the fall occurring at the end of exposure (the last flower cohort was considered). Leaves and stems of all plants (more than 70 kg for each treatment) were harvested and immediately placed in an oven at 75 °C for three days until reaching a constant weight to evaluate their total dry biomass (hereafter reported as 'biomass').

2.5. Determination of Leaf Chlorophyll Content

The leaf Chl *a* and *b* contents were determined according to [46], with minor modifications. Fifteen mL of ethanol were added to 50 mg of leaf tissue and maintained in the dark, until samples were colorless. The extracts were directly used for quantification, which was carried out by measuring the absorbance at 665 and 649 nm (A665 and A649, respectively) using a UV/Vis spectrophotometer (Model V-530, Jasco Corporation, Tokyo, Japan). The obtained data were processed by calculating the Chl *a* and *b* concentrations according to the following equations:

$$\text{Chl } a = 13.95 \times \text{A665} - 6.88 \times \text{A649} \quad (1)$$

$$\text{Chl } b = 24.96 \times \text{A649} - 7.32 \times \text{A665}. \quad (2)$$

The total Chl content was calculated as Chl *a* + *b* and expressed as mg g^{-1} fresh weight (FW).

2.6. Determination of Leaf Flavonoid Content

The total flavonoid content ($Flav_{TOT}$) of leaves was determined according to [47]. Around 50 mg of leaf tissue was extracted with a 2 mL mixture of HPLC-grade methanol/ddH_2O/formic acid/trifluoroacetic acid (70:27:2:1, *v*/*v*/*v*/*v*) and maintained for 24 h in the dark at 4 °C. The extract was shaken manually every 8 h and finally centrifuged for 10 min at 2000× *g* at 4 °C. The supernatant was filtered through 0.22 µm PTFE syringe filters (Jinteng, Tianjin, China), and analyzed in an HPLC system (Model 1100, Agilent Technologies, Santa Clara, CA, USA) equipped with a photodiode array detector (PAD-100, Agilent) and a reverse-phase C18 column (5 µm particle size, 250 mm length × 4.6 mm internal diameter; Waters corporation, Milford, MA, USA), kept at 25 °C. Ten µL of extract were eluted in 1 mL min^{-1} ddH_2O:HPLC grade acetonitrile/formic acid/trifluoroacetic acid (62.9:35:2:0.1, *v*/*v*/*v*/*v*; mobile phase A) and ddH_2O/formic acid/trifluoroacetic acid (97.9:2:0.1, *v*/*v*/*v*; mobile phase B), and separation was performed by gradually changing the percentage of A, as follows: 0–20 min, 30–53%; 20–40 min, 53%; 40–45 min, 53–30%; and 45–50 min, 30%. Flavonoids were measured at 350 nm and determined by correlating the peak area with the concentration of a standard curve prepared using a pure amount of rutin (1–500 µg mL^{-1}). The LOD and LOQ were determined at signal-to-noise (S/N) ratios of 3 and 10, respectively, and correspond to 2.05 and 8.1 mg mL^{-1}. Data were analyzed using the software ChemStation version B.01.01C (Agilent Technologies, Santa Clara, CA, USA). The sum of all flavonoids identified was used to calculate the $Flav_{TOT}$.

2.7. Statistical Analyses

The normality of data was preliminarily assessed by the Shapiro–Wilk test. The effects of "Variety", "O_3", and their interaction on biomass, Chl_{TOT}, $Flav_{TOT}$, and their ratio $Chl_{TOT}/Flav_{TOT}$ were evaluated by two-way analysis of variance (ANOVA). For the parameters in which the "Variety × O_3" effect resulted not statistically significant, i.e., FD and FL, the effect of the single factor "O_3" was evaluated in each variety by one-way ANOVA. Differences among means were evaluated by Tukey's post hoc test. The lack of OTC replication may raise concerns about pseudo-replication [48], but this limitation was here overcome regularly by reversing the O_3 concentrations among OTCs, as reported above [49]. Relations among the investigated flower and leaf parameters were evaluated using Pearson's correlations. Effects with $p \leq 0.05$ were considered statistically significant. Statistical analyses were performed using JMP version 13.5 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Visible Injuries

The onset of O_3-induced symptoms was checked throughout the whole experiment and occurred under both elevated O_3 concentrations (i.e., AA + 60 and AA + 120) as interveinal chlorosis on the upper surfaces of leaves (Figure S3). However, in both varieties, foliar symptoms occurred firstly under AA + 120 (at an AOT40 of 7.7 ppm·h, with an O_3

peak of 183 ppb), around 10 days from the beginning of the exposure and subsequently under AA + 60 (at an AOT40 of 4.2 ppm·h, with an O_3 peak of 117 ppb). Visible injuries were not observed under AA (at an AOT40 of 1.1 ppm·h, with an O_3 peak of 60 ppb).

3.2. Flower Diameter and Longevity and Biomass

The one-way ANOVA (factor "O_3") for F_D and F_L was significant for all petunia varieties except the rose-red one. The flower diameter of the pink and red varieties was reduced only under AA + 120 (8.5 ± 0.06 vs. 7.9 ± 0.3, and 8.3 ± 0.2 vs. 7.7 ± 0.0404, respectively, for AA vs. AA + 120 for the pink and red varieties, corresponding to −8 and −7%; Figure 1A), while, in the white variety, FD similarly decreased under both AA + 60 and AA + 120 (7.6 ± 0.01 vs. 7 ± 0.01, corresponding to −7%, on average). Similarly, the FL of the pink and red varieties was reduced only under AA + 120 (8.3 ± 0.3 vs. 7.2 ± 0.2, and 9 ± 0.4 vs. 8.2 ± 0.2, corresponding to −11% on average), while, in the white variety, FL decreased under AA + 60 and decreased even more under AA + 120 (7.8 ± 0.25 vs. 7.12 ± 0.2 and 6.5 ± 0.2, corresponding to −6 and −14%, respectively; see Figure 1B). No significant O_3 effects were reported on either the FD or FL of the rose-red variety.

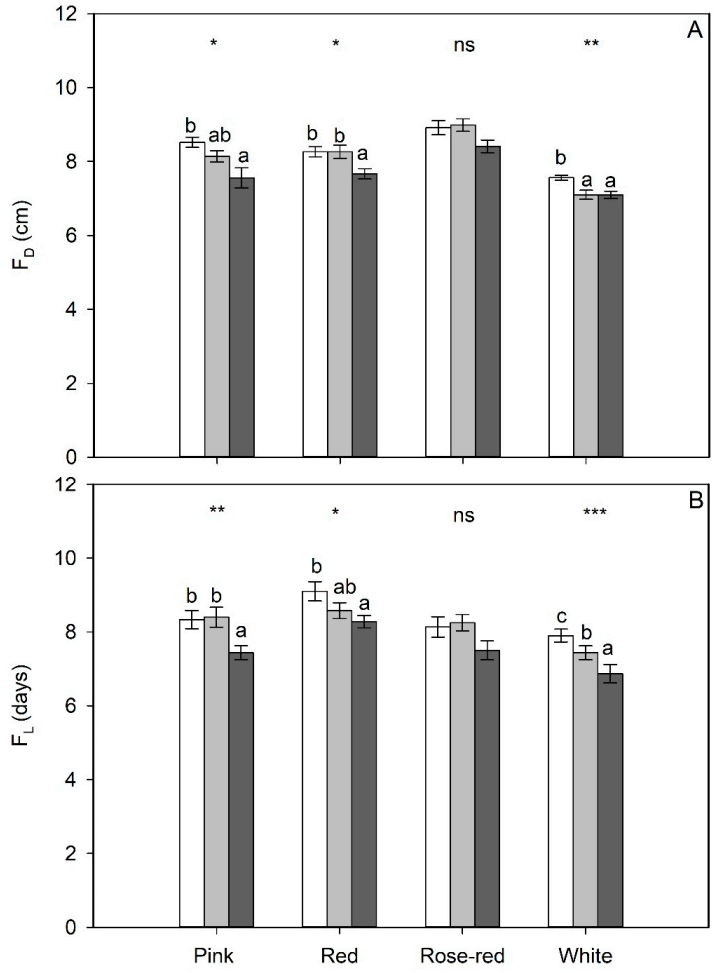

Figure 1. Flower diameter (F_D, **A**) and longevity (F_L, **B**) of pink, red, rose-red, and white petunia varieties exposed, in open-top chambers, to ambient air (AA; *white bar*); AA increased by 60 ppb (AA + 60; *light-grey bar*), and AA increased by 120 ppb (AA + 120; *dark-grey bar*) in ozone (O_3) concentration for 85 consecutive days (9 h day^{-1}). Data are shown as mean ± standard error (n = 5). p levels assessed for each variety by a one-way ANOVA (factor "O_3") are reported (***, $p \leq 0.001$; **, $p \leq 0.01$, *, $p \leq 0.05$; ns, $p > 0.05$. According to Tukey's post hoc test, different letters indicate significant differences among means.

The two-way ANOVA for biomass resulted statistically significant for the singular factors "Variety" and "O_3" and their interaction. Inter-variety effects were observed firstly under environmental conditions and then under different O_3 concentrations. Specifically, under AA, the biomass (range of 20–26 g DW) differed only in the rose-red variety in comparison to the white one (−23%; Figure 2); under AA + 60, the biomass (range 17–21 g DW) of the red variety was the lowest (−34% on average, in comparison with the other varieties); under AA + 120 (range of 16–20 g DW), only the biomass of the rose-red variety was statistically lower than the pink one (−23%) and the minimum value detected. Intra-variety differences due to increasing O_3 concentrations were also observed. Under AA + 60, the biomass decreased in the pink, red, and white varieties (−24, −30 and −23%, in comparison to the respective AA; throughout the whole text, percentage variations are given in comparison with controls under AA), and these values were also maintained at a higher O_3 concentration. In the rose-red variety, the loss of biomass occurred only under AA + 120 (−24%).

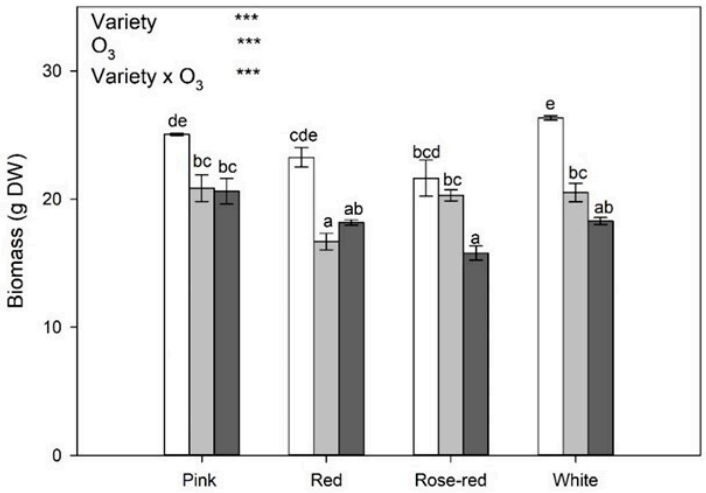

Figure 2. Total biomass (leaves and stems) in pink, red, rose-red, and white petunia varieties exposed in open-top chambers to ambient air (AA; *white bar*), AA increased by 60 ppb (AA + 60; *light-grey bar*), and AA increased by 120 ppb (AA + 120; *dark-grey bar*) in ozone (O_3) concentration for 85 consecutive days (9 h day^{-1}). Data are shown as mean ± standard error (n = 5). p levels assessed by two-way ANOVA (factors "Variety" and "O_3") are reported (***: $p \leq 0.001$). According to Tukey's post hoc test, different letters represent significant differences among means. DW, dry weight.

3.3. Leaf Biochemistry

3.3.1. Total Chlorophyll Content

According to two-way ANOVA, the singular "Variety" and "O_3" effects and the "Variety × O_3" interactive one were significant for Chl_{TOT} (Figure 3A). Specifically, under AA, the red variety showed the highest Chl_{TOT} value in comparison to the rose-red and white ones (1.14 ± 0.01 vs. 0.86 ± 0.01, and 0.91 ± 0.01 mg g^{-1} FW, respectively). In the pink and rose-red varieties, the Chl_{TOT} content decreased only under AA + 60 (0.93 ± 0.02 vs. 0.64.0.02 mg g^{-1} FW, and 0.86 ± 0.01 vs. 0.6 ± 0.05, corresponding to −32 and −31%, respectively), while, in the red one, Chl_{TOT} levels were similarly reduced under both AA + 60 and AA + 120 (1.14 ± 0.02 vs. 0.57 ± 0.05, and 0.56 ± 0.05 mg g^{-1} FW, corresponding to −50%, on average). In the white variety, Chl_{TOT} decreased under AA + 60 and decreased even more under AA + 120 (0.91 ± 0.03 vs. 0.63 ± 0.02 ± 0.05, and 0.40 mg g^{-1} FW, corresponding to −32 and −57%, respectively).

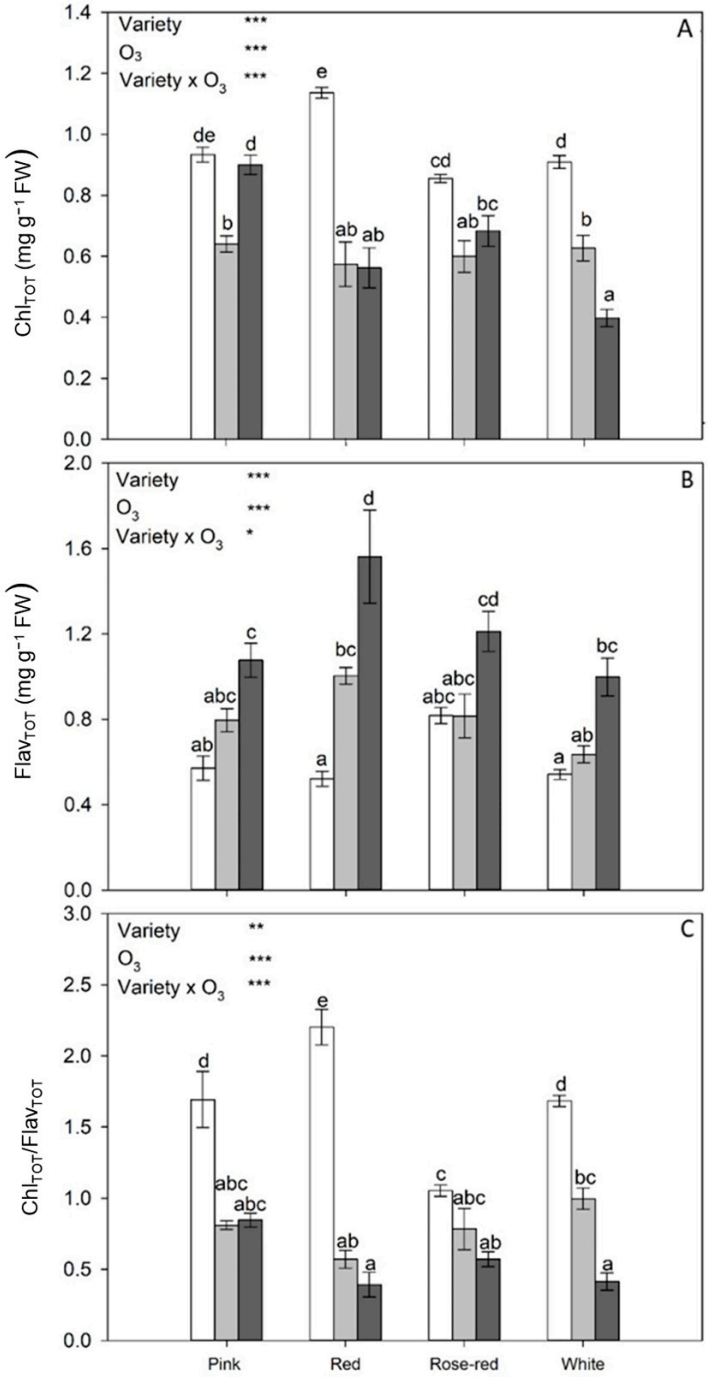

Figure 3. Total chlorophyll content (Chl_{TOT}, **A**), total flavonoid content ($Flav_{TOT}$, **B**), and total chlorophyll content/total flavonoid content ($Chl_{TOT}/Flav_{TOT}$, **C**) in pink, red, rose-red, and white petunia varieties exposed in open-top chambers to ambient air (AA, *white bar*), AA increased by 60 ppb (AA + 60, *light-grey bar*) and AA increased by 120 ppb (AA + 120, *dark-grey bar*) in ozone (O_3) concentrations for 85 consecutive days (9 h day^{-1}). Data are shown as mean ± standard error ($n = 5$). p levels assessed by a two-way ANOVA (factors "Variety" and "O_3") are reported (***: $p \leq 0.001$; **: $p \leq 0.01$; *: $p \leq 0.05$). According to Tukey's post hoc test, different letters significant differences among means. FW, fresh weight.

3.3.2. Total Flavonoid Content

According to the two-way ANOVA, the singular "Variety" and "O_3" effects, as well as the "Variety × O_3" interactive effect, were significant in $Flav_{TOT}$ (Figure 3B). Inter-variety effects were only observed under the highest O_3 concentration. Specifically, the

red variety showed the higher $Flav_{TOT}$ value under AA + 120 (the maximum detected during the experiment), in comparison with the pink and red ones (1.56 ± 0.4 vs. 1.08 ± 0.2, and 1.0 ± 0.2 mg g^{-1} FW, respectively, corresponding to +30%, on average). Intra-variety effects were observed due to the different O_3 concentrations. In the pink and white varieties, $Flav_{TOT}$ increased only under AA + 120 (0.57 ± 0.02 vs. 1.08 ± 0.05, and 0.54 ± 0.02 vs. 1.0 ± 0.04 mg g^{-1} FW, corresponding to +87 and +83%, respectively), while, in the red one, $Flav_{TOT}$ increased under AA + 60 and increased even more under AA + 120 (0.52 ± 0.03 vs. 1.00 ± 0.03, and 1.56 ± 0.2 mg g^{-1} FW, corresponding to +92% and two-fold higher, respectively), reaching the highest values herein reported. No significant changes in $Flav_{TOT}$ content were reported in the rose-red variety. Qualitative alterations in flavonoid composition were observed in relation to the variety and increasing O_3 concentration. In the pink, red, and rose-red varieties (Figure S4), four peaks are identified (i.e., PF1, PF2, PF3, and PF4), all eluted between 19 and 24 min: PF3 was the most abundant flavonoid under AA, PF2 was the highest under AA + 60 ppb, and PF2 and PF4 were the major peaks under AA + 120. Four peaks were also identified for the white variety (PF_{W1}, PF_{W2}, PF_{W3}, and PF_{W4}; see Figure S5), but the HPLC profile of this variety was very different from the others. The flavonoids of the white variety were eluted starting from the 8th min, and, under different O_3 concentrations, PFW3 was always the highest compound, while PFW1 was the lowest. It is worth noting that PF2 corresponded to PFW3 and PF4 corresponded to PFW4.

3.3.3. $Chl_{TOT}/Flav_{TOT}$ Ratio

According to two-way ANOVA, the singular "Variety" and "O_3" effects, as well as the "Variety × O_3" interactive effect, were significant in the $Chl_{TOT}/Flav_{TOT}$ ratio (Figure 3C). Inter-variety effects were observed only for environmental conditions. Specifically, under AA, the red variety showed the highest $Chl_{TOT}/Flav_{TOT}$ ratio, while the rose-red showed the lowest (2.20 ± 0.1 and 1.05 ± 0.2, respectively).

The ratio similarly decreased under both AA + 60 and AA + 120 in the pink variety (1.63 ± 0.2 vs. 0.81 ± 0.05, and 0.84 ± 0.06, corresponding to −52%, on average) and the red variety (2.20 ± 0.1 vs. 0.57 ± 0.05, and 0.39 ± 0.01, corresponding to −75%, on average), while, in the white one, it decreased by 42% and by 76% under AA + 60 and under AA + 120, respectively (1.68 ± 0.05 vs. 0.99 ± 0.05, and 0.41 ± 0.05). In the rose-red variety, a reduction in $Chl_{TOT}/Flav_{TOT}$ occurred only under AA + 120 (1.05 ± 0.2 vs. −0.57 ± 0.05, corresponding to −46%).

3.4. Relationships among Flower and Leaf Parameters

All results (except visible injuries) are summarized in Figure S6.

Several significant relationships among the investigated flower and leaf parameters were reported, taking into account all the varieties together, as well as with them separated (Figure 4, Table S1). Considering all the varieties, strong positive correlations (i.e., $r > 0.6$) were reported for Chl_{TOT} with $Chl_{TOT}/Flav_{TOT}$, and biomass and $Chl_{TOT}/Flav_{TOT}$ with biomass (+0.83, +0.64 and +0.75, respectively; see Figure 4). Strong negative correlations (i.e., $r < -0.6$) were reported for $Flav_{TOT}$ with $Chl_{TOT}/Flav_{TOT}$, and biomass (−0.73 and −0.62, respectively).

In the pink petunia, a strong positive correlation was reported only for biomass with $Chl_{TOT}/Flav_{TOT}$ (+0.71). Conversely, strong negative correlations were reported for Flav-TOT with FD and $Chl_{TOT}/Flav_{TOT}$ (−0.64 and −0.78, respectively). In the red petunia, strong positive correlations were observed for biomass with Chl_{TOT} and $Chl_{TOT}/Flav_{TOT}$, and also for $Chl_{TOT}/Flav_{TOT}$ with FL and Chl_{TOT} (+0.91, +0,87, +0.65 and +0.95, respectively). Strong negative correlations were reported for $Flav_{TOT}$ with FD, Chl_{TOT}, and $Chl_{TOT}/Flav_{TOT}$ (−0.85, −0.73, and −0.82, respectively). In the rose-red petunia, strong positive correlations were reported for $Chl_{TOT}/Flav_{TOT}$ with biomass, FD, and Chl_{TOT} (+0.66, +0.62, and +0.69, respectively), while strong negative ones were reported for $Flav_{TOT}$ with biomass, FL, and $Chl_{TOT}/Flav_{TOT}$ (−0.66, −0.74, and −0.80, respectively). In the

white petunia, several very strong positive correlations (mostly > 0.75) were detected for all crossed parameters except for $Flav_{TOT}$ with FD, FL, Chl_{TOT}, and $Chl_{TOT}/Flav_{TOT}$. Regarding these last two parameters, strong negative correlations were reported (−0.82 and −0.88, respectively).

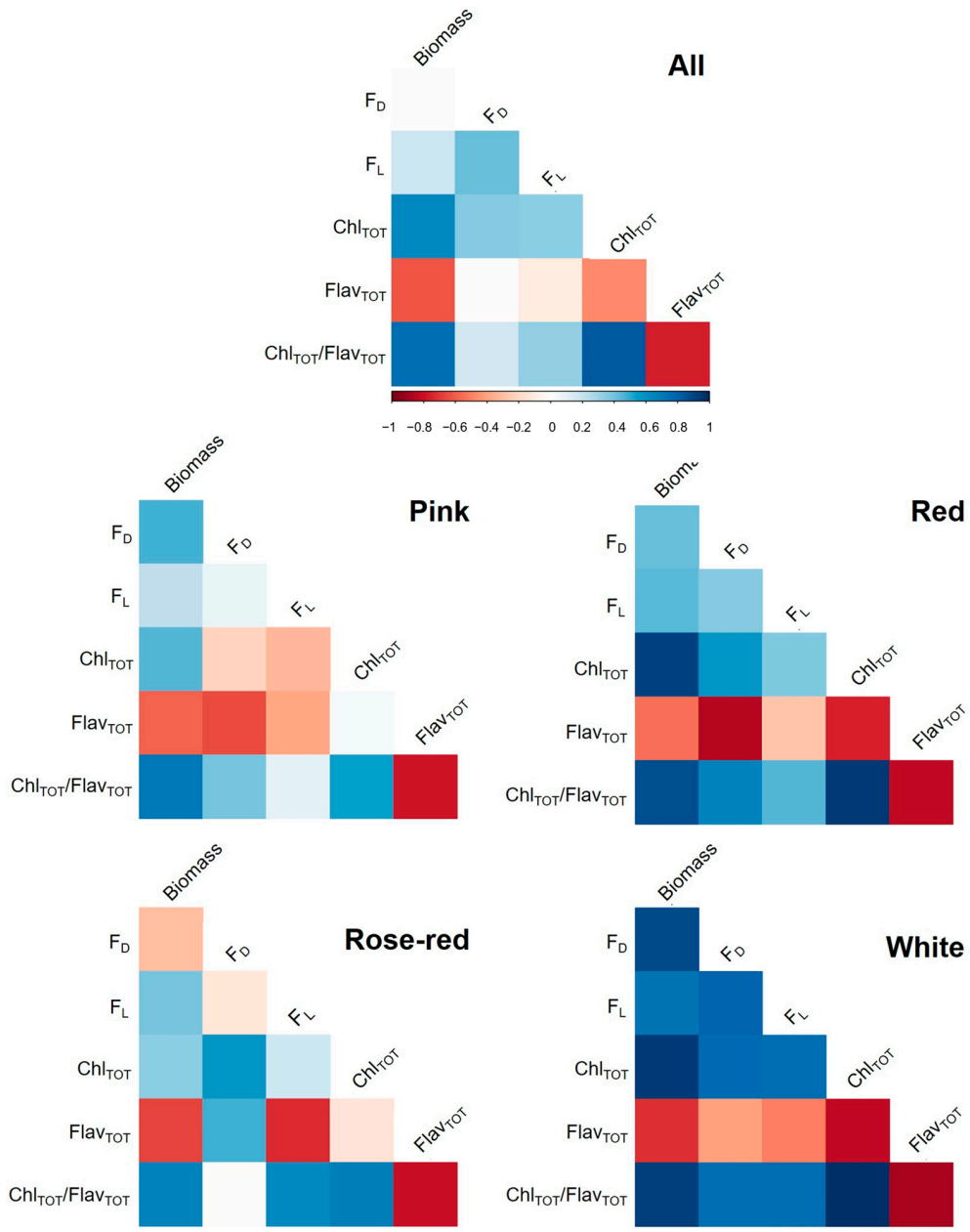

Figure 4. Pearson's correlations (*r*, from −1 to 1; see the reported color scale) among the investigated parameters (i.e., total biomass (biomass), flower diameter and duration (F_D and F_L), and leaf chlorophyll and total flavonoid contents and their ratio (Chl_{TOT}, $Flav_{TOT}$, and $Chl_{TOT}/Flav_{TOT}$ ratio, respectively) calculated taking all petunia varieties together and then separated (pink, red, rose-red, and white).

4. Discussion

The first aim of the present study was to screen and elucidate the responses (at both the leaf and flower levels) of four petunia varieties with different floral pigmentation to increasing O_3 concentrations. At a macroscopic level, O_3 induced the development of typical visible injuries on leaf surfaces, independent of the variety. Indeed, the AOT40 values (and peaks before analyses) reached in the OTCs were extreme and were far be-

yond the O_3 critical levels for potential damage to plants, established at an AOT40 of >3–5 ppm h [50], thus leading to symptomatic responses regardless of the effective degree of plant sensitivity.

FD and FL were compromised in the white variety under AA + 60, and in the pink and red varieties under AA + 120 only, while these parameters never changed in the rose-red variety, thus suggesting that different flower pigmentation might influence the effects of increasing O_3 concentrations on flowering development. A previous work [32] found that an exposure to 120 ppb of O_3 for five days (6 h day^{-1}) accelerated the flowering of wild-type *Sinapis arvensis* (Brassicaceae). Similar findings were reported by Hayes et al. [34] in *Lotus corniculatus* (Fabaceae) exposed to 30–70 ppb of O_3 for six days. Since flowering is a critical stage in the lifecycle of a plant, any type of alteration to this process requires attention, since the consequences could be reflected in the ecosystem community (microbial, plant, and insect species [32,34,51]).

According to the development of symptoms (the timing and extension of which are often indicative for determining plant sensitivity [27]), Chl_{TOT} decreased under AA + 60 in all varieties, but only in the red and white varieties did it also show statistically different values at AA + 120. It is known that visible injuries, such as leaf chlorosis and necrosis, and Chl loss are the most indicative effects of O_3 on leaves [52], even if several structural and ultrastructural changes usually occur after O_3 penetration through the stomata [13].

Flavonoids are secondary metabolites included in the group of polyphenol compounds and synthesized by the general phenylpropanoid pathway in plants, abundantly present in different organs and responsible for color (especially anthocyanins, from pink to blue), fragrance, and flavor characteristics. In plants, these compounds act in different ways, by regulating cell growth, attracting pollinator insects, scavenging ROS, and protecting tissues from the direct and detrimental effects of both biotic and abiotic stresses [53]. In this work, the different floral pigmentation did not influence the initial $Flav_{TOT}$ content. However, under AA + 60, $Flav_{TOT}$ increased only in the red variety, while under AA + 120, it increased in all varieties, showing the highest accumulation in the red variety (with levels notably higher than in the white one). Flavonoids could constitute an effective shield against the penetration of UV-B radiation into sensitive leaf tissues [54]. In the literature, the stimulation of flavonoid production by increasing O_3 concentration is widely reported (e.g., [54,55]), and this is related to the fact that when plants are exposed to O_3, their antioxidant defense mechanism is commonly triggered by the expression of defense genes (e.g., phenylpropanoid pathway genes), followed by an accumulation of ROS scavengers. The increase in antioxidant-related enzyme activity and phenylpropanoid metabolites, along with the formation of various phenolic compounds, is known as one of the main responses of plants under oxidative stress [56]. This phenomenon has even favored and encouraged the use of O_3 as a new technology for crop protection during post-harvest management [57,58]. In our work, we did not identify specific flavonoids, but, according to the literature, we certainly hypothesized the presence of quercetin and kaempferol among the peaks observed [59]. Specifically, these two are flavonols, which differ from each other only in the degree of hydroxylation on the B-ring of the flavonoid skeleton, with quercetin being dihydroxylated and kaempferol being monohydroxylated, and their ratio can be subject to change in *Petunia* species as a response to stimuli [60]. The evaluation of the $Chl_{TOT}/Flav_{TOT}$ ratio is not so frequent in the literature, but it is often expressed as the Nitrogen Balance Index (NBI), where data are derived from non-destructive optical measurements [61]. The NBI allows us to understand how N is preferentially distributed or allocated among tissues. Here, the trends observed for Chl_{TOT} and $Flav_{TOT}$ are confirmed by the $Chl_{TOT}/Flav_{TOT}$ values, since in all varieties, this ratio decreased under AA + 60, except for in the rose-red variety, where it occurred only under AA + 120, thus suggesting that varieties with a higher degree of sensitivity enforced physiochemical barriers through the accumulation of N-containing compounds to balance the effects of O_3 on the photosynthetic apparatus. Furthermore, this trend was also observed in biomass

reduction, another important indicator of plant sensitivity to O_3 [27,62] and a factor that might influence the esthetic value of an ornamental species.

Another major and novel achievement of the present study was the elucidation of the variety-specific relationships among leaf biochemical responses and morphological and phenological changes in flowers. As reported above, few studies have focused on the direct effects of O_3 on flowers. To the best of our knowledge, this is the first work where biochemical changes due to increasing O_3 concentrations are correlated to flower macroscopic responses. Considering all the petunia varieties together, no significant correlations were found among the investigated parameters. This is mainly due to the fact that varieties with a red percentage tended to show a similar response. Indeed, we found the strongest relationships in the white petunia, where all parameters were positively related with F_D and F_L (except for $Flav_{TOT}$). Consequently, it is possible to speculate that the smaller flower sizes observed under elevated O_3 concentrations significantly contributed to the reduction in biomass. Similarly, the repartitioning of N in the leaf (i.e., $Chl_{TOT}/Flav_{TOT}$ ratio) might also have effects on the flower duration and be related to a premature fall. In the other varieties, FD and FL did not alter the biomass of plants exposed in OTCs. Conversely, the smaller flower diameter reported in the pink and red varieties was associated with a $Flav_{TOT}$ increase. The arrangement of biochemical parameters was correlated to FD and FL in rose-red petunias, which effectively never changed under increased O_3 concentrations. Flower longevity was proven to be sensitive to other environmental factors, such as water availability and air temperature [63–65], and since flower development and maintenance demand carbon, nutrients, and water resources [63,66,67], extended longevity may bring high energetic and transpiration costs to the plant in order to maintain flower function [68]. Therefore, flower longevity should be optimized by selection to maximize fitness (i.e., an increase in secondary metabolites at leaf levels to protect leaf tissues) at minimum costs (e.g., reducing flower size and plant biomass). This preliminary study offers only a general view of plant-variety-dependent responses. With the aim of preserving the urban green environment, and considering our results, we suggest to perform a specific and more accurate screening in plant metabolites (also at a genetic level), to show how they are stimulated or suppressed by increasing O_3 concentrations, including the potential detrimental changes in volatile organic compounds (responsible for the floral scent). Choosing the optimal ornamental variety, able to tolerate high O_3 levels, is a fundamental step that may help in establishing a good ecosystem balance by maintaining trophic relations among components (e.g., the plant–insect pollinator relationship).

5. Conclusions

In conclusion, the present study highlighted different responses to increasing O_3 stress conditions among petunia verities with various floral pigmentation and showed that variety-specific changes in chlorophyll and flavonoid contents at the leaf level can influence the morphology and duration of flowers: the rose-red variety was shown to be the most O_3-tolerant, the pink and red ones showed a moderate degree of sensitivity, and, finally, the white variety was confirmed as the most sensitive due to both leaf biochemical and flower morphological impairments. Overall, these outcomes highlight the importance of investigating the effects of air pollution on the flowers of ornamental plants, as their pigmentation can affect the sensitivity/responses to O_3 challenge. Further investigations focusing on other plant species and flower parameters would be helpful for a more complete understanding of these mechanisms.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14092027/s1, Figure S1: Flowers of *Petunia hybrida* with pink (Spreading Petunia Easy Wave® pink, #PAS3189), red (Spreading Petunia Easy Wave® red, #PAS1016994), rose-red (Spreading Petunia Easy Wave® Red Velour, #PAS933560), and white (Spreading Petunia Easy Wave® white, #PAS760712) floral pigmentation; Figure S2: Experimental design, non-destructive and destructive analyses conducted on pink, red, rose-red, and white petunia varieties exposed in open-top chambers to ambient air (AA; *white bar*), AA increased by 60 ppb

(AA + 60; *light-grey bar*) and AA increased by 120 ppb (AA + 120; *dark grey bar*) in ozone (O_3) concentration for 85 consecutive days (9 h day^{-1}); Figure S3: Visible injuries on leaves of *Petunia hybrida* plants exposed in open-top chambers to ambient air (AA; *white bar*), AA increased by 60 ppb (AA + 60; *light-grey bar*) and AA increased by 120 ppb (AA + 120; *dark-grey bar*) in ozone (O_3) concentrations for 85 consecutive days (9 h day^{-1}); Figure S4: Flavonoid profiles obtained by high-performance liquid chromatography of *Petunia hybrida* leaves (pink, red, and rose-red varieties) exposed in open-top chambers to ambient air (AA), AA increased by 60 ppb (AA + 60) and AA increased by 120 ppb (AA + 120) in ozone concentration for 85 consecutive days (9 h day^{-1}). PF1-4 indicates quantified flavonoid components; Figure S5: Flavonoid profiles obtained by high-performance liquid chromatography for *Petunia hybrida* leaves (white variety) exposed in open-top chambers to ambient air (AA), AA increased by 60 ppb (AA + 60) and AA increased by 120 ppb (AA + 120) in ozone concentrations for 85 consecutive days (9 h day^{-1}). PFW1-4 indicates quantified flavonoid components; Figure S6: Summary of the results for flower diameter (F_D), flower longevity (F_L), chlorophyll total content (Chl_{TOT}), flavonoid total content ($Flav_{TOT}$), and chlorophyll total content–flavonoid total content ratio ($Chl_{TOT}/Flav_{TOT}$) for pink, red, rose-red, and white petunia varieties exposed in open-top chambers to ambient air (AA; *white bar*), AA increased by 60 ppb (AA + 60; *light-grey bar*), and AA increased by 120 ppb (AA + 120; *dark-grey bar*) in ozone (O_3) concentration for 85 consecutive days (9 h day^{-1}). Symbols (up and down arrows and equals signs) indicate differences in each variety due to the different O_3 concentrations. Colors indicate differences among the varieties compared at the same O_3 concentration: from the darkest to the lightest orange, the highest to lowest statistically different values are shown; a white color means no statistical difference; striped orange denotes that statistical difference exists only with darker or lighter orange. Table S1. *p*-values of correlations among the parameters.

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