The Effects of Elevated Tropospheric Ozone on Carbon Fixation and Stable Isotopic Signatures of Durum Wheat Cultivars with Different Biomass and Yield Stability

Limin Ma 1,2,*,†, Chong Chen 1,2,*,†, Lorenzo Cotrozzi 3,4,†, Chengcheng Bu 1,2, Jiahong Luo 1,2, Guodong Yao 1,2, Guangyao Chen 1,2, Weiwei Zhang 1,2, Cristina Nali 3,4 and Giacomo Lorenzini 3,4

1 State Key Laboratory of Pollution Control and Resources Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, China
2 Key Laboratory of Yangtze River Water Environment, Ministry of Education, Tongji University, Shanghai 200092, China
3 Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto, 80, 56124 Pisa, Italy
4 CIRSEC, Centre for Climate Change Impact, University of Pisa, Via del Borghetto, 80, 56124 Pisa, Italy
* Correspondence: lmma@tongji.edu.cn (L.M.); cchen@tongji.edu.cn (C.C.); Tel.: +86-21-65980439 (L.M.); +86-17-721061327 (C.C.)
† These authors contributed equally to this work.

Abstract: Tropospheric ozone (O₃) enrichment caused by human activities can reduce important crop yields with huge economic loss and affect the global carbon cycle and climate change in the coming decades. In this study, two Italian cultivars of durum wheat (Claudio and Mongibello) were exposed to O₃ (80 ppb, 5 h day⁻¹ for 70 consecutive days), with the aim to investigate the changes in yield and biomass, ecophysiological traits, and stable carbon and nitrogen isotope values in plants, and to compare the stable isotope responses under environmental stressors. Both cultivars showed a relative O₃ tolerance in terms of photosynthetic performance, but in cultivar Mongibello, O₃ was detrimental to the grain yield and plant biomass. The δ¹³C values in the leaves of plants identified that the impact of O₃ on CO₂ fixation by RuBisCO was dominant. The δ¹⁵N value showed significant differences between treatments in both cultivars at seven days from the beginning of the exposure, which could be considered an early indicator of ozone pollution. Under increasingly frequent extreme climates globally, the relationships among stable isotope data, ecophysiological traits, and agronomic parameters could help breed future cultivars.

Keywords: air pollution; oxidative stress; yield; ecophysiological parameters; stable carbon isotope; stable nitrogen isotope

1. Introduction

Tropospheric ozone (O₃) is a major secondary air pollutant [1]. Despite efforts to reduce the emission of O₃ precursors (e.g., nitrogen oxides and volatile organic compounds), concentrations of this pollutant are still elevated in many areas worldwide and are expected to rise further due to both anthropogenic activities and climate change [2,3]. Background O₃ instantaneous levels in the Northern Hemisphere are estimated to increase from 35–50 ppb to 42–84 ppb in 2100, depending on the seasonal and spatial variability (1 ppb = 1.96 µg m⁻³, at 25 °C and 101.325 kPa) [4–6]. This increase is expected in hot-spot regions such as East Asia and the Mediterranean [7–9], although uncertain or even opposite tendencies have been predicted in other regions [10–12].

Ozone can severely affect plants by entering through the open stomata and producing reactive oxygen species (ROS), which react with all biological macromolecules [13–15]. Although plants can detoxify ROS [16], O₃-induced oxidative stress commonly alters physiological processes such as carbon assimilation by inducing partial stomatal closure or less efficient stomatal control, reducing foliar pigment content, impairing the electron
transport chain, contracting the efficiency of Calvin Cycle enzymes like RuBisCO, lowering the availability of photosynthates for reallocation, and increasing respiration rates \[12, 17\]. Consequently, crop yield and grain quality would decline. For example, yield loss due to O\(_3\) has been predicted to range from 3 to 4% for rice, from 3 to 5% for maize, from 6 to 16% for soybean and from 7 to 12% for wheat \[18\].

Wheat is one of the most important stable crops worldwide and one of the most O\(_3\)-sensitive crops \[19\]. \textit{Triticum durum} Desf. (durum wheat) is a major member of the genus Triticum, being largely used for its relatively high gluten content \[20, 21\]. It is mainly grown in the Mediterranean area, the Northern Plains between the USA and Canada, and in the desert of southwest of the USA and Northern Mexico, Turkey, Syria, North Africa, and other Asian countries \[22, 23\]. Although most studies have focused on O\(_3\) effects on common wheat (\textit{T. aestivum} L.), durum wheat has also been reported as O\(_3\) sensitive (e.g., Chen et al. \[24\]), even if somewhat contrasting results have been highlighted depending on concentration and duration of O\(_3\) exposure, as well as on genotypic differences in O\(_3\) tolerance (e.g., Gerosa et al. \[25\]).

Breeding of higher grain yield in wheat (and other crops) has been frequently related to increased stomatal conductance (\(g_s\)), which generally fosters higher photosynthetic rates and cools down plants. However, the higher \(g_s\) can also potentially increase cultivar O\(_3\)-sensitivity, as they absorb higher amounts of the pollutant \[17\]. Although O\(_3\)-induced effects on plants are commonly investigated by discrete gas exchange measurements \[17, 26\], photosynthetic/transpiration responses to environmental stress conditions may be better elucidated through the analysis of the stable carbon (\(\delta^{13}C\)) and nitrogen (\(\delta^{15}N\)) isotope compositions \[27, 28\]. The physiological mechanism underlying \(^{13}C\) enrichment in stressed plants is usually attributed to a decrease in \(g_s\) and an increase in water use efficiency \[29\]. The \(\delta^{15}N\) values in plant tissues are instead primarily associated with the isotopic composition of the nitrogen (N) sources (soil, precipitation, \(N_2\) fixation, fertilization) and forms (\(NH_4^+, NO_3^-, \) organic N). It could be further influenced by the N-fixing bacteria and mycorrhizal fungi \[30\], and the \(\delta^{15}N\) values will change during N uptake, translocation, assimilation, and reallocation within the plant \[31\]. Although all these processes are influenced by environmental conditions \[29\], only a few studies have reported the effects of O\(_3\) stress on plant \(\delta^{13}C\) and \(\delta^{15}N\) (e.g., Saurer et al. \[27\]). Since \(^{13}C\) and \(^{15}N\) contents vary with leaf age and plant development, studies investigating variations of these parameters in response to O\(_3\) should include leaves at different phenological stages \[29\].

In our previously published research \[24\], we investigated the responses of two Italian durum wheat cultivars, i.e., Claudio and Mongibello, exposed to chronic O\(_3\) exposure (80 ppb, 5 h day\(^{-1}\), for 70 consecutive days), reporting that Claudio showed a higher O\(_3\) tolerance than Mongibello, and characterizing the cultivar-specific phenolic profiles. The present work represents a follow-up aimed at investigating the relationships between the ecophysiological responses and the \(\delta^{13}C\) and \(\delta^{15}N\) variations and elucidating the effects of O\(_3\) pollution on biomass and yield parameters of the investigated durum wheat cultivars. Outcomes from the present study could be useful for breeding wheat cultivars with a high tolerance to environmental stress.

2. Results and Discussion

2.1. Biomass and Yield Variations Induced by Ozone

Variations of biomass and yield parameters induced by O\(_3\) are shown in Table 1. The interaction cultivar (\(Cv\)) \(\times\) O\(_3\) was significant for total aboveground biomass, ear and grain dry weight (DW), thousand-grain weight, and the number of grains and spikelets per ear. A significant O\(_3\) effect was reported on leaf and stem DW and the number of grains per spikelet. No significant effects were reported on the number of ears per plant. Total aboveground biomass, ear and grain DW, and thousand-grain weight were higher in controls of Mongibello than in Claudio (+32, +49, +54 and +51%, respectively). In Claudio and even more in Mongibello, O\(_3\) decreased total aboveground biomass (–30 and –40%, respectively), ear DW (–29 and –40%, respectively), and the number of grains per ear (–29
and −40%). Only in Mongibello, O₃ also significantly decreased grain DW, thousand-grain weight, and the number of spikelets per ear (−38, −37 and −15%, respectively). Similarly, between cultivars, O₃ reduced leaf and stem DW and the number of grains per spikelet (−32, −37 and −28%, as average, respectively).

### Table 1. Biomass and yield parameters in wheat cultivars Claudio and Mongibello exposed to 0 (control) or 80 ppb of ozone (O₃, 5 h day⁻¹) for 70 consecutive days.

|                          | Claudio Control | O₃ | Mongibello Control | O₃ | Cv | O₃ | Cv × O₃ |
|--------------------------|-----------------|----|-------------------|----|----|----|--------|
| Leaf biomass (g DW plant⁻¹) | 2.1 ± 1.3       | 1.5 ± 0.4 | 2.3 ± 1.0       | 1.5 ± 0.4 | 0.833 | <0.001 | 0.833 |
| Stem biomass (g DW plant⁻¹) | 3.5 ± 1.2       | 2.4 ± 0.7 | 4.3 ± 1.4       | 2.5 ± 0.7 | 0.523 | <0.001 | 0.542 |
| Total aboveground biomass (g DW plant⁻¹) | 10.11 ± 4.11 b | 7.1 ± 2.3 a | 13.3 ± 5.1 c | 8.0 ± 2.4 a | <0.001 | <0.001 | 0.015 |
| Ears plant⁻¹ (n)          | 3.7 ± 1.3       | 4.0 ± 1.3 | 4.2 ± 1.1       | 3.7 ± 1.1 | 0.888 | 0.888 | 0.577 |
| Ear weight (g DW plant⁻¹) | 4.5 ± 1.6 b     | 3.2 ± 1.2 a | 6.7 ± 2.6 c | 4.0 ± 1.3 ab | <0.001 | <0.001 | 0.014 |
| Grain weight (g DW plant⁻¹) | 2.4 ± 1.1 a     | 2.2 ± 1.0 a | 3.7 ± 0.9 b    | 2.3 ± 0.6 a | 0.018 | 0.007 | 0.026 |
| Thousand grain weight (g) | 24.7 ± 3.0 a    | 20.3 ± 1.4 a | 37.4 ± 3.5 b   | 23.4 ± 1.4 a | <0.001 | <0.001 | 0.024 |
| Grains ear⁻¹ (n)          | 42.0 ± 9.0 c    | 30.0 ± 6.1 b | 40.0 ± 7.9 c   | 24.0 ± 9.0 a | 0.003 | <0.001 | 0.013 |
| Spikelets ear⁻¹ (n)       | 16.4 ± 2.3 b    | 15.5 ± 1.9 b | 16.0 ± 1.8 b   | 13.6 ± 2.0 a | 0.018 | <0.001 | 0.025 |
| Grains spikelet⁻¹ (n)     | 2.8 ± 0.6       | 2.0 ± 0.6 | 2.5 ± 0.6       | 1.8 ± 0.9 | 0.536 | <0.001 | 0.551 |

Note: Data are shown as mean ± standard deviation. p-values of two-way ANOVA for the effects of the cultivar (Cv), ozone exposure (O₃), and their interactions on yield parameters of wheat are shown. Significant values (p ≤ 0.05) are shown in bold. According to the Tukey HSD post hoc test, different letters in each row indicate significant differences (p ≤ 0.05). Abbreviations: df, degrees of freedom; DW, dry weight.

The different behavior of these two cultivars in relation to O₃ exposure showed that Mongibello was more sensitive, while Claudio was more resistant. Claudio showed a reduction in the ear DW but not in grain DW, although there was a decrease in the number of grains per ear. Therefore, Claudio produced ears with less but larger caryopsis, and the thousand-grain weight under O₃ was also not significantly different from that in controls. Moreover, only in Mongibello O₃ was detrimental to the most important agronomic parameter, the grain yield. This may be due to the greater sensitivity between inflorescence emission and anthesis during the critic period and decreased photosynthetic activity in Mongibello [32]. Another possible reason is that the photosynthetic pigments may not be exploited as antioxidants, and the delay in the activation of the xanthophyll de-epoxidation cycle has greater photoinhibition-related damages [33–35]. Similar conclusions were also carried out by Pleijel et al. [19], who reported that O₃ (35.6 ± 10.6 ppb) had significant negative effects on grain yield (−8%), grain mass (−4%), harvest index (−2%), total aboveground biomass (−5%), starch concentration (−3%), starch yield (−11%), and protein yield (−6%) of 19 wheat cultivars, compared with charcoal filtered air (13.7 ± 8.8 ppb of O₃). A more quantitative relationship between O₃ concentrations and wheat yields was observed by Harmens et al. [15], who reported that wheat yield and thousand-grain weight declined linearly with increasing phytotoxic O₃ dose above a flux threshold of Y (PODY). However, the wheat in different regions worldwide showed variant responses to O₃. Pleijel et al. [36] compared the influence of O₃ on the grain yield, average grain mass, and harvest index of wheat in Europe, Asia, and North America. They found that North American wheat was less sensitive than European and Asian ones, which responded similarly. The variation in responses across all three continents was smallest for the harvest index, followed by grain mass and yield.
2.2. Leaf Area (La) and Ecophysiological Parameters

The effects of Cv, O₃, growth stage (Gs), and their interactions on leaf area (La), CO₂ assimilation rate (A), gₛ, and maximum RuBP-saturated rate of carboxylation (Vₖmax) are reported in Table 2. No significant Cv effect and Cv × O₃ × Gs interaction were found for these four indicators, whereas significant O₃ × Gs interactions were reported on La and A. Meanwhile, significant Cv × Gs interactions were observed on La, gₛ, and Vₖmax, and a significant Cv × O₃ interaction was reported on A. The singular O₃ and Gs effects showed significant effects on all of the four indicators.

Table 2. p-values of three-way ANOVA for the effects of the cultivar (Cv; Claudio and Mongibello), growth stage (Gs; 7, 28, 50 and 70 days), ozone treatment (O₃; 0 and 80 ppb, 5 h day⁻¹) and their interactions on leaf area (La), CO₂ assimilation rate (A), stomatal conductance (gₛ) and maximum RuBP-saturated rate of carboxylation (Vₖmax) of durum wheat.

| df | La  | A   | gₛ  | Vₖmax |
|----|-----|-----|-----|-------|
| Cv | 1   | 0.894 | 0.160 | 0.103 | 0.687 |
| O₃ | 1   | <0.001 | 0.002 | 0.017 | <0.001 |
| Gs | 3   | <0.001 | <0.001 | <0.001 | <0.001 |
| Cv × O₃ | 1 | 0.478 | 0.019 | 0.053 | 0.724 |
| Cv × Gs | 3 | 0.050 | 0.195 | 0.021 | 0.003 |
| O₃ × Gs | 3 | 0.001 | 0.046 | 0.057 | 0.120 |
| Cv × O₃ × Gs | 3 | 0.866 | 0.351 | 0.258 | 0.160 |

Note: Significant values (p ≤ 0.05) are shown in bold. Abbreviation: df, degrees of freedom.

Variations of La and A induced by O₃ are shown in Figure 1, and variations of gₛ and Vₖmax are shown in Figure 2. Ozone significantly decreased La at 50 days from the beginning of exposure (FBE) in both cultivars (−42% in Claudio and −52% in Mongibello; Table 3). The differences in La between Claudio and Mongibello at all growth stages were not significant in controls or exposed to O₃ (Table 3). The La in both cultivars increased at 28 and 50 days FBE and decreased at 70 days FBE due to the senescence of plants. A significant reduction of A was observed at 28 and 50 days FBE in Mongibello exposed to O₃ (−45% and −16%, respectively; Table 3), and A was lower at 70 days FBE in both cultivars compared with the other three growth stages [24]. Similarly to A, O₃ decreased gₛ at 28 and 50 days FBE in Mongibello (−66% and −34%, respectively; Table 3). The gₛ in Claudio decreased at 70 days FBE compared with the previous growth stage in both groups (controls and O₃-treated groups), while the difference in Mongibello was only observed in controls (Table 3). The significant differences between treatments were shown at 7 and 28 days FBE in Claudio and 28 days FBE in Mongibello for Vₖmax (Table 3). In Claudio, the Vₖmax in O₃-treated plants was 18% and 21% lower than that in controls at 7 and 28 days FBE, respectively, and in Mongibello, the Vₖmax in O₃-treated plants was 18% lower than that in controls at 28 days FBE. The Vₖmax decreased at 70 days FBE in both treatments for both cultivars.
Figure 1. Leaf area (La) (a,b) and CO$_2$ assimilation rate (A) (c,d) in durum wheat cultivars Claudio (circle, left) and Mongibello (square, right) exposed to 0 (open) or 80 (closed) ppb of ozone (5 h day$^{-1}$) at different growth stages (7, 28, 50, 70 days from the beginning of exposure). Data are shown as mean ± standard deviation (n = 3). A vertical dashed line separates the cultivars.

Figure 2. Stomatal conductance ($g_s$) (a,b) and maximum RuBP-saturated rate of carboxylation ($V_{\text{max}}$) (c,d) in durum wheat cultivars Claudio (circle, left) and Mongibello (square, right) exposed to 0 (open) or 80 (closed) ppb of ozone (5 h day$^{-1}$) at different growth stages (7, 28, 50, 70 days from the beginning of exposure). Data are shown as mean ± standard deviation (n = 3). A vertical dashed line separates the cultivars.
Table 3. Three-way ANOVA analysis describing significant differences among leaf area (La), CO$_2$ assimilation rate (A), stomatal conductance (gs), maximum RuBP-saturated rate of carboxylation ($V_{cmax}$), $\delta^{15}$N and $\delta^{13}$C in durum wheat cultivars Claudio (CLAU) and Mongibello (MONG) exposed to 0 (CTR) or 80 (O$_3$) ppb of ozone (5 h day$^{-1}$) at different growth stages (7, 28, 50, 70 days from the beginning of exposure).

|                  | p Values | La   | A    | gs   | $V_{cmax}$ | $\delta^{15}$N | $\delta^{13}$C |
|------------------|----------|------|------|------|------------|----------------|----------------|
|                  |          | 0.991| 0.278| 0.515| 0.178      | <0.001         | 0.216          |
| CTR 7 days       | CLAU     | 0.790| 0.278| 0.214| 0.019      | <0.001         | 0.012          |
| 28 days          | MONG     |      |      |      |            |                |                |
| 50 days          | CLAU     | 0.159| 0.122| 0.646| 0.466      | <0.001         | <0.001         |
| 70 days          | MONG     | 0.055| 0.094| 0.008| 0.161      | <0.001         | 0.001          |
| O$_3$ 7 days     | CLAU     | 1.000| 0.632| 0.160| 0.009      | <0.001         | 0.002          |
| 28 days          | MONG     | 0.833| 0.025| 0.097| 0.142      | <0.001         | 0.154          |
| 50 days          | CLAU     | 0.067| 0.341| 0.057| 0.822      | 0.004          | <0.001         |
| 70 days          | MONG     | 0.634| 0.177| 0.084| 0.165      | <0.001         | 0.907          |
| CLAU 7 days      | CTR      | 0.991| 0.539| 0.735| 0.024      | <0.001         | 0.487          |
| 28 days          | O$_3$    | 0.317| 0.249| 0.291| 0.001      | 0.067          | 0.431          |
| 50 days          | CTR      |      |      |      |            |                |                |
| 70 days          | O$_3$    | 0.642| 0.891| 0.744| 0.416      | 0.530          | 0.116          |
| MONG 7 days      | CTR      | 0.982| 0.223| 0.663| 0.335      | 0.001          | 0.147          |
| 28 days          | O$_3$    | 0.292| <0.001| 0.717| 0.378      | 0.155          | 0.226          |
| 50 days          | CTR      |      |      |      |            |                |                |
| 70 days          | O$_3$    | 0.307| 0.837| 0.484| 0.051      | <0.001         | <0.001         |
| O$_3$ 7 days     | CTR      | 0.047| 0.945| 0.772| 0.008      | <0.001         | 0.727          |
| 28 days          | CTR      |      |      |      |            |                |                |
| 7 days 28 days   | O$_3$    | 0.003| <0.001| 0.003| <0.001     | <0.001         | <0.001         |
| 28 days 50 days  | CTR      |      |      |      |            |                |                |
| 28 days 70 days  | CTR      | 0.237| <0.001| 0.001| <0.001     | <0.001         | <0.001         |
| 50 days 70 days  | CTR      |      |      |      |            |                |                |
| O$_3$ 7 days     | CTR      | 0.297| 0.539| 0.663| 0.095      | <0.001         | 0.259          |
| 28 days 50 days  | CTR      |      |      |      |            |                |                |
| 28 days 70 days  | CTR      | 0.001| <0.001| 0.014| 0.004      | <0.001         | <0.001         |
| 50 days 70 days  | CTR      |      |      |      |            |                |                |

Note: The p values are given for the comparison of leaf area (La), CO$_2$ assimilation rate (A), stomatal conductance (gs), maximum RuBP-saturated rate of carboxylation ($V_{cmax}$), $\delta^{15}$N and $\delta^{13}$C in durum wheat cultivars Claudio (CLAU) and Mongibello (MONG) exposed to 0 (CTR) or 80 (O$_3$) ppb of ozone (5 h day$^{-1}$) at different growth stages (7, 28, 50, 70 days from the beginning of exposure).
Table 3. Cont.

|                       | La    | A     | g₅₀ | V_{cmax} | δ¹³C | δ¹⁵N |
|-----------------------|-------|-------|-----|----------|------|------|
| 7 days 28 days        | 0.027 | 0.945 | 0.373 | 0.314    | <0.001 | 0.298 |
| 7 days 50 days        | <0.001 | 0.008 | 0.348 | 0.115    | <0.001 | 0.022 |
| 7 days 70 days        | <0.001 | <0.001 | 0.275 | <0.001   | 0.002 | <0.001 |
| 28 days 50 days       | <0.001 | 0.007 | 0.961 | 0.013    | <0.001 | 0.184 |
| 28 days 70 days       | 0.006 | <0.001 | 0.053 | <0.001   | <0.001 | <0.001 |
| 50 days 70 days       | <0.001 | <0.001 | 0.047 | <0.001   | 0.564 | <0.001 |
| 7 days 28 days        | 0.212 | 0.002 | 0.001 | 0.016    | <0.001 | 0.001 |
| 7 days 50 days        | <0.001 | 0.054 | 0.128 | 0.242    | <0.001 | <0.001 |
| 7 days 70 days        | <0.001 | 0.004 | 0.031 | <0.001   | <0.001 | <0.001 |
| 28 days 50 days       | 0.001 | <0.001 | 0.051 | 0.001    | <0.001 | <0.001 |
| 28 days 70 days       | 0.006 | 0.732 | 0.193 | <0.001   | <0.001 | <0.001 |
| 50 days 70 days       | 0.596 | <0.001 | 0.492 | <0.001   | <0.001 | <0.001 |

Note: Significant values (p ≤ 0.05) are shown in bold.

|                       | La    | A     | g₅₀ | V_{cmax} | δ¹³C | δ¹⁵N |
|-----------------------|-------|-------|-----|----------|------|------|
|                       | CTR   | MONG  |     |          |      |      |

Notably, positive correlations were found among the ecophysiological traits in both Claudio and Mongibello (Figure 3). The correlation coefficients for A with g₅₀ and V_{cmax} were 0.96 and 0.87 in Claudio, and 0.78 and 0.88 in Mongibello, while that for g₅₀ with V_{cmax} were 0.90 and 0.59 in Claudio and Mongibello, respectively. The correlation coefficients for La with A, g₅₀, and V_{cmax} were relatively small, and they were 0.45, 0.43, and 0.34 in Claudio and 0.37, 0.19, and 0.09 in Mongibello.

![Figure 3](image-url)  

La, A, g₅₀, and V_{cmax} are all widely used photo-oxidative stress markers [37]. In this study, the reductions of La in both cultivars were only observed at 50 days FBE, and the reductions of A and g₅₀ were only observed in Mongibello at 28 and 50 days FBE. The reductions of V_{cmax} were shown at 7 and 28 days FBE in Claudio and 28 days FBE in Mongibello. All these traits later recovered the same levels of control. These results indicate a relative O₃ tolerance of durum wheat in photosynthetic performance, and the photosynthetic performance between the two cultivars was similar. Similar conclusions were reached in previous studies on other cultivars of wheat [24,25,38,39]. However, the
temporary decrease of photosynthetic activity during the critic period (at 7, 28, and 50 days FBE) was demonstrated, and as mentioned above, this may be an important reason for the O₃-induced reductions of some biomass and yield parameters in wheat cultivars.

2.3. δ¹³C Values

Ozone induced an increase of δ¹³C values at 28 days FBE (+1.070‰) and a significant decrease of δ¹³C values at 50 and 70 days FBE in Mongibello (−1.387‰ and −1.739‰, respectively; Figure 4b; Table 3), while the δ¹³C differences between treatments in Claudio at all of the growth stages and in Mongibello at seven days FBE were not significant (Figure 4a,b; Table 3). The δ¹³C values changed gently at 7, 28, and 50 days FBE in both cultivars, but the values increased significantly at 70 days FBE with the senescence of plants (Figure 4a,b). Significant Cv × O₃ × Gs interactions were found for both δ¹³C and δ¹⁵N (actually, only the Cv × O₃ effect on δ¹³C was not significant; Table 4).

![Figure 4. δ¹³C (a,b) and δ¹⁵N (c,d) values in durum wheat cultivars Claudio (circle, left) and Mongibello (square, right) exposed to 0 (open) or 80 (closed) ppb of ozone (5 h day⁻¹) at different growth stages (7, 28, 50, 70 days from the beginning of exposure). Data are shown as mean ± standard deviation (n = 3). A vertical dashed line separates the cultivars.](image)

1²CO₂ is preferred to plants compared with ¹³CO₂ during photosynthetic CO₂ assimilation, and both CO₂ diffusion across the stomata and CO₂ fixation by RuBisCO could contribute to the discrimination of ¹³CO₂ in favor of ¹²CO₂ in C₃ plants [27,40]. The metabolic capacity decreases with the senescence of plants, and the δ¹³C values in the plants had an upward trend through time, especially at 70 days FBE (Figure 4a,b). The δ¹³C value in the air (δ¹³C_air) is about −8‰, and that in the C₃ plants (δ¹³C_plant) ranges from −36‰ to −22‰ [41,42], the plant is isotopically lighter than atmospheric CO₂, and their relationship could be quantitatively described using the following equation [27,43,44].

\[
\delta^{13}\text{C}_{\text{plant}} = \delta^{13}\text{C}_{\text{air}} - a - (b-a)(p_i/p_a)
\]

where a is the fractionation occurring due to diffusion, and the value is about +4.4‰; b is the net fractionation caused by carboxylation (mainly by RuBisCO), and the value is about +27‰; \(p_i\) is the partial pressure of CO₂ in the mesophyll; \(p_a\) is the partial pressure of CO₂ in ambient air.
Table 4. p-values of three-way ANOVA for the effects of the cultivar (Cv; Claudio and Mongibello), growth stage (Gs; 7, 28, 50 and 70 days), ozone treatment (O₃; 0 and 80 ppb, 5 h day⁻¹) and their interactions on δ¹³C and δ¹⁵N values of durum wheat.

|                | df | δ¹³C     | δ¹⁵N     |
|----------------|----|----------|----------|
| Cv             | 1  | <0.001   | <0.001   |
| O₃            | 1  | 0.001    | <0.001   |
| Gs            | 3  | <0.001   | <0.001   |
| Cv × O₃       | 1  | 0.090    | <0.001   |
| Cv × Gs       | 3  | <0.001   | <0.001   |
| O₃ × Gs       | 3  | <0.001   | <0.001   |
| Cv × O₃ × Gs  | 3  | 0.001    | <0.001   |

Note: Significant values (p ≤ 0.05) are shown in bold. Abbreviation: df, degrees of freedom.

As is shown in Equation (1), when the gₛ limits photosynthesis, the pᵢ/pₐ value is relatively low, leading to a relatively small fractionation and less δ¹³C negative values compared to controls [44]. The other situation is that when the impact on CO₂ fixation by RuBisCO is dominant, the increase in pᵢ/pₐ value will result in larger fractionation and more negative δ¹³C values [43]. Therefore, the carbon isotopic composition of plants is a good indicator to show how some environmental conditions (e.g., air pollutants, water availability, temperature) affect the uptake and fixation of CO₂. In this study, A and gₛ at 28 and 50 days FBE in Mongibello decreased significantly, and these traits returned to control values (Figures 1d and 2b). Vₑmax showed significant reductions only at 28 days FBE (Figure 2d). However, the grain yield and biomass of plants still decreased significantly (Table 1). The δ¹³C values could better explain this phenomenon under the long-term accumulated O₃ exposure condition, and the impact of O₃ on CO₂ fixation by RuBisCO was dominant. In other words, the process of CO₂ fixation by RuBisCO is more sensitive to O₃ than the stomatal conductance in Mongibello. Correlation analysis further verified this conclusion (Figure 3). In Mongibello, the correlation coefficients for δ¹³C with Vₑmax and A were −0.80 and −0.71, respectively, while that for δ¹³C with the stomatal conductance was only −0.33. In Claudio, the correlation for δ¹³C with Vₑmax (−0.73) was stronger than with gₛ (−0.67), and the correlation coefficient for δ¹³C with A was −0.64. Therefore, the δ¹³C values well explained why the temporary decrease of photosynthetic activity during the critical period (at 28 and 50 days FBE) affected the O₃-induced reductions of some yield parameters.

2.4. δ¹⁵N Values

In Claudio, O₃ only induced a significant reduction of δ¹⁵N values at seven days FBE (−2.252‰; Table 3), and the δ¹⁵N values increased through time in controls and O₃-treated plants, especially at 28 and 70 days FBE (Figure 4c). In Mongibello, significant differences in δ¹⁵N values between treatments were observed at all of the growth stages (Table 3). O₃ increased the δ¹⁵N values at 7, 28, and 50 days FBE (+1.105‰, +11.402‰, and +6.224‰, respectively) and decreased the values at 70 days FBE (−1.895‰). The δ¹⁵N values in both treatments increased at 28 days FBE and then decreased at 50 and 70 days FBE, which was especially noticeable in O₃-treated plants (Figure 4d).

The δ¹⁵N value is also a commonly applicable ecotoxicological indicator of O₃ pollution early on, as significant differences between treatments were observed in both cultivars at seven days FBE (Table 3). Claudio recovered later, while O₃-treated Mongibello showed a significant increase in the δ¹⁵N values at 7, 28, and 50 days FBE compared with the control treatment (Figure 4c,d; Table 3). Previous studies proved the most significant changes in the isotope ratios in the non-protein nitrogen fraction, followed by the soluble protein and the structural protein fractions [45,46], and this enrichment could be explained by accelerated N metabolism. Furthermore, wheat leaves exposed to O₃ increased their permeability to soluble substances such as amino acids and proteins before the visible injury, caused
by membrane alterations [47]. Such a leakage led to the enrichment of the proportion of N-containing substances with larger $\delta^{15}N$ values according to general isotope effects [48]. In addition, plants react to O$_3$ stress by narrowing their stomatal openings at the following growth stages, N fixation is changed from the air more towards the soil, which has higher $\delta^{15}N$ values compared with N in the air [49]. The correlation coefficient of $-0.48$ for $\delta^{15}N$ with the $g_s$ in Mongibello could verify this inference (Figure 3). It should also be noted that O$_3$ exposure was not the only reason causing the stable isotope responses. Other stresses, such as heat and drought, might give similar responses to those shown in this study. Therefore, more information is needed to determine wheat’s characteristic stable isotope responses of wheat under different stress conditions.

The stable isotope data in plants is a good indicator and recorder of historical environmental information. Under increased frequent extreme climates globally [50], the $\delta^{13}C$ and $\delta^{15}N$ values in wheat can tell humans what kind of climatic conditions they have encountered, and the relationships among stable isotope data, ecophysiological traits, and agronomic parameters could help screen more tolerant crops quickly and efficiently in the early stage and eventually benefit breeding future cultivars.

3. Materials and Methods

3.1. Plant Material and Ozone Exposure

Details of plant material and O$_3$ exposure used in the experimental activity are already reported by Chen et al. [24]. In short, seeds of durum wheat cultivars Claudio and Mongibello were sown in plastic pots, which were then maintained in a greenhouse under charcoal-filtered air until the stage of “first leaf unfolded/second leaves unfolded” (BBCH-Code [BBCH-C] 11–12, i.e., 20-day-old seedlings) [51]. Uniform-sized seedlings were then distributed among four had hoc built perspex fumigation chambers ($595 \times 540 \times 1975$ mm; two chambers per treatment; twelve seedings per chamber (three plants for each collection period)) and exposed to charcoal-filtered air (controls, assumed as 0 ppb of O$_3$) or a target concentration of 80 $\pm$ 10 ppb of O$_3$ (ca. 160 $\mu$g m$^{-3}$ at 20 $^\circ$C and 101.3 kPa, 5 h day$^{-1}$, in the form of a square wave between 10:00 and 15:00 h) for 70 consecutive days. For further details about fumigation facilities, plant management, and environmental conditions, see Landi et al. [52] and Chen et al. [24].

Ecophysiological analyses and leaf (the third fully expanded mature leaves) collections for $\delta^{13}C$ and $\delta^{15}N$ investigations of control and O$_3$-treated plants were performed at seven (BBCH-C 13), 28 (BBCH-C 23–32), 50 (BBCH-C 59) and 70 (BBCH-C 65–69) days FBE. For each combination of cultivar, O$_3$ treatment, and time, two completely expanded leaves were collected, mixed, divided into aliquots, instantly frozen in liquid nitrogen, and stored at $-80 ^\circ$C until $\delta^{13}C$ and $\delta^{15}N$ analyses. At the end of the exposure, biomass and yield parameters were also assessed (see Section 3.4).

3.2. Leaf Area (La) and Ecophysiological Analyses

La was measured using a Leaf Area Meter (YMJ-G, Fengtu, Shandong, China). Ecophysiological analyses were carried out between 11:00 and 13:00 h (i.e., when maximum gas exchanges usually occur) on one completely expanded leaf per plant [24]. The A and the $V_{\text{max}}$ were measured under light-saturated conditions (photosynthetic active radiation of about 1200 $\mu$mol photons m$^{-2}$ s$^{-1}$), ambient CO$_2$ concentration (ca. 390 $\mu$mol mol$^{-1}$) and 60% of relative humidity, using an Infrared Gas Analyzer (CIRAS-1; PP-Systems, Hitchin, Hertfordshire, UK). The acclimation time for the leaves before the measurements were 40 min. One fully expanded mature leaf per plant and three plants per treatment were chosen for the measurements. Stomatal conductance was measured using a Stomatal Conductance Meter (FS-3080C, Fangsheng, Hebei, China).
3.3. $\delta^{13}C$ and $\delta^{15}N$

The C isotopic composition of a sample is expressed on the $\delta^{13}C$ scale, defined as Equation (2).

$$\delta^{13}C = \frac{\left(\frac{^{13}C}{^{12}C}\right)_{\text{sample}} - \left(\frac{^{13}C}{^{12}C}\right)_{\text{standard}}}{\left(\frac{^{13}C}{^{12}C}\right)_{\text{standard}}} \times 1000(\text{‰}) \quad (2)$$

where $^{13}C/^{12}C$ is the ratio of the number of $^{13}C$ atoms to the number of $^{12}C$ atoms in the sample or standard. Vienna Pee Dee Belemnite (V-PDB) was the standard, defined as 0‰ [53].

The N isotopic composition of a sample is expressed on the $\delta^{15}N$ scale, defined as Equation (3).

$$\delta^{15}N = \frac{\left(\frac{^{15}N}{^{14}N}\right)_{\text{sample}} - \left(\frac{^{15}N}{^{14}N}\right)_{\text{standard}}}{\left(\frac{^{15}N}{^{14}N}\right)_{\text{standard}}} \times 1000(\text{‰}) \quad (3)$$

where $^{15}N/^{14}N$ is the ratio of the number of $^{15}N$ atoms to the number of $^{14}N$ atoms in the sample or standard. The atmospheric air was used as the standard, defined as 0‰ [54].

For each combination of cultivar, $O_3$ treatment, and time, 400 $\mu$g of leaves were collected and wrapped in a tin foil cup. Triplicates of $\delta^{13}C$ and $\delta^{15}N$ analyses of each sample were performed by an Element Analyzer-Isotope Ratio Mass Spectrometer (EA-IRMS, Thermo Fisher, Waltham, MA, USA). The EA was set as follows: the helium pressure (99.999%) was 250 kPa, and the flow rate was 100 mL min$^{-1}$. The reference flow rate of the Thermal Conductivity Detector was 80 mL min$^{-1}$, and the temperature of the Oxidation Furnace and Reduction Furnace was 960 $^\circ$C and 680 $^\circ$C, respectively. The chromatographic column was a packed column whose temperature was 55 $^\circ$C. The pressure of oxygen (99.999%) was 300 kPa, and the flow rate was 180 mL min$^{-1}$. The oxygen injection time was 1 s. The IRMS was operated at an accelerating potential of 10 kV. Ions were generated by an electron impact of 70 eV. The emitted energy for $\delta^{13}C$ analysis was 1.5 mA, and it was 2.0 mA for $\delta^{15}N$ analysis.

The reference materials for the calibration of $^{13}C$ were caffeine (IAEA-600), carbamide (UREA), and L-glutamic acid (USGS 41). Their stable carbon isotope ratios relative to V-PDB were $-1.17\text{‰}$, $-48.63\text{‰}$, and $37.626\text{‰}$, respectively. The reference materials for calibration of $^{15}N$ were caffeine (IAEA-600), potassium nitrate (IAEA-NO-3), carbamide (UREA), and L-glutamic acid (USGS 41). Their stable nitrogen isotope ratios relative to atmospheric air were $37.83\text{‰}$, $4.70\text{‰}$, $-0.30\text{‰}$, and $47.6\text{‰}$, respectively. All reference materials were from Sigma-Aldrich (Shanghai, China).

3.4. Determinations of Biomass and Yield Parameters

The leaves, stems, ears, and grains of the durum wheat in fully ripe and developed condition were placed in an oven at 103 $^\circ$C for 24 h and weighed to calculate the biomass (g DW). The biomass sum of the leaves, stems, and ears was calculated to obtain the total above-ground biomass (g DW). The number (n) of ears plant$^{-1}$, grains ear$^{-1}$, spikelets ear$^{-1}$, and grains spikelet$^{-1}$ were obtained by manual counting. Thousand-grain weight (g) of the durum wheat was given by:

$$\text{Thousand grain weight (g)} = \frac{\text{Grain weight plant}^{-1}(\text{g DW})}{\text{Ears plant}^{-1}(n) \times \text{Grains ear}^{-1}(n)} \times 1000 \quad (4)$$

3.5. Statistics

The normal distribution of data was preliminary analyzed following the Shapiro-Wilk test. The effects of the cultivar ($Cv$), growth stage ($Gs$), $O_3$ exposure ($O_3$), and their interactions on ecophysiological traits and $\delta^{13}C$ and $\delta^{15}N$ values were tested using a three-way analysis of variance (ANOVA). The effects of $Cv$ and $O_3$ and their interaction on biomass and yield parameters investigated at the end of the exposure were tested
using a two-way ANOVA. The Tukey HSD test was used as the post hoc test. Relations among ecophysiological traits and $\delta^{13}$C and $\delta^{15}$N values were evaluated using Pearson's correlations. Effects with $p \leq 0.05$ were considered statistically significant. Statistical analyses were carried out in SPSS version 25 (IBM, New York, NY, USA).

4. Conclusions

The tropospheric O$_3$ enrichment caused by human activities in the coming decades can reduce the yields of important crops with a huge economic loss worldwide. Though O$_3$ is unlikely to be the only stress during plants’ growth and development, the variation of stable carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) isotope values in the leaves is a useful tool to understand the response of the carbon sequestration mechanism of plants, and it was also found to be a potential marker for the ecophysiological traits of plants at all the stages of growth. In this study, the durum wheat cultivar Mongibello showed a significant reduction in grain yield and plant biomass, and the cultivar Claudio showed a relative O$_3$ tolerance. The $\delta^{13}$C values explained the mechanism of the O$_3$-induced reductions of some yield parameters, and the $\delta^{15}$N values played an important role in the early indication of oxidative stress.

Author Contributions: Conceptualization, L.M., C.N. and G.L.; methodology, L.M. and C.C.; software, C.C.; validation, L.C.; formal analysis, C.B.; investigation, L.M. and C.C.; resources, C.N. and G.L.; data curation, J.L.; writing—original draft preparation, C.B.; writing—review and editing, L.M. and L.C.; visualization, G.Y.; supervision, G.C.; project administration, W.Z.; funding acquisition, C.N. and G.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science and Technology Major Project of China (2018YFC1803100) and the Natural Science Foundation of China (21377098).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: This research was also supported by the University of Pisa, Italy. Thanks are due to Andrea Parrini for his technical support to carry out the experiments and to Elisa Pellegrini for helpful discussion of results.

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

References

1. Monks, P.S.; Archibald, A.T.; Colette, A.; Cooper, O.; Coyle, M.; Derwent, R.; Fowler, D.; Granier, C.; Law, K.S.; Mills, G.E.; et al. Tropospheric ozone and its precursors from the urban to the global scale from air quality to short-lived climate forcer. *Atmos. Chem. Phys.* 2015, 15, 8889–8973. [CrossRef]

2. Lefohn, A.S.; Malley, C.S.; Smith, L.; Wells, B.; Hazucha, M.; Simon, H.; Naik, V.; Mills, G.; Schultz, M.G.; Paoletti, E.; et al. Tropospheric ozone assessment report: Global ozone metrics for climate change, human health, and crop/ecosystem research. *Elem. Sci. Anthr.* 2018, 6, 28. [CrossRef] [PubMed]

3. Sage, R.F. Global change biology: A primer. *Glob. Chang. Biol.* 2020, 26, 3–30. [CrossRef] [PubMed]

4. Field, C.B.; Barros, V.R.; Mach, K.J.; Mastrandrea, M.D.; Aalst, M.V.; Adger, W.N.; Arent, D.J.; Barnett, J.; Betts, R.; Bilir, T.E.; et al. International Panel on Climate Change (IPCC) Climate change 2014: Impacts, adaptation, and vulnerability. Part A: Global and sectoral aspects. In *Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Cambridge University Press: Cambridge, UK, 2014; pp. 1–32.

5. Yang, N.; Cotrozzoli, L.; Zhang, Y.; Wang, X.; Ouyang, Z.; Zheng, F. First defense mechanisms of ornamental species suitable for urban green spaces in China exposed to ozone. *Urban For. Urban Green.* 2018, 36, 68–75. [CrossRef]

6. Cotrozzoli, L.; Pellegrini, E.; Nali, C.; Lorenzini, G. Climate change, ozone and plant life. *Agrochimica* 2019, 62, 181–188.

7. Verstraeten, W.W.; Neu, J.L.; Williams, J.E.; Bowman, K.W.; Worden, J.R.; Boersma, K.F. Rapid increases in tropospheric ozone production and export from China. *Nat. Geosci.* 2015, 8, 690–695. [CrossRef]

8. Ochoa-Hueso, R.; Munzi, S.; Alonso, R.; Arroniz-Crespo, M.; Avila, A.; Bermejo, V.; Bobbink, R.; Branquinho, C.; Concostrina-Zubiri, L.; Cruz, C.; et al. Ecological impacts of atmospheric pollution and interactions with climate change in terrestrial ecosystems of the Mediterranean Basin: Current research and future directions. *Environ. Pollut.* 2017, 227, 194–206. [CrossRef]
9. Ridley, D.A.; Cain, M.; Methven, J.; Arnold, S.R. Sensitivity of tropospheric ozone to chemical kinetic uncertainties in air masses influenced by anthropogenic and biomass burning emissions. Geophys. Res. Lett. 2017, 44, 7472–7481. [CrossRef]

10. Watson, L.; Lacressonnière, G.; Gauss, M.; Engardt, M.; Andersson, C.; Josse, B.; Marécal, V.; Nyiri, A.; Sobolowski, S.; Siour, G.; et al. Impact of emissions and +2 °C climate change upon future ozone and nitrogen dioxide over Europe. Atmos. Environ. 2016, 142, 271–285. [CrossRef]

11. Sicard, P.; Anay, A.; De Marco, A.; Paolletti, E. Projected global ground-level ozone impacts on vegetation under different emission and climate scenarios. Atmos. Chem. Phys. 2017, 17, 12177–12196. [CrossRef]

12. Ainsworth, E.A. Understanding and improving global crop response to ozone pollution. [PubMed]

13. Ahmed Bhuiyan, M.; Rashid Khan, H.U.; Zaman, K.; Hishan, S.S. Measuring the impact of global tropospheric ozone, carbon dioxide and sulfur dioxide concentrations on biodiversity loss. Environ. Res. 2018, 160, 398–411. [CrossRef] [PubMed]

14. Nuvolone, D.; Petri, D.; Voller, F. The effects of ozone on human health. Environ. Sci. Pollut. Res. Int. 2018, 25, 8074–8088. [CrossRef]

15. Harmens, H.; Hayes, F.; Sharps, K.; Osborne, S.; Pleijel, H. Wheat yield responses to stomatal uptake of ozone: Peak vs. rising background ozone conditions. Atmos. Environ. 2018, 173, 1–5. [CrossRef]

16. Burkey, K.O.; Neufeld, H.S.; Souza, L.; Chappelka, A.H.; Davison, A.W. Seasonal profiles of leaf ascorbic acid content and redox state in ozone-sensitive wildflowers. Environ. Pollut. 2006, 143, 427–434. [CrossRef] [PubMed]

17. Chang-Espino, M.; González-Fernández, I.; Alonso, R.; Araus, J.L.; Bermejo-Bermejo, V. The effect of increased ozone levels on the stable carbon and nitrogen isotopic signature of wheat cultivars and landraces. Atmosphere 2021, 12, 883. [CrossRef]

18. Van Dingenen, R.; Dentener, F.J.; Raes, F.; Krol, M.C.; Emberson, L.; Cofala, J. The global impact of ozone on agricultural crop physiology and yield, quality and climate scenarios. Atmos. Environ. 2009, 43, 604–618. [CrossRef]

19. Pleijel, H.; Broberg, M.C.; Udmpling, J.; Mills, G. Current surface ozone concentrations significantly decrease wheat growth, yield and quality. Sci. Total Environ. 2018, 613–614, 687–692. [CrossRef]

20. Šramková, Z.; Gregová, E.; Šturdík, E. Chemical composition and nutritional quality of wheat grain. Acta Chim. Slovaca 2009, 2, 115–138.

21. Cotrozi, L.; Lorenzini, G.; Nali, C.; Pisutti, C.; Pampanga, S.; Pellegrini, E. Transient waterlogging events impair shoot and root physiology and reduce grain yield of durum wheat cultivars. Plants 2021, 10, 2357. [CrossRef]

22. Kadkol, G.P.; Sissons, M. Durum wheat: Overview. Encycl. Food Grain Sci. 2004, pp. 153–175.

23. Li, L.; Niu, Y.; Ruan, Y.; DePauw, R.; Singh, A.; Gan, Y. Agronomic advancement in tillage, crop rotation, soil health, and genetic gain in durum wheat cultivation: A 17-year Canadian study. Agronomy 2018, 8, 193. [CrossRef]

24. Chen, C.; Chen, S.; Jha, R.K.; Cotrozi, L.; Nali, C.; Lorenzini, G.; Ma, L. Phenol metabolism of two cultivars of durum wheat (Triticum durum Desf.) as affected by ozone and flooding stress. J. Agron. Crop Sci. 2020, 206, 338–351. [CrossRef]

25. Gerosa, G.; Marzuoli, R.; Finco, A.; Menga, R.; Fusaro, I.; DePauw, R.; Singh, A.; Gan, Y. Contrasting effects of water salinity and ozone concentration on two cultivars of durum wheat (Triticum durum Desf.) in Mediterranean conditions. Environ. Pollut. 2014, 193, 13–21. [CrossRef]

26. Cotrozi, L.; Remorini, D.; Pellegrini, E.; Guidi, L.; Nali, C.; Lorenzini, G.; Massai, R.; Landi, M. Living in a Mediterranean city in 2050: Broadleaf or evergreen ‘citizens’? Environ. Sci. Pollut. Res. Int. 2018, 25, 8161–8173. [CrossRef] [PubMed]

27. Saurer, M.; Fuhrer, J.; Siegenthaler, U. Influence of ozone on the stable carbon isotope composition, δ13C, of leaves and grain of spring wheat (Triticum aestivum L.). Plant. Physiol. 1991, 97, 313–316. [CrossRef] [PubMed]

28. Araus, J.L.; Cabrera-Bosquet, L.; Serret, M.D.; Bort, J.; Nieto-Taladriz, M.T. Comparative performance of Triticum aestivum L. in a Mediterranean climate. Environ. Sci. Pollut. Res. Int. 2018, 25, 29208–29218. [CrossRef]

29. Bogers, P.; Tansley review no 95—15N natural abundance in soil-plant systems. New Phytol. 1997, 137, 179–203. [CrossRef]

30. Evans, R.D. Physiological mechanisms influencing plant nitrogen isotope composition. Trends Plant Sci. 2001, 6, 121–126. [CrossRef]

31. Reichenauer, T.G.; Goodman, B.A.; Kostelcky, P.; Soja, G. Ozone sensitivity in Triticum durum and T. aestivum with respect to leaf injury, photosynthetic activity and dry matter content. Physiol. Plant. 2010, 140, 681–686. [CrossRef]

32. Bai, R.; Agrawal, S.B. Effects of ambient O3 on wheat during reproductive development: Gas exchange, photosynthetic pigments, chlorophyll fluorescence, and carbohydrates. Photosynthetica 2011, 49, 285–294. [CrossRef]

33. Feng, Z.; Jiang, L.; Calatayud, V.; Dai, L.; Paolletti, E. Intraspecific variation in sensitivity of winter wheat (Triticum aestivum L.) to ambient ozone in northern China as assessed by ethylene degradability (EDU). Environ. Sci. Pollut. Res. Int. 2018, 25, 29208–29218. [CrossRef] [PubMed]

34. Latowski, D.; Grzyb, J.; Strzałka, K. The xanthophyll cycle—Molecular mechanism and physiological significance. Acta Physiol. Plant. 2004, 26, 197–212. [CrossRef]

35. Pleijel, H.; Broberg, M.C.; Uddling, J. Ozone impact on wheat in Europe, Asia and North America—A comparison. Sci. Total Environ. 2019, 664, 908–914. [CrossRef]

36. Fenollosa, E.; Munne-Bosch, S. Photoprotection and photo-oxidative stress markers as useful tools to unravel plant invasion success. In Advances in Plant Ecophysiology Techniques; Sánchez-Moreiras, A., Reigosa, M., Eds.; Springer: Cham, Switzerland, 2018; pp. 153–175.
38. Herbinger, K.; Tausz, M.; Wonisch, A.; Soja, G.; Sorger, A.; Grill, D. Complex interactive effects of drought and ozone stress on the antioxidant defence systems of two wheat cultivars. *Plant. Physiol. Biochem.* **2002**, *40*, 691–696. [CrossRef]

39. Monga, R.; Marzuoli, R.; Alonso, R.; Bermejo, V.; González-Fernández, I.; Faoro, F.; Gerosa, G. Varietal screening of ozone sensitivity in Mediterranean durum wheat (*Triticum durum*, Desf.). *Atmos. Environ.* **2015**, *110*, 18–26. [CrossRef]

40. Hare, V.J.; Loftus, E.; Jeffrey, A.; Ramsey, C.B. Atmospheric CO2 effect on stable carbon isotope composition of terrestrial fossil archives. *Nat. Commun.* **2018**, *9*, 252. [CrossRef]

41. Lomax, B.H.; Knight, C.A.; Lake, J.A. An experimental evaluation of the use of C3 δ13C plant tissue as a proxy for the paleoatmospheric δ13CO2 signature of air. *Geochem. Geophys. Geosystems* **2012**, *13*, Q0A103. [CrossRef]

42. Zhang, T.; Wang, X. Stable carbon isotope and long-chain alkane compositions of the major plants and sediment organic matter in the Yellow River estuarine wetlands. *J. Ocean. Univ. China* **2019**, *18*, 735–742. [CrossRef]

43. Iniguez, C.; Capo-Bauca, S.; Niinemets, U.; Stoll, H.; Aguilo-Nicolau, P.; Galmes, J. Evolutionary trends in RuBisCO kinetics and their co-evolution with CO2 concentrating mechanisms. *Plant. J.* **2020**, *101*, 897–918. [CrossRef] [PubMed]

44. Twohey, R.J., III; Roberts, L.M.; Studer, A.J. Leaf stable carbon isotope composition reflects transpiration efficiency in *Zea mays*. *Plant J.* **2019**, *97*, 475–484. [CrossRef] [PubMed]

45. Hofmann, D.; Jung, K.; Bender, J.; Gehre, M.; Schuurmann, G. Using natural isotope variations of nitrogen in plants as an early indicator of air pollution stress. *J. Mass Spectrom.* **1997**, *32*, 855–863. [CrossRef]

46. Pandey, A.K.; Ghosh, A.; Agrawal, M.; Agrawal, S.B. Effect of elevated ozone and varying levels of soil nitrogen in two wheat (*Triticum aestivum* L.) cultivars: Growth, gas-exchange, antioxidant status, grain yield and quality. *Ecotoxicol. Environ. Saf.* **2018**, *158*, 59–68. [CrossRef]

47. Alwi, N.A.; Ali, A. Dose-dependent effect of ozone fumigation on physiological characteristics, ascorbic acid content and disease development on bell pepper (*Capsicum annuum* L.) during storage. *Food Bioprocess Technol.* **2014**, *8*, 558–566. [CrossRef]

48. Trandel, M.A.; Vigardt, A.; Walters, S.A.; Lefticariu, M.; Kinsel, M. Nitrogen isotope composition, nitrogen amount, and fruit yield of tomato plants affected by the soil-fertilizer types. *ACS Omega* **2018**, *3*, 6419–6426. [CrossRef]

49. Chen, C.; Li, J.; Wang, G.; Shi, M. Accounting for the effect of temperature in clarifying the response of foliar nitrogen isotope ratios to atmospheric nitrogen deposition. *Sci. Total Environ.* **2017**, *609*, 1295–1302. [CrossRef]

50. Anand, A.; Kaur, G.; Bammidi, S.; Mathur, D.; Battu, P.; Sharma, K.; Tyagi, R.; Pannu, V.; Bhanushali, D.; Limaye, N. Primer for mainstreaming Mind-Body techniques for extreme climates-insights and future directions. *Medicines* **2020**, *7*, 12. [CrossRef]

51. Lancashire, P.D.; Bleiholder, H.; Boom, T.V.D.; Langeluddeke, P.; Stauss, R.; Weber, E.; Witzenberger, A. A uniform decimal code for growth stages of crops and weeds. *Ann. Appl. Biol.* **1991**, *119*, 561–601. [CrossRef]

52. Landi, M.; Cotrozzi, L.; Pellegrini, E.; Remorini, D.; Tonelli, M.; Trivellini, A.; Nali, C.; Guidi, L.; Massai, R.; Vernieri, P.; et al. When “thirsty” means “less able to activate the signalling wave trigged by a pulse of ozone”: A case of study in two Mediterranean deciduous oak species with different drought sensitivity. *Sci. Total Environ.* **2019**, *657*, 379–390. [CrossRef]

53. Davidson, J.A.; Cantrell, C.A.; Tyler, S.C.; Shetter, R.E.; Cicerone, R.J.; Calvert, J.G. Carbon kinetic isotope effect in the reaction of CH4 with HO. *J. Geophys. Res. Atmos.* **1987**, *92*, 2195–2199. [CrossRef]

54. Esmeijer-Liu, A.J.; Kurschner, W.M.; Lotter, A.F.; Verhoeven, J.T.; Goslar, T. Stable carbon and nitrogen isotopes in a peat profile are influenced by early stage diagenesis and changes in atmospheric CO2 and N deposition. *Water Air Soil Pollut.* **2012**, *223*, 2007–2022. [CrossRef] [PubMed]