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Wood Distillate Mitigates Ozone-Induced Visible and Photosynthetic Plant Damage: Evidence from Ozone-Sensitive Tobacco (*Nicotiana tabacum* L.) BelW3

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Abstract: The use of wood distillate (WD) is emerging as a valuable strategy for protecting horticultural crops from the oxidizing effects of ozone (O_3). To fully understand its effectiveness, extensive testing on different plant species is needed. As a viable interim measure, an assessment of WD efficacy in model plants can be made until species-specific results become available. The aim of this study is to evaluate the ability of WD to protect the ozone-sensitive tobacco plant (*Nicotiana tabacum* L.) BelW3 from the oxidizing effects of O_3, using the ozone-resistant tobacco plant BelB as a benchmark. The protective effect was evaluated during treatment applications and three weeks after these were completed. Ten BelW3 and five BelB plants were grown just outside Parma from June to October 2023, a period when average maximum O_3 concentrations were at least 120 ppb. Starting from July, five BelW3 plants were sprayed weekly with WD at 0.2% for two months. Morphometric and photosynthetic measurements were then taken after six and 11 weeks from the beginning of treatments and three weeks after the end to assess protection persistence (if any). BelW3 showed a significant effect of O_3 compared to BelB plants for both morphometric and photosynthetic measurements, exhibiting increased necrotic areas on the leaf blade, reduced number of viable leaves, reduced average plant height, together with reduced chlorophyll content and impaired photosynthetic system functionality. BelW3 plants also showed a significant decrease in the efficiency of parameters related to PSII and PSI when compared to BelB. Wood distillate application, however, successfully mitigated O_3 effects on BelW3, as revealed by morphometric and photosynthetic values, which were in line with those observed in BelB. Notably, WD protective effect persisted 3 weeks after treatment cessation, highlighting the short-term protective capacity of the distillate against the oxidative action of O_3.

Keywords: leaf injury; leaves number; model species; ozone-sensitive plants; photosynthesis; plant height

Citation: Vannini, A.; Petraglia, A. Wood Distillate Mitigates Ozone-Induced Visible and Photosynthetic Plant Damage: Evidence from Ozone-Sensitive Tobacco (*Nicotiana tabacum* L.) BelW3. *Horticulturae* **2024**, *10*, 480. https://doi.org/10.3390/horticulturae10050480

Academic Editors: Begoña Miras-Moreno and Esther Novo-Uzal

Received: 5 April 2024
Revised: 2 May 2024
Accepted: 6 May 2024
Published: 7 May 2024

1. Introduction

Ozone (O_3) poses a significant threat to vegetation [1]. By generating reactive oxygen species (ROS) such as -OH, -O_2, and H_2O_2 [2], O_3 disrupts the functionality of plant photosynthetic systems, retarding their development and accelerating senescence. This effect is particularly important in agriculture as it leads to reduced crop yields [3,4] which result in significant economic losses [5]. Crops affected by the phytotoxic effects of O_3 include wheat (*Triticum aestivum*) [6], maize (*Zea mays*), soybean (*Glycine max*) [7], and even rice, potato, and barley [5]. Wheat, in particular, stands out as one of the agronomic plants most sensitive to O_3 [8]. In 2019, wheat production in Europe was severely impacted by O_3, resulting in an average reduction of 20,000–35,000 tons in Italy alone [9]. In certain Italian regions, such as Emilia-Romagna, the reduction reached 60,000 tons, equivalent to 1.5% of national production (estimated at 4 million tons) [10], a significant datum considering that in this region O_3 levels are high, often reaching up to 200 parts per billion (ppb) during the summer [11]. Future increases in CO_2 may potentially mitigate the negative effects of O_3 on crop yields [12] which are expected to worsen with rising ground-level concentrations [5]. Even though short-term effects are hard to observe, reducing atmospheric concentrations of O_3 precursors—such as NO_X and CH_4—remains of crucial importance [13]. Until such time, further studies are needed to assess potential protective strategies to counteract the negative effects that the constant rise in summer O_3 concentrations in Europe may have on plants.

To date, numerous molecules have been tested which could protect plants from the oxidative effects of O_3 [14–21]; however, some of them are synthetic, with their environmental toxicity still poorly understood [21]. In this context, natural or circular economy-based solutions would, therefore, be more ecologically sound. In agriculture, the environmental benefits associated with such solutions are being increasingly recognized, especially those related to the pyrolysis of woody biomass, such as pyroligneous acid [22,23]. Pyroligneous acid, also known as wood vinegar or wood distillate (hereafter WD), is a product obtained from the distillation of wood gases produced during the thermal treatment of wood under anoxic conditions [22]. The resulting WD consists of a complex mixture of organic substances, including acetic acid, methanol, formaldehyde, acetone, and other compounds, and is widely used in various industries due to its solvent and preservative properties. From an agronomic perspective, its application reduces oxidative stress in plants [24], increases yields [16], and protects the photosynthetic system of common vegetable plants exposed to ecologically relevant O_3 concentrations (60 ppb)—a benefit evidenced in studies on lettuce [25]. This protective effect can probably be attributed to the high content of antioxidant molecules in WD—such as tannins or polyphenols in general [26]—which are able to protect plants' photosynthetic systems. However, to fully understand WD effectiveness in crop protection, thorough species-specific testing is needed, a process requiring years of experimentation, and which is already underway in current research. The evaluation of WD efficacy in model species, therefore, represents a valid interim measure to be pursued until such comprehensive species-specific results are available.

With regard to recent studies, Saitanis et al. [17] investigated the potential of O_3 sensitive *Nicotiana tabacum* L. plants (BelW3 mutants) [27] in order to provide insights into the effectiveness of different agrochemicals in protecting plants from the oxidizing effects of O_3. Of the seven biocides tested, penconazole, hexaconazole, and kresoxim-methyl were found to significantly protect the photosynthetic system of BelW3 plants and reduce leaf senescence, suggesting that these compounds would be suitable candidates for species-specific studies. The use of BelW3 plants as a model species to test the efficacy of synthetic anti-ozonant molecules could also provide important indications for an assessment of the overall protective effect of WD on economically important crops. Wood distillate application on plants could also be extended after treatment cessation in order to investigate the short-term durability of its effect, thus providing new insights regarding the duration of WD's protective effect for which information is still lacking.

The present research investigates the effectiveness of WD in protecting the photosynthetic system of horticultural plants, using BelW3 (ozone-sensitive) tobacco plants as a model organism. The main aim was to evaluate the ability of WD derived from forest wood to protect BelW3 tobacco plants against the oxidizing effects of O_3. A field experiment was carried out in the urban area of Parma. A comparative analysis of the physiological results was made, comparing results obtained on BelW3 with those of BelB, i.e., tobacco plants known for their resistance to this pollutant [28]. The research also aimed to investigate the persistence of the protective effect (if any), by carrying out measurements three weeks after the end of treatments. We hypothesized that WD application would reduce leaf senescence in BelW3 plants and protect their photosynthetic apparatus from the phytotoxic effects of O_3, both during the treatments and three weeks after their cessation. We also hypothesized that BelW3 and BelB would exhibit similar morphological and physiological responses to treatments.

2. Materials and Methods

2.1. Experimental Design

Seeds of ozone-resistant (BelB) and ozone-sensitive (BelW3) *Nicotiana tabacum* L. varieties were purchased from a certified European producer (NiCoTa; www.NiCoTa.de, accessed on 20 January 2023). For each variety, 20 seeds were germinated in a germination box (relative humidity = 95%) located in a warm (24 °C), dark climate chamber. After two weeks, the tallest seedlings (>1 cm) were transplanted into 50 mL volume phytocells containing a suitable commercial soil substrate and left to grow in a climate chamber at 24 °C and 1000 Photosynthetic Photon Flux Density (PPFD) and 10/14 h light/dark period. After one month (1 June 2023), five BelB and 10 BelW3 seedlings of 5 cm each in height were transplanted into 1.2 L pots containing commercial soil substrate (Table 1) and left to grow in the peri-urban area of Parma (Montanara district to the south of the city) for 14 weeks, until 3 October 2023.

Table 1. Main characteristics of the substrate used for the cultivation of BelW3 and BelB tobacco plants.

Parameters	Values
Peat	50%
Organic carbon (dw)	25%
Humic and fulvic carbon (dw)	7%
Organic nitrogen (dw)	80% of total nitrogen
Salinity	1.20 dS/m

To simulate the most common type of cultivation, plants were exposed to ambient rainfall and were watered daily with well water, with no artificial watering being carried out on rainy days. Furthermore, no fertilizer was applied. Starting on 29 June, five randomly selected BelW3 plants were labeled and sprayed weekly with 100 mL of a 1:500 *v*/*v* (0.2%) WD solution derived from forest wood, including *Pinus* spp., *Abies* spp., *Cedrus* spp., *Castanea sativa* Mill., *Alnus* spp., *Quercus* spp., *Acacia* spp., and *Robinia pseudoacacia* L., on both the lower- and upper-leaf surface of each plant. WD was produced from a reducing oxide plant, using countercurrent steam extraction with controlled temperature gradients (Endotech RM Energy Solutions). Throughout the experiment, the three groups of plants—BelB, untreated BelW3 (positive control), and treated BelW3 (BelW3(wd))—were kept at a distance of five meters from each other to prevent the wood distillate being absorbed by the untreated BelW3. Treatments were completed 11 weeks later (12 September). All three groups of plants were then left to grow for a further three weeks (until 3 October). During the field trial, the average temperature was around 24 °C. The highest temperatures were recorded at the end of August (39.9 °C), while the lowest at the end of September (11.1 °C). Temperatures were obtained from the weather station located approx. 5 km from the BelW3 and BelB tobacco planting area (San Pancrazio; Parma). In addition, the average O_3 concentration was 122 ± 18 ppb. In particular, the following mean O_3 concentration was recorded for each plant-growth period: 119 ± 25 ppb during the pre-treatment period (1 June–28 June); 122 ± 18 ppb during the treatment period (weekly spraying with wood distillate 1:500 *v/v* from 29 June to 6 September); and 83 ± 22 ppb during the post-treatment period (6 September–3 October). Ozone concentrations were obtained from the ARPAE station located approx. 1.5 km from the BelW3 and BelB tobacco planting area (Cittadella Park; Parma; coordinates: 44.791311, 10.329581). An overview of the climatic conditions and O_3 concentration during the test period is shown in Figure 1.

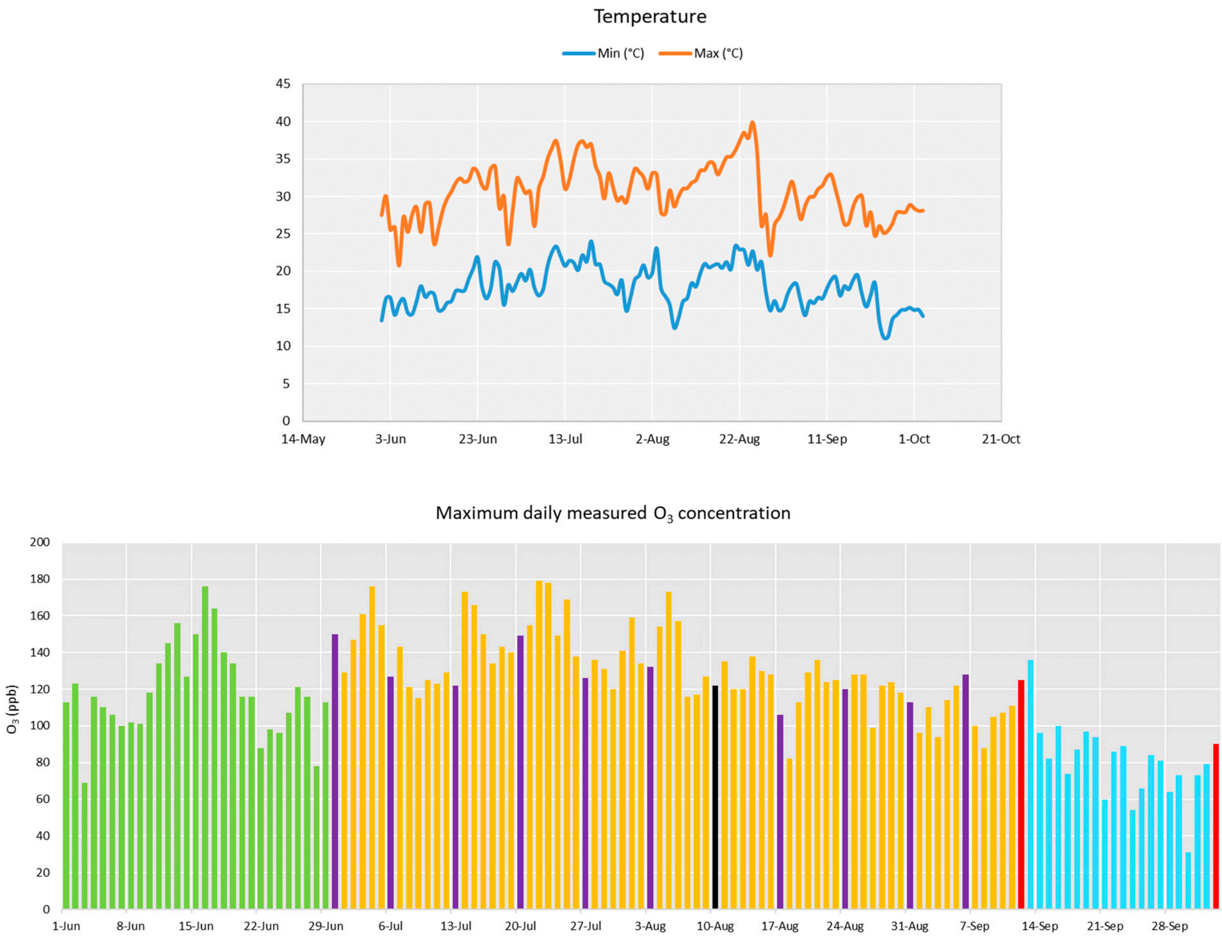


Figure 1. (**Top graph**) Maximum and minimum daily air temperature (°C) recorded during the experiment. (**Bottom graph**) Maximum daily ozone concentration (ppb) recorded at the ARPAE station. Green bars: pre-treatment period of plant growth in the peri-urban area of Parma. Orange bars: treatment period, with weekly spraying using wood distillate 1:500 *v/v*. Blue bars: post-treatment period of plant growth, without WD. Violet bars: treatment days. Black bar: treatment and plant measurement day. Red bars: plant measurement days.

2.2. Tobacco Plant Measurements

Measurements on tobacco plants were carried out at 6, 11, and 14 weeks after the beginning of WD treatments.

2.3. Measurements of Plant Height and Leaf Number

Plant height and leaf number are two of the most important parameters associated with plant yield and development [29–31]. Plant height (cm) was measured as the length of the plant from the soil surface to the base of the highest leaf, whether fully developed or not. Leaf number included only viable leaves, i.e., those with <50% necrotic area on the leaf surface, with only >6 cm leaves being counted. Counting was carried out from top to bottom.

2.4. Measurements of Photosynthetic System Functionality

The chlorophyll content and the functionality of the photosynthetic system are key parameters to assess the effectiveness of WD in protecting the plant photosynthetic machinery from the oxidizing action of O_3 [25]. The chlorophyll content of plant leaves was estimated using an atLEAF+ chlorophyll meter (Wilmington, DE, USA); data are expressed as atLEAF values [32]. The functionality of the photosynthetic system was recorded using the Handy PEA instrument (Handy PEA, Hansatech Ltd., Norfolk, UK), set with a single

1-s light flash at an intensity of 3000 PPFD. Results were expressed using the parameters described in Table 2.

Table 2. Photosynthesis-related parameters used to assess photosynthetic system functionality. For details, see Strasser and Tsimilli-Michael [33] and Stefanov et al. [34].

Parameters	Description
F_V/F_M	Maximum quantum efficiency of Photosystem II
PI_{TOT}	Performance Index for energy conservation from excitation to the reduction of PSII and PSI
RC/CS_0	Density of reaction centers (Q_A reducing PSII RC)
TR_0/CS_0	Maximum trapped exciton flux per excited cross section
ET_0/CS_0	Electron transport flux from Q_A to Q_B per excited cross section
RE_0/CS_0	Electron transport flux until PSI acceptors per excited cross section

As plants did not have the same number of leaves, we chose not to perform analysis on entire plants but only on a specific leaf, ensuring it was the same for each plant. The selected leaf was the one corresponding to the last vital leaf (visually damaged by less than 50%) of the BelW3 plant with the fewest leaves. Counting was carried out from top to bottom. The selected leaves were number six and seven for the 6- and 11-week monitoring, respectively, while leaf number eight was selected for the 3-week post-treatment monitoring. The criterion used for the choice of the measured leaf is summarized in Figure S1. For each leaf and monitoring session, five measurements were taken on the outer part of the leaf blade (Figure 2).

Figure 2. Area where photosynthetic measurements were carried out.

2.5. Statistical Analysis

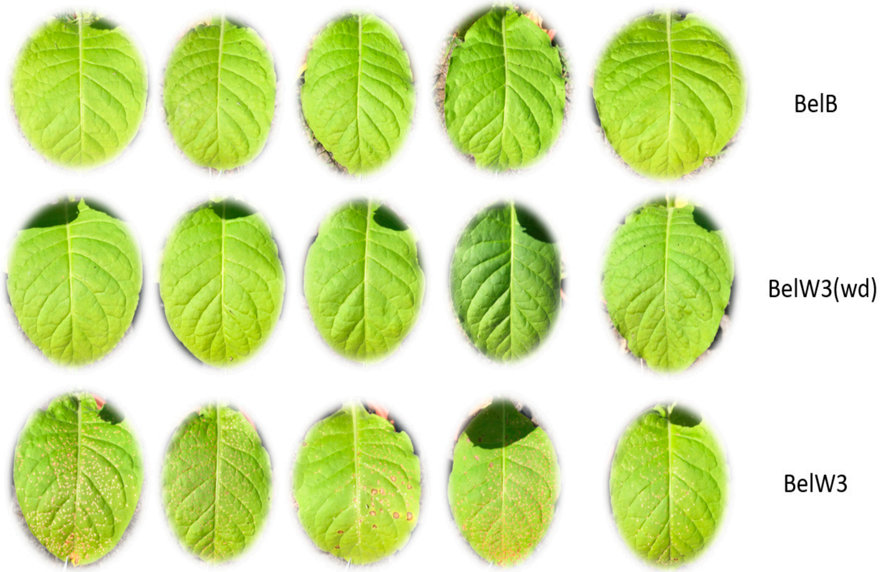
Differences in the percentage of necrotic areas between BelW3, BelB, and BelW3(wd) leaves were tested using the Mann–Whitney U test. To compare differences in chlorophyll content and chlorophyll fluorescence between treated (BelW3(wd)) and untreated (BelW3 and BelB) plants, a linear mixed-effect model (using the 'lme4' package) was employed for each variable. Treatment was considered as a fixed effect, while individual plants were treated as random effects. Model validation included checking for homoscedasticity through scatterplots of residual and fitted values; normality was assessed using normal probability (qqnorm) plots together with the Shapiro–Wilk test. Models were fitted using restricted maximum likelihood (REML) estimation, and their significance was determined via ANOVA (using the 'car' package). Tukey's post hoc ('multicomp' package) was used to search for statistically significant differences between treatments. All analyses were conducted using the open-source R-4.4.0 software [35].

3. Results

Results are presented on a temporal basis in order to highlight the protective effect of WD on ozone-sensitive plants both during and after the treatments.

3.1. Tobacco Growth and Vitality Six Weeks after Treatment with WD

After 10 weeks of growth in the peri-urban area of Parma, untreated BelW3 tobacco plants showed numerous small necrotic areas on the surface of their leaves (estimated mean necrotic damage = 9.4%). In contrast, there were no obvious signs of leaf necrosis on BelW3 leaves treated with WD for six weeks, the same outcome as for BelB leaves (Figure 3). Statistically significant differences were observed between BelW3 leaves and BelB, and between BelW3 and BelW3(wd) ($p < 0.01$). In contrast, no differences were observed between BelB and BelW3(wd) ($p > 0.05$).



Compared treatments	*p*-value
BelB:BelW3(wd)	0.11
BelB:BelW3	0.007
BelW3:BelW3(wd)	0.007

Figure 3. (**Top**) Photographs of leaf number six taken after 10 weeks of growth in the urban area of Parma. Leaves taken from each of the five plants in each group: BelB, BelW3(wd) (BelW3 plants treated with WD), and BelW3 (untreated). (**Bottom**) Statistical results of Mann–Whitney U test.

After 11 weeks, the leaves of untreated BelW3 plants showed a statistically significant reduction in all photosynthesis-related parameters evaluated compared to the control BelB leaves ($p < 0.05$; Figure 4).

Specifically, the chlorophyll content was reduced by 29%, the number of reaction centers by 46% (RC/CS_0), photosynthetic efficiency (F_V/F_M) by 25%, and the total performance index (PI_{TOT}) by 87%; the parameters related to trapping flux (TR_0/CS_0), energy transmission (ET_0/CS_0), and electron transmission from PSII to PSI (RE_0/CS_0) were reduced by 21%, 44%, and 43%, respectively. However, the investigated parameters of BelW3(wd) plants treated for six weeks with foliar WD applications showed significantly higher values than BelW3 plants ($p < 0.001$): chlorophyll value (+34%), RC/CS_0 (+62%), F_V/F_M (+33%), PI_{TOT} (+389%), TR_0/CS_0 (+25%), ET_0/CS_0 (+67%), and RE_0/CS_0 (+92%). Only the chlorophyll content, the F_V/F_M, the TR_0/CS_0, and the ET_0/CS_0 of these plants showed similar values to those measured in BelB.

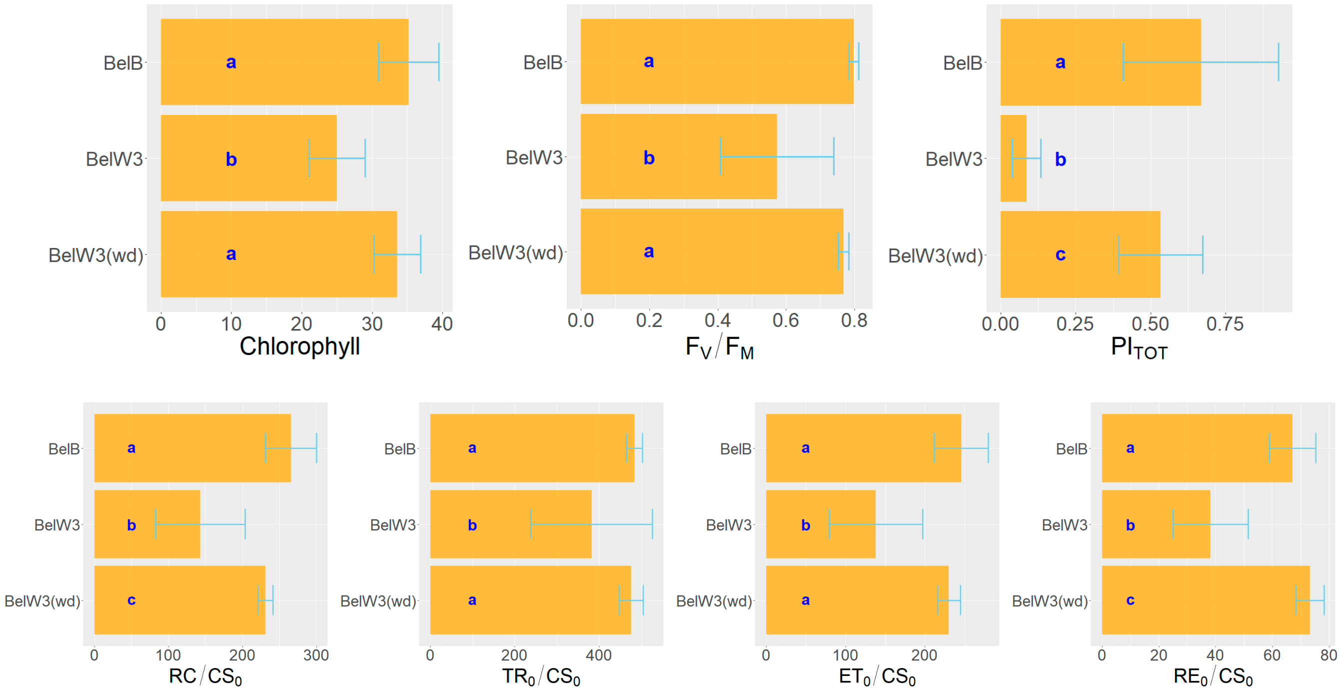


Figure 4. Expression (mean ± standard deviation) of atLEAF values (Chlorophyll), photosynthetic efficiency (F_V/F_M), total performance index (PI_{TOT}), number of reaction centers per excited cross section (RC/CS_0), trapping flux per excited cross section (TR_0/CS_0), energy transmission per excited cross section (ET_0/CS_0), and electron transmission from PSII to PSI per excited cross section (RE_0/CS_0). Parameters refer to leaf number six of BelB, BelW3 (untreated), and BelW3(wd) (BelW3 plants treated with WD). Different letters indicate statistically significant ($p < 0.05$) differences between treatments.

3.2. Tobacco Growth and Vitality 11 Weeks after Treatment with WD

After 15 weeks of growth in the peri-urban area of Parma, BelW3 leaves showed extensive necrotic areas on their surface (estimated mean necrotic damage = 24.6%). There was less visual evidence of necrotic areas on BelW3 leaves treated with WD for 11 weeks (estimated mean necrotic damage = 6.8%), although certain leaves were more damaged than others. BelB plants showed no signs of necrosis (Figure 5). Statistically significant differences were only observed between BelW3 and BelB leaves ($p < 0.01$); in contrast, no differences were observed between BelW3 and BelW3(wd) or between BelW3(wd) and BelB ($p > 0.05$).

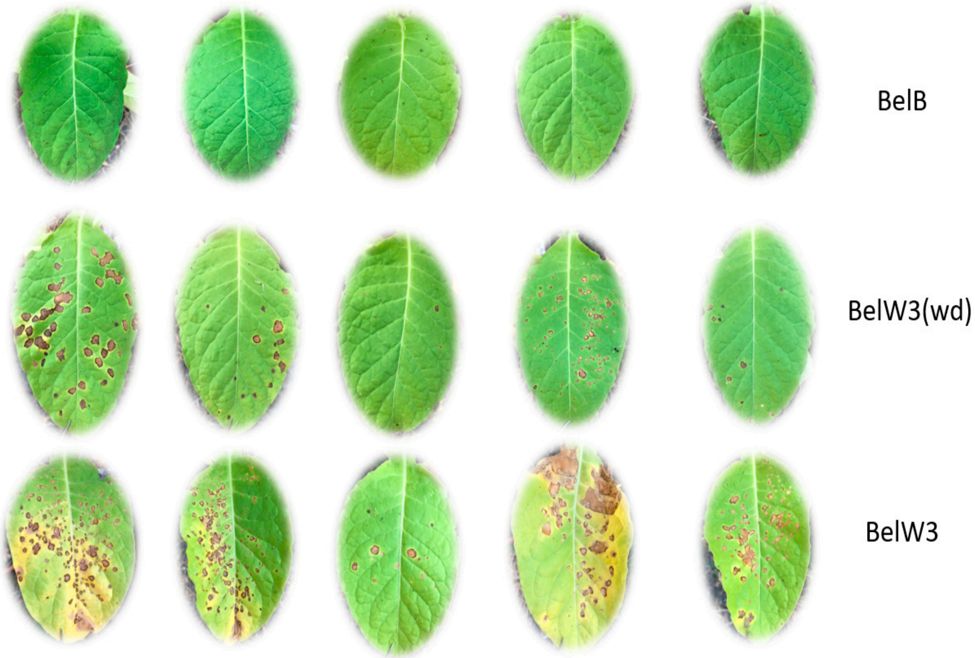
BelW3 plants showed a significant reduction in leaf number and plant height compared to BelB ($p < 0.05$) with percentage reductions of 20% and 7%, respectively (Figure 6).

As expected, BelW3 plants treated with WD showed higher values ($p < 0.001$) than BelW3 by 13% and 33%, respectively. For both leaf number and plant height, BelB and BelW3(wd) plants differed significantly, with BelW3(wd) plants showing only a 10% reduction in leaf number compared to BelB plants, while plant height was 24% higher ($p < 0.05$).

Leaf number seven of untreated BelW3 plants showed a statistically significant reduction in all photosynthesis-related parameters evaluated compared to the control BelB ($p < 0.05$) (Figure 7).

Specifically, chlorophyll content was reduced by 37%, the number of reaction centers by 51% (RC/CS_0), photosynthetic efficiency (F_V/F_M) by 21%, and the total performance index (PI_{TOT}) by 66%; the parameters related to trapping flux (TR_0/CS_0), energy transmission (ET_0/CS_0), and electron transmission from PSII to PSI (RE_0/CS_0) were reduced by 35%, 47%, and 45%, respectively. However, the investigated parameters of BelW3(wd) plants showed significantly higher values than untreated BelW3 plants ($p < 0.001$): chlorophyll value (+55%), RC/CS_0 (+92%), F_V/F_M (+27%), PI_{TOT} (+212%), TR_0/CS_0 (+38%), ET_0/CS_0

(+79%), and RE_0/CS_0 (+74%). Only the total performance index (PI_{TOT}) showed statistically significant differences when compared to BelB plants ($p = 0.048$).



Compared treatments	*p*-value
BelB:BelW3(wd)	0.07
BelB:BelW3	0.007
BelW3:BelW3(wd)	0.07

Figure 5. (**Top**) Photographs of leaf number seven taken after 15 weeks of growth in the urban area of Parma. Leaves taken from each of the five plants in each group: BelB, BelW3(wd) (BelW3 plants treated with WD), and BelW3 (untreated). (**Bottom**) Statistical results of Mann–Whitney U test.

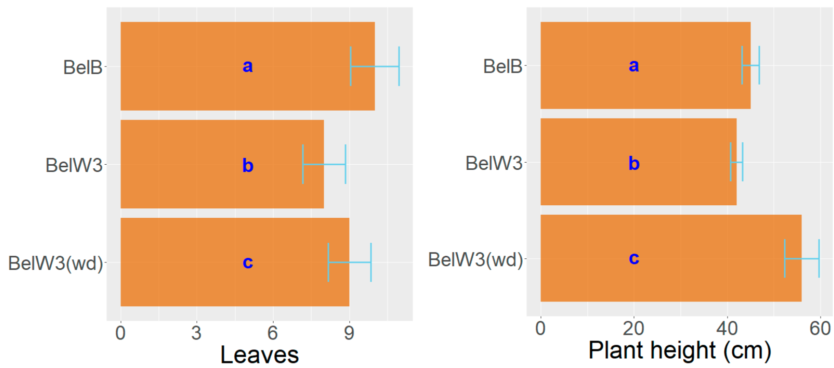


Figure 6. Leaf number (Leaves) and plant height (mean ± standard deviation) of BelB, BelW3 (untreated), and BelW3(wd) (BelW3 plants treated with WD). Different letters indicate statistically significant ($p < 0.05$) differences between treatments.

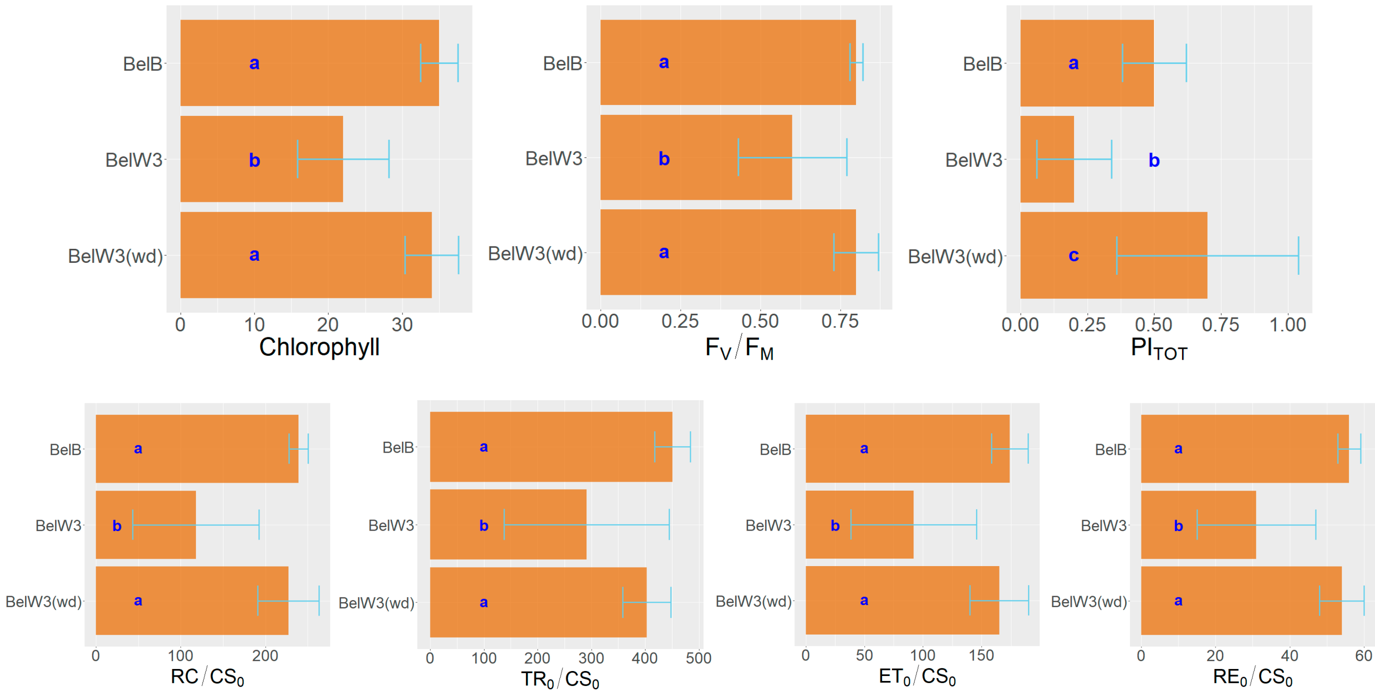


Figure 7. Expression (mean ± standard deviation) of atLEAF values (Chlorophyll), photosynthetic efficiency (F_V/F_M), total performance index (PI_{TOT}), number of reaction centers per excited cross section (RC/CS_0), trapping flux per excited cross section (TR_0/CS_0), energy transmission per excited cross section (ET_0/CS_0), and electron transmission from PSII to PSI per excited cross section (RE_0/CS_0). Parameters refer to leaf number seven of BelB, BelW3 (untreated), and BelW3(wd) (BelW3 plants treated with WD). Different letters indicate statistically significant ($p < 0.05$) differences between treatments.

3.3. Tobacco Growth and Vitality Three Weeks after the End of the Treatments with WD

After 18 weeks of growth in the peri-urban area of Parma, BelW3 tobacco plants showed reduced signs of phytotoxicity (estimated mean necrotic damage = 21.2%), with only three leaves showing evidence of damage. Compared to the previous measurement session (at 11 weeks), the effect of O_3 on BelW3 plants appears to have been slightly reduced (Figure 8). In general, BelW3 leaves treated with WD showed a reduction in damage compared to BelW3 leaves (estimated mean necrotic damage = 2.2%). Statistically significant differences were observed between BelB and BelW3 and between BelB and BelW3(wd) leaves ($p < 0.05$). However, no differences were observed between BelW3 and BelW3(wd) ($p > 0.05$).

In line with these results, BelW3 plants showed a significant reduction in leaf number and plant height compared to BelB plants ($p < 0.05$) with percentage reductions of 38% and 7%, respectively (Figure 9).

In contrast, BelW3 plants treated with WD exhibited higher values than BelW3 plants for these parameters, by 25% and 27%, respectively ($p < 0.001$). For both leaf number and plant height, BelB and BelW3(wd) plants differed significantly, with BelW3(wd) plants exhibiting a 21% reduction in leaf number compared to BelB plants, while plant height was 18% higher ($p < 0.05$).

Leaf number eight of untreated BelW3 plants showed a statistically significant reduction in all photosynthesis-related parameters evaluated compared to the control BelB ($p < 0.05$) (Figure 10).

Compared treatments	p-value
BelB:BelW3(wd)	0.02
BelB:BelW3	0.02
BelW3:BelW3(wd)	0.11

Figure 8. (**Top**) Photographs of leaf number eight taken after 18 weeks of growth in the urban area of Parma. Leaves taken from each of the five plants in each group: BelB, BelW3(wd) (BelW3 plants treated with WD), and BelW3 (untreated). (**Bottom**) Statistical results of Mann–Whitney U test.

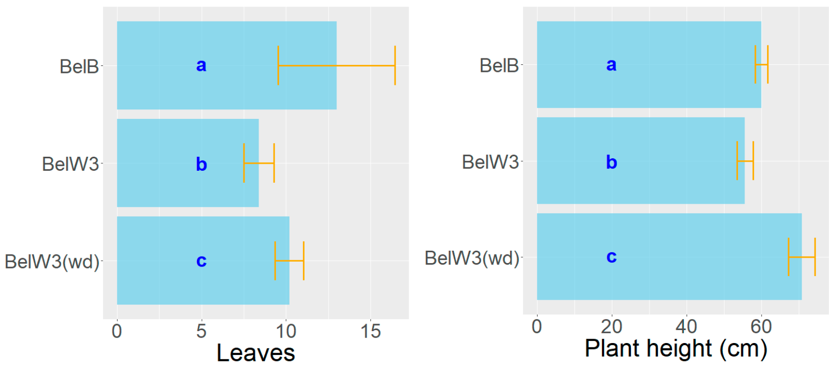


Figure 9. Leaf number (Leaves) and plant height (mean ± standard deviation) of BelB, BelW3 (untreated), and BelW3(wd) (BelW3 plants treated with WD). Different letters indicate statistically significant ($p < 0.05$) differences between treatments.

Specifically, chlorophyll content was reduced by 33%, the number of reaction centers by 24% (RC/CS_0), photosynthetic efficiency (F_V/F_M) by 21%, and the total performance index (PI_{TOT}) by 46%; the parameters related to trapping flux (TR_0/CS_0), energy transmission (ET_0/CS_0), and electron transmission from PSII to PSI (RE_0/CS_0) were reduced by 18%, 11%, and 17%, respectively. However, 3 weeks after WD treatment cessation, the investigated parameters of BelW3(wd) plants still showed significantly higher values than untreated BelW3 plants ($p < 0.001$): chlorophyll value (+54%), RC/CS_0 (+48%), F_V/F_M (+27%), PI_{TOT} (+67%), TR_0/CS_0 (+26%), ET_0/CS_0 (+38%), and RE_0/CS_0 (+27%). No differences ($p > 0.05$) were found between BelW3(wd) and BelB for any of the above parameters.

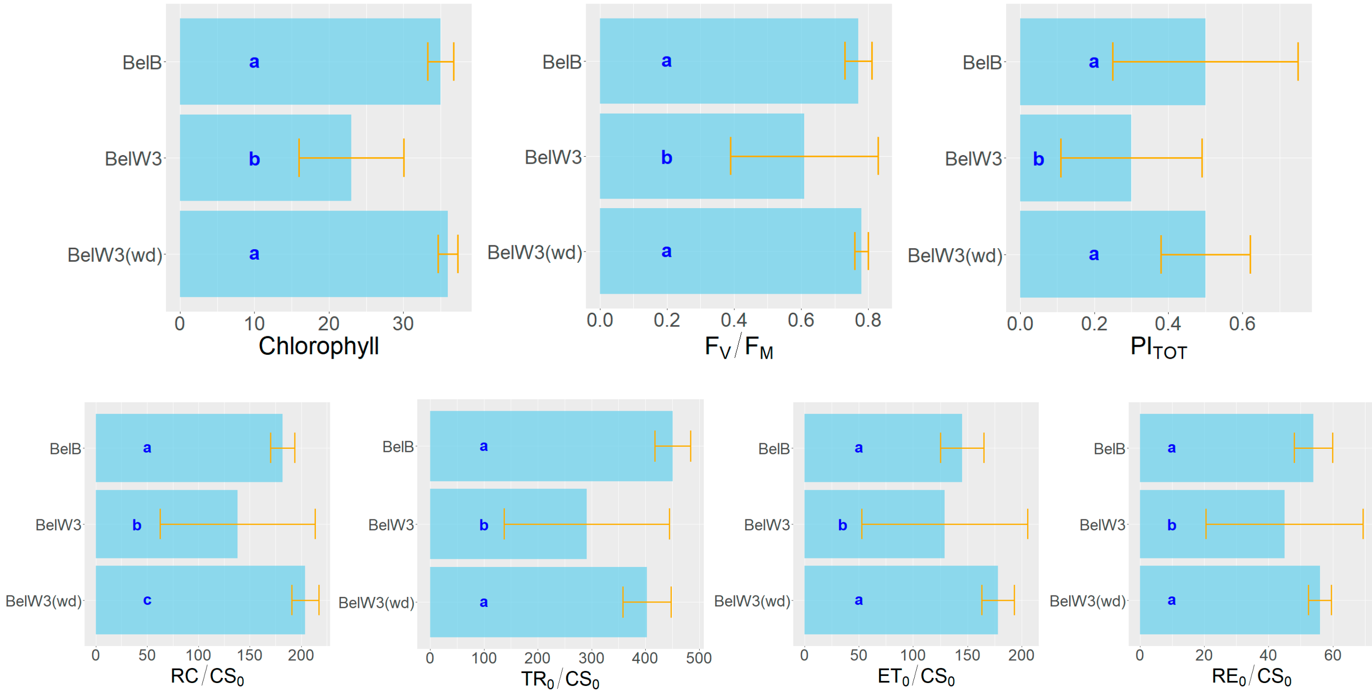


Figure 10. Expression (mean ± standard deviation) of atLEAF values (Chlorophyll), photosynthetic efficiency (F_V/F_M), total performance index (PI_{TOT}), number of reaction centers per excited cross section (RC/CS_0), trapping flux per excited cross section (TR_0/CS_0), energy transmission per excited cross section (ET_0/CS_0), and electron transmission from PSII to PSI per excited cross section (RE_0/CS_0). Parameters refer to leaf number eight of the BelB, BelW3 (untreated), and BelW3(wd) (BelW3 plants treated with WD). Different letters indicate statistically significant ($p < 0.05$) differences between treatments.

4. Discussion

BelW3 tobacco plants have been found to be highly sensitive to O_3 and their exposure to relatively low airborne concentrations (90 or 135 ppb) in controlled environments promotes leaf senescence [36]. Genetic defects seem to reduce the ability of the photosynthetic system to fully recover from damage [37], resulting in programmed cell death [38], evidenced by the appearance of necrotic zones on the leaf surface. These outcomes were confirmed by the present study, where BelW3 plants growing in the peri-urban area of Parma showed significant leaf damage, reduced photosynthetic efficiency, reduced leaf number, and reduced plant height in comparison with BelB plants.

Foliar WD applications were able to successfully mitigate the negative effects of O_3, with morphometric and photosynthetic values of BelW3 close, or almost equal, to those recorded for BelB. Specifically, foliar WD applications reduced the visual presence on BelW3 leaves of necrotic areas together with yellowish areas where chlorophyll had apparently oxidized (approx. 15%). This effect was evident after both 6 and 11 weeks of treatment. After 6 weeks, WD was able to reduce the presence of necrotic areas on the leaf surface to a minimum, with treated BelW3 leaves visually resembling those of BelB. On the other hand, after 11 weeks, WD failed to fully protect the leaves of BelW3 plants, probably a result of persistently high O_3 concentrations. The antioxidant capacity of WD may, in fact, have been exceeded by ROS generated by stomatal O_3 uptake. In line with this assumption, damage exhibited by leaves of BelW3 plants, photographed during the 11-week monitoring, was consistently greater than that in leaves photographed during the 6-week monitoring period. However, it cannot be excluded that more frequent WD applications or more concentrated dilutions, i.e., a higher amount of spray-applied antioxidants, may prove more effective in protecting leaves from the phytotoxic effects of the contaminant. This effect should be tested to be confirmed. Three weeks after the end of treatments, there was a tendency for the visual differences between untreated BelW3 and treated BelW3(wd) leaves to be less marked. In spite of this, some BelW3(wd) plants exhibited fewer necrotic areas than BelW3 and, in some cases, showed an apparently similar number (see leaf number three of BelW3(wd), Figure 8). In line with this result, no significant differences between BelW3 and BelW3(wd) were observed. Overall, results suggest that the protective effect of WD was only partially maintained. In fact, two BelW3 leaves still showed very high O_3 damage, with almost 40% of the leaf area exhibiting necrosis, whereas in BelW3(wd) plants this percentage was much less, estimated at approx. <5%. A higher number of replicates should have been employed to clarify this effect.

In addition, foliar application of WD significantly reduced leaf senescence in BelW3 plants (approx. 17%). However, their leaf number never reached that of BelB plants. This result was observed after 11 weeks of WD spraying and also three weeks after treatment cessation, highlighting the ability of WD to protect plant leaves from ozone-induced senescence both during and after treatments. Since ROS overproduction is the main cause of O_3 phytotoxicity [2], the protective effect of WD observed during treatments could mainly be due to the direct protection of WD on the photosynthetic machinery of the treated leaves, whereas the effect observed three weeks after treatment cessation could result from the stimulatory effect of antioxidant-like molecules produced by the treatment itself. As evidence of this, after four weeks of weekly treatments with 0.2% foliar application of chestnut WD, the expression of total antioxidant defenses increased by 140% (values measured using the DPPH assay). The content of caffeic acid and quercetin also increased by approx. 400% and 105%, respectively [25]. These results are fully in line with those reported in other studies which found a protective effect of antioxidant-like molecules, such as melatonin, on leaf senescence of different plant species [38,39]. As suggested by Khan et al. [40], this effect might be related to the overstimulation of enzymatic antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, and enzymes involved in the ascorbate–glutathione cycle resulting from the treatment.

The exposure to biologically relevant O_3 concentrations (90, 135, and 180 ppb) is also known to cause a reduction in the height of BelW3 tobacco plants when compared to control (unexposed) plants [41]. This was also recorded in our untreated BelW3 plants, which exhibited a similar pattern, with both 19- and 22-week-old BelW3 plants showing a significant reduction in plant height compared to BelB plants. This was probably the result of the above-mentioned effects of O_3 at the level of the photosynthetic system. Plant height is, in fact, positively correlated with photosynthesis [42]. Impairments in the latter may, therefore, lead to reductions in the former. In the present study, however, not only did WD treatments significantly impede this height loss, but plant height exceeded that of BelB plants—in all likelihood due to the stimulating effect of WD on plant phytohormones, cell growth, and chlorophyll synthesis and/or protection. Such effects inevitably lead to an increase in sugar and amino acid synthesis, resulting in increased plant yields [22,25,43]. In the agronomic field, the success of WD mainly stems from this ability to increase crop yield [16,22]. Increases in plant height after foliar application of WD (from 1% to 10%) have been observed in crops such as aman rice (BRRI dhan34), rockmelon (*Cucumis melo* L. cv. 'Golden Langkawi'), and soybean (*Glycine max* L.) [44–46].

Photosynthesis is the first target of O_3 [47], so the exposure of BelW3 plants to this pollutant inevitably leads to a reduction in chlorophyll content and in the efficiency of the whole photosynthetic machinery [36]. As chlorophylls represent the main antennae pigments for both PSII and PSI [48], their amount/concentration can be used as a general indicator of antenna pigment density within these photosystems. Within PSII, chlorophyll is contained both in the antennae complex—an area indispensable for photon-harvesting and the transmission of an electron to the photochemical reaction center (RC)—and inside the RC itself, with the role of generating a high-energy electron to produce chemical energy [49]. The decrease in chlorophyll in PSII following O_3 exposure may generate reductions in both the amount of antennae pigments and the chemical efficiency of the RC itself, with impairments potentially limiting the efficiency of the whole photosynthetic machinery—an effect that was particularly evident in this study.

The breakdown of the two photosystem subunits is mainly caused by the overproduction of ROS such as $^{-}O_2$. However, this stimulates the activation of the repair mechanism, where the damaged subunits are immediately degraded by proteases and subsequently replaced by new subunits produced by stromal lamellae [50]. Although BelW3 plants lack this repair mechanism on account of genetic mutations [37], foliar WD applications would appear to compensate for this, with WD probably acting as a shield against increased ROS production generated by the oxidizing action of O_3. In this way, the entire photosynthetic system is protected, and damage is limited [25]. In fact, following WD applications, all studied photosynthesis-related parameters (i.e., those involved in the energy transfer from PSII and PSI) were equal to those measured in BelB leaves. Moreover, it is worth noting that WD treatments temporarily overstimulated the expression of overall photosynthetic system functionality (see PI_{TOT} results after 11 weeks of treatments). This represents an extremely important result because it improves our understanding of the way in which foliar WD application can effectively protect the photosynthetic machinery of O_3-sensitive plants/crops, including their yield, from the oxidizing effects of this pollutant. Research is ongoing to understand the mechanisms involved in this photosynthetic protection. However, as suggested by the leaf senescence results, this protective effect may be related to the antioxidant activity of WD against the phytotoxic activity of O_3 or to its stimulatory effect on the synthesis of antioxidants routinely produced by the photosynthetic machinery to protect itself from oxidative stress, as previously indicated by Vannini et al. [25]. In line with this hypothesis, Chen et al. [51] reported that exogenous application of antioxidants—i.e., ascorbic acid—to tomato (*Lycopersicon esculentum* L. cv. Ligeer87-5) plants increased chlorophyll synthesis, alleviated oxidative stress damage to chloroplasts, increased the stability of PSII, and promoted energy transfer from PSII and PSI.

Considering that cultivated plants in most parts of the world are exposed to O_3 concentrations well above their pollution tolerance threshold (around 40 ppb) [52–55], the protective effect of WD on horticultural plants exposed to average O_3 concentrations even half of those recorded in this study (around 120 ppb) could be complete, as previously observed for lettuce plants [25].

5. Conclusions

In the present study we found that foliar WD application successfully mitigated O_3 effects on both the morphometric and photosynthetic characteristics of BelW3 plants exposed to ecologically relevant O_3 concentrations (120 ppb). WD applications reduced leaf injury by approx. 15% and leaf senescence by approx. 19%. Additionally, WD applications enhanced the overall photosynthetic machinery (i.e., the PI_{TOT}) by approx. 300% when compared to BelW3 plants. Notably, the WD protective effect persisted for (at least) 3 weeks after the end of treatments, highlighting the protective capacity of the distillate, albeit short-term, to counter the oxidating effects of O_3. This preliminary research provides important insights into the potential usefulness of WD in mitigating ozone-induced damage in summer crops, with wider implications for sustainable agriculture. It also underlines the advantages of using BelW3 tobacco plants as a model with which to evaluate the effectiveness of anti-ozonants for protecting crops against the oxidizing effects of this harmful pollutant. The biochemical mechanisms involved in the protective effect of WD on this plant remain to be elucidated.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10050480/s1, Figure S1: Example of the choice of leaf on which to measure photosynthesis. All plants have a leaf number (viable and non-viable: those with more than 50% leaf damage) of 11 to 12. BelW3 plants show a vital leaf number of eight to nine. The choice of leaf therefore falls to number eight, as this is the leaf that is vital in all cultivated plants.

Author Contributions: Conceptualization, A.V.; methodology, A.V.; validation, A.V.; formal analysis, A.V.; investigation, A.V.; resources, A.V. and A.P.; data curation, A.V.; writing—original draft preparation, A.V.; writing—review and editing, A.V. and A.P.; visualization, A.V.; supervision, A.V. and A.P.; project administration, A.V. and A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: We thank Michele Carbognani (University of Parma) for reading the draft of this article. We thank T'ai G. W. Forte (University of Parma) for the English revision of the manuscript. We thank Elisa Pellegrini (University of Pisa) for suggesting the supplier of the seeds of BelW3 and BelB plants. This work has benefited from the equipment and framework of the COMP-HUB and COMP-R Initiatives, funded by the 'Departments of Excellence' program of the Italian Ministry for University and Research (MIUR, 2018–2022 and MUR, 2023–2027).

Conflicts of Interest: The authors declare no conflicts of interest.

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