UNIVERSITAT DE BARCELONA

The effects of ozone on Mediterranean wheat in a changing environment

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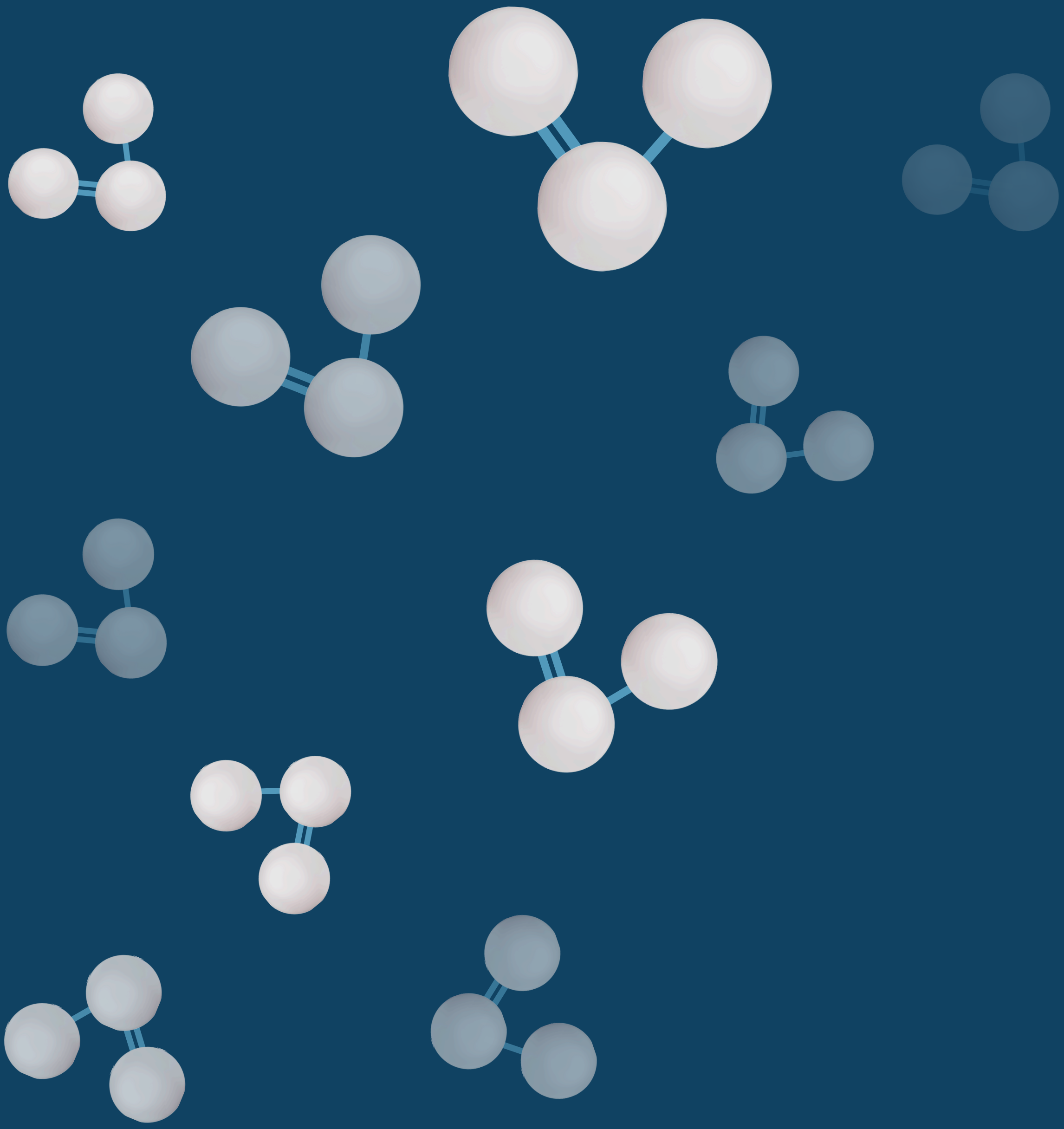

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The effects of ozone on Mediterranean wheat in a changing environment

This dissertation was presented by Melissa Chang Espino to obtain the degree of Doctor from the Universitat de Barcelona.

This thesis was performed for the doctoral program in "Ecology, Environmental Sciences, and Plant Physiology" of the Department of Evolutionary Biology, Ecology and Environmental Sciences of the Faculty of Biology of the Universitat de Barcelona.
This work was performed as part of the Integrative Crop Ecophysiology Group research group and in collaboration with the Unit of Ecotoxicology of Air Pollution of the Centre of Energy, Environmental and Technological Research (CIEMAT).
This thesis was written under the direction of Dr. Victoria Bermejo-Bermejo and Dr. Jose Luis Araus Ortega.
In Spanish, the thesis is titled: Los efectos del ozono sobre el trigo mediterráneo en un ambiente cambiante: Implicaciones para la seguridad alimentaria y la creación de políticas.

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June 17th 2024

Acknowledgments

The first person I need to thank is one of my directors, Jose Luis, for answering an email back in 2017 and giving my idea for a doctoral project a chance, and for his patience with me ever since. The second person, is my other director, Vicky, because without her I wouldn't have been able to go through this. She was always a great guide and supporter, especially through the toughest moments. I also want to thank my CIEMAT friends, for all your help with the experiments and for always making those speedy trips to Madrid both informative and fun.

I want to thank the people from HZM and GU for allowing me to work with you, it was a great and informative experience and I got to meet some amazing people.

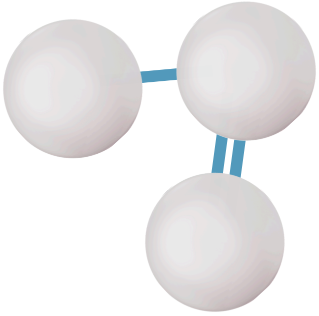
I also want to thank all of my friends, old and new, in Barcelona and away. To my amazing group of Barcelona friends (Claudia, Maite, Maria, Cristiano, Barna, Anna, Jairo, Chiara, Andras, Gosha, and Oli), who made my time here so much fun and were always there to play volleyball, party, eat, listen to music on terraces and to chat the night away. My girls, Fi, Jazz, Sho and Lore, you always manage to make me feel at home no matter where we are. Fati and Luisa, thank you for all the help, advice, cathartic sessions and amazing lunches.

I also need to thank my parents, who have always supported my dreams and crazy curiosity, no matter where it took me. Su apoyo ha sido todo para mí, gracias por siempre creer que podía hacer lo que me propusiera. To my siblings, who are always there to chat, rant, talk about our dreams and projects, and for whom nothing is ever too crazy.

To Sansa, thank you for the cuddles. And finally, I'd like to thank Florent, my husband-to-be. Thank you for always being there, for all those fun times, for cheese nights and amazing trips, for all your patience with my random presentation practices and idea storms, for post-its on the table and for all your support.

I couldn't have done this without you.

ABSTRACT



Abstract

The assays were conducted in the *La Higueruela/MNCN-CSIC* Agricultural Research Station, situated within a climate typical of the Iberian Peninsula, where controlled exposure to ambient and elevated ozone levels was facilitated through open-top chambers. Chapter 1 focused on evaluating the impact of ozone fumigation on different aged cultivars, while Chapters 2 and 3 explored the interactive effect of ozone and nitrogen fertilization. Additionally, Chapter 4 examined the effect of ozone on the fungal pathogen *Puccinia striiformis*.

To assess the impact of ozone on wheat, various factors were studied that are pertinent for crop management and policy-making. Chapter 1 delved into the effects breeding has had on wheat sensitivity to tropospheric ozone pollution, noting that the selection for a higher harvest index in the more modern varieties has led to an increased susceptibility to ozone, though the benefits of breeding mostly offset the effects of the pollutant. However, as landraces have a more extensive genetic base to improve ozone tolerance, they should be considered for future breeding processes.

Expanding upon crop management, Chapter 2 investigated the potential mitigative effects of additional nitrogen fertilization on ozone-induced yield and quality reductions. While findings indicated a beneficial impact from the fertilizer, this effect was only observed until the highest levels of ozone concentration, beyond which the fertilizer effect was entirely eliminated, resulting in both yield and growth values resembling those of the lower fertilizer treatment under the same fumigation. This resulted in lower critical levels under the higher nitrogen treatment, prompting a policy-making-oriented consideration of the economic and environmental consequences associated with ineffective fertilizer use at elevated ozone levels.

However, as additional nitrogen does provide some compensation at certain elevated ozone levels, the mechanisms through which this could be happening were explored in Chapter 3. It was noted that the additional nitrogen allowed the plant to invest more resources into the photosynthetic machinery, thereby enhancing assimilation to a certain extent. This can be considered for future research aimed at selecting varieties for a higher nitrogen use efficiency, particularly given the distinct nitrogen uptake and storage mechanisms of modern cultivars compared to landraces. Finally, Chapter 4 addressed another factor that could be essential for both crop management and policy-making, pathogens. The effect of ozone on a yellow rust infection was analyzed, noting its mitigation on yellow rust infection. While ozone exhibited beneficial effects at its current levels, higher concentrations – albeit lower than those reducing nitrogen use efficiency – proved detrimental to plant health.

In Chapters 1 and 3, isotopic signatures were also investigated. For both, and regardless of nitrogen fertilization treatment, the grain carbon isotope proved to be an excellent indicator of chronic ozone stress, while nitrogen isotopes in both leaf and grain provided valuable insights into nitrogen remobilization in wheat. However, the interpretation of these findings depended on correlations with other measurements due to the intricate nature of nitrogen dynamics within the plant.

In conclusion, this thesis underscores the importance of considering cultivar-specific tolerance mechanisms in breeding programs, acknowledging the limitations of crop management practices such as nitrogen fertilization, and recognizing the adaptation of modern wheat cultivars to current ozone levels, which provide them with protection against pathogens such as *Puccinia striiformis*.

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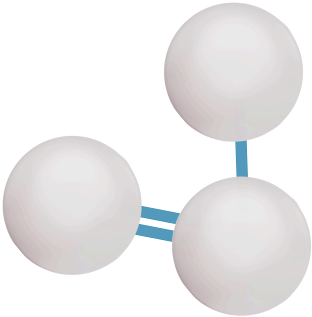

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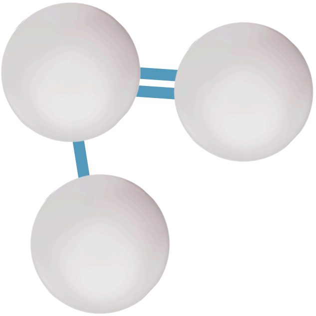
ABBREVIATIONS



Abbreviations

O_3 – Ozone

ROS – Reactive Oxygen Species

OTC – Open Top Chamber

AOT40 – Accumulated Ozone over the Threshold of 40 nL L^{-1}

POD6 – Phytotoxic Ozone Dose over the threshold of 6 mmol m^{-2} PLA s^{-1}

UNECE – United Nations Economic Commission for Europe

CLRTAP – Convention on Long-Range Transboundary Air Pollution

MITECO – Ministry for the Ecological Transition and the Demographic Challenge

MAPA – Ministry of Agriculture, Fishery and Food

EEA – European Environment Agency

ppb – parts per billion, equal to nL L^{-1}

$\delta^{13}C$ – Carbon isotopic signature

$\delta^{15}N$ – Nitrogen isotopic signature

GY – Grain Yield

GNY – Grain Nitrogen Yield

FL – Flag Leaf

PAR – Photosynthetically Available Radiation

NUE – Nitrogen Use Efficiency

PNUE – Photosynthetic Nitrogen Use Efficiency

g_s – stomatal conductance

A – Photosynthetic rate

WUE – Water Use Efficiency

g_m – mesophyll CO_2 diffusion conductance

C_i – Intercellular CO_2 concentration

C_a – Ambient CO_2 concentration

C_i/C_a – Ratio of intercellular to ambient CO_2 concentration

Low-N – 100 kg ha-1 N fertilizer supplementation

High-N – 200 kg ha-1 N fertilizer supplementation

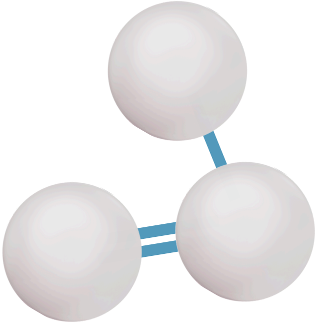
FA – Charcoal-Filtered Air

NFA – Non-Filtered Air, reproducing ambient levels

NFA+ – Non-Filtered Air + 20 nL L^{-1} of added O_3

NFA++ – Non-Filtered Air + 40 nL L^{-1} of added O_3

GENERAL INTRODUCTION



General Introduction

1. Tropospheric ozone

Ozone (O_3) is both a natural and anthropogenic gas, where 90% exists in the upper atmosphere (stratosphere) and 10% at ground level (troposphere) (Crutzen, 1988; Dewan and Lakhani, 2022). While in the stratosphere O_3 is a natural component and plays a crucial role in absorbing harmful ultraviolet radiation from the sun (de Gruijl and van der Leun, 2000), in the Northern Hemisphere tropospheric O_3 is mostly caused by anthropogenic activities (Lelieveld and Dentener, 2000; Fowler et al., 2020).

More specifically, even though some O_3 is naturally transported from the stratosphere to the troposphere, most of the O_3 found at ground level, which is about 7-15 times what is moved from the stratosphere (Cooper et al., 2014), is a secondary pollutant produced by the photochemical reaction of anthropogenic gases driven by industrial and urban emissions in the atmosphere (Monks et al., 2015; EEA, 2022a), such as carbon monoxide, methane, nitrogen oxides, and non-methane volatile organic compounds (EEA, 2019). These anthropogenic pollutants are known as tropospheric O_3 precursors and, although they are also emitted from natural sources (fires, volcanos, biogenic VOCs emissions, etc.), most come from human activities and have caused an increase in tropospheric O_3 levels since the industrial revolution, paired with increases in CO_2 (Fowler et al., 2020). O_3 is formed when carbon monoxide and Volatile Organic Compounds (VOCs) photochemically degrade in the presence of NO_2 (Archibald et al., 2020), increasing O_3 levels. Atmospheric photochemical processes increase under high temperatures and solar radiation, explaining why the highest O_3 levels in Europe are found in the Southern countries of the Mediterranean basin (European Environment Agency, 2019a). Tropospheric O_3 levels currently surpass the plant-sensitivity threshold of 40 nL L^{-1} in the Northern Hemisphere causing negative effects on both wild plant communities and crops (Mills et al., 2007; CLRTAP, 2017; Pleijel et al., 2018; Li et al., 2021; EEA, 2022b).

As these O_3 precursors can travel great distances within local, regional, and intercontinental air masses, they can reach high O_3 levels far away from the site where the precursors were generated (Dewan and Lakhani, 2022) affecting extended rural areas and natural ecosystems despite local efforts to maintain low precursor emission levels (Monks et al., 2015). Long-range O_3 transport can be seen at regional and continental scales in the Northern Hemisphere. In the Iberian Peninsula, an eastern coastal breeze with a constant O_3 level acts as background O_3 and moves towards the inner Peninsula following the valleys oriented in the same direction (Millán et al., 2000). For two of the regions with the highest O_3 levels in Spain, Querol and Monsfort (2023) describe that anabatic winds on the southeastern slope of the Sierra de Guadarrama and northwest of the Cuenca, recirculate vertically, moving air masses that have been trapped between the mountains, while in the Vic plains, winds move from the coast up two main valleys (Llobregat and Besòs), and, by mixing with precursors produced in Barcelona city, can reach 160km inland, and move up the mountains to 2000-3000 m.a.s.l. These movements can also happen in the Western Mediterranean Basin due to regional transport of air masses within the basin (Gangoiti, 2001), within Europe (Derwent, 1990), from North America to Europe (Stohl and Trickl, 2006), and in other places around the world like the Arctic

(Eckhardt et al., 2003) and Asia (Xu et al., 2021; Li et al., 2023). Some atmospheric processes also contribute to the intercontinental atmospheric transport of both O_3 and its precursors. In Europe, the North Atlantic Oscillation (NAO) causes surface tropospheric O_3 anomalies in Northern Europe due to increased western wind inflows from the United States to Europe (Russo et al., 2023). In contrast, O_3 precursors that accumulate in the northern hemisphere during winter move to lower latitudes during the spring through pollution transport pathways (Eckhardt et al., 2003).

2. *Ozone and climate change*

Since the atmospheric production of O_3 depends on solar radiation, temperature, humidity and wind stability, the warmer and drier summers expected with climate change (Zhao, Stevenson and Bollasina, 2019; Dewan and Lakhani, 2022) along with more frequent heat waves (Meehl et al., 2018) would increase O_3 levels (Ainsworth et al., 2012; Zanis et al., 2022), particularly in the warmer Mediterranean region of Europe (Sicard et al., 2013; Prieto-Benítez et al., 2023). In fact, the expected warmer temperatures due to climate change will exacerbate the photochemical formation of surface O_3 , a process known as the O_3 climate penalty (Dewan and Lakhani, 2022; Zanis et al., 2022). This could lead to increases in summertime concentrations of 1-5 nL L^{-1} in Southern Europe, 4-10 nL L^{-1} in eastern United States, and 3-6 nL L^{-1} in North-Eastern Asia by the 2100s compared to the 2000s (Gong et al., 2022). Particularly in the Mediterranean basin, which is considered a hot spot for climate change (Giorgi and Lionello, 2008; Paeth et al., 2017), modeling exercises predict a possible 4 ºC increase by the end of the 21st century (Sicard et al., 2013; Paeth et al., 2017; Archibald et al., 2020). This is expected to counteract O_3 mitigation strategies, causing increases of up to 5ppbv in Europe by the end of the century (Colette et al., 2015). Despite the complexity of atmospheric chemistry, the increase or reduction of future O_3 levels will depend on the regulation of precursor emissions. O_3 future projections vary greatly for the end of the 21st century depending on the different IPCC scenarios considered, ranging from a 16% decrease under RCP 2.6 (a very stringent pathway) to increasing by 18% by RCP 8.5 (where emissions continue to rise) compared to the year 2000 (Young et al., 2013).

Predicting future O_3 levels is complex, since they depend on precursor emissions which are also linked to the potential boost that climate change exerts on them, like the expected increase in wildfires that would release more NOx, the increase of N-compounds emissions from soils or the augmentation of biogenic VOCs emissions and wetland CH_4 emissions (Archibald et al., 2020; Dewan and Lakhani, 2022; Gong et al., 2022). Although there are also some uncertainties related to methane production, some modeling exercises show that future CH_4 emissions will double from their current values, which could cause surface O_3 to remain almost unchanged due to the warmer climate and even with emissions reductions (Jaidan et al., 2018).

So far, despite efforts to reduce O_3 precursor emissions, O_3 surface levels remain high, posing challenges for human health, crops, and ecosystems in the Northern Hemisphere (Tai et al., 2014; McGrath et al., 2015). In the last 11 years, EU background O3 levels have not shown a clear decreasing trend, based on EEA measurements available since 2013. Twentytwo European countries, particularly those located in Eastern Europe and the Mediterranean, have levels surpassing the EU target value for O_3 of 60 nL L^{-1} (2008/50/EU). The tropospheric O_3 levels foreseen in the Zero Pollution Action Plan of the European Green Deal for 2030 will not be reached (EEA, 2023).

3. *Ozone absorption by the plant and plant response*

The following Figure shows the chronic effects of O_3 on wheat based on Emberson et al. (2018):

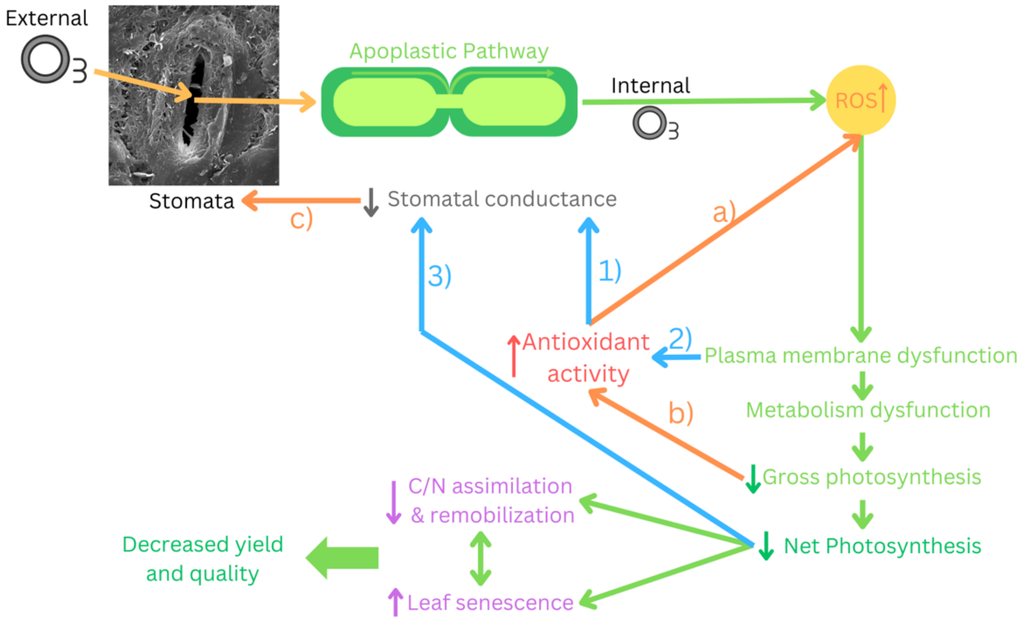


Figure 1. The green arrows show the movement of O_3 and its products as described in Section 3; the orange arrows show the limitations to O_3 absorption; the blue arrows show feedback on stomatal conductance. The feedback mechanisms on stomatal conductance are depicted with arrows labeled a-c, and defense mechanisms with arrows labeled 1-3, and will be elaborated in Section 6.2.

O_3 enters the plant through the stomatal pores when the plant is functioning for gas exchange and carbon fixation, following a diffusive gradient that is similar to that of CO_2 but with a specific diffusive constant. Inside the plant, the O_3 molecules react in the apoplastic fluid producing reactive oxygen species (ROS). As O_3 enters the plant, the ROS reactions trigger an oxidative burst that causes O_3-damage at a cellular scale by altering the cell membrane permeability. As ROS react with the plasma membrane, other compounds are created that are less reactive than O_3 but have a longer half-life, allowing them to infiltrate even deeper into the cell and causing metabolic shifts (Emberson et al., 2018).

The accumulation of ROS in the extracellular space affects the chloroplasts (Vainonen and Kangasjärvi, 2015), and the secondary ROS activate interorganelle signaling pathways that trigger defense mechanisms to the stressor, such as hormone signaling, protecting the photosynthetic machinery by enhancing the catabolic system and increasing the production of antioxidant response compounds (Krasensky et al., 2017). One example of these pathways is associated with an increased carbohydrate breakdown which can lead NADPH to accumulate within the chloroplast and boost detoxification (Gandin et al., 2021). Eventually, if the plant is exposed to O_3 chronically or to very high levels, it can overtake the apoplastic antioxidant response and ROSs can accumulate excessively, further intensifying the cascade effects, surpassing mitochondria metabolism and leading to mitochondrial rupture and cell death (Emberson et al., 2018; Gandin et al., 2021).

At the physiological level, O_3 impacts important processes related to plant gas exchange: it alters stomatal opening (closure or sluggishness) (Calatayud et al., 2010; Hoshika et al., 2015), decreases rubisco content and carboxylation activity (Calatayud et al., 2002; Emberson et al., 2018), disrupts the electron transport chain (Gillespie et al., 2012; Hoshika et al., 2020a), or decreases chlorophyll content (Pleijel et al., 2006; Pandey et al., 2018). These alterations in turn affect photosynthetic function decreasing carbon assimilation (Ainsworth et al., 2012; Emberson et al., 2018). Photosynthate redistribution and biomass partitioning within the plant are also affected (Sanz et al., 2005; Calatayud et al., 2011; Pleijel et al., 2014). By altering these mechanisms, plant senescence is accelerated, shortening the plant lifespan (Sanz et al., 2015; Osborne et al., 2019), whereas overall growth and yield are reduced (Pleijel et al., 2006; Avnery, 2013; Calvete-Sogo et al., 2014; Hayes et al., 2020; Schucht et al., 2021; Effah et al., 2022) as well as the reproductive capacity of the plant (Gimeno et al., 2004; Gillespie et al., 2015). In some cases, depending on the plant's response, the ROS reaction and plant defense mechanisms can produce cell death, causing visible symptoms to appear on the leaf blade (Gimeno et al., 1999; Bermejo et al., 2003).

To prevent O_3 from entering the plant, the most common mechanism of plant protection is decreasing stomatal conductance through stomatal closure (Hoshika et al., 2020b; Hasan et al., 2021). A decreased mesophyll diffusion conductance caused by an increase in cell wall resistance may also prevent O_3 from entering the protoplast. To achieve this, mesophyll cell walls can thicken with callose depositions (Ljubešić and Britvec, 2006). Most plants also activate defense mechanisms such as antioxidant response to block the extra O_3-induced ROS through the increased production of antioxidant enzymes like peroxidase, superoxide dismutase, or antioxidants substrates like ascorbic acid or glutathione (Liu et al., 2015; Feng et al., 2016; Fatima et al., 2018; Li et al., 2021). An increase or redirection of resources toward repair has also been seen (Fatima et al., 2018).

Factors such as canopy structure and height, leaf size, shape or roughness, and stomatal density can all affect O_3 uptake (McNaughton and Van Den Hurk, 1995; CLRTAP, 2017). In turn, these factors are also related to the conditions under which the plant grows, which also determine plant responses to the pollutant. Especially, environmental conditions that regulate stomatal conductance, such as atmospheric vapor pressure deficit (based on air temperature and RH), soil moisture determining the hydric status of the plant, CO_2 concentration in the leaf, nutrient availability, and photosynthetic active radiation (PAR) can also affect O_3 absorption by the plant and therefore the intensity of O_3-damage (Calatayud et al., 2011; Mills et al., 2011; CLRTAP, 2017; González-Fernández et al., 2017). The relationship between growing conditions and O_3 absorption and its effects, increase the complexity of the plant response to the pollutant under future Global Change scenarios, because plant response would depend not only on ambient O_3 levels but also on changing environmental conditions. For example, in the case of increasing drought periods, predicted by the models in the Mediterranean basin in the range 12-25% for the period 2021-2060 (Essa et al., 2023), would lead to stomatal closure and therefore less O_3 absorption (González Fernández et al., 2023). On the contrary, CO_2 levels, which are expected to increase in the future (Calvin et al., 2023) could also offset some of the detrimental effects of O_3, although they pose other threats such as decreasing grain quality in wheat (Leung et al., 2022).

4. Ozone risk indicators and Environmental policies

At the international level, protection and regulation protocols are in place to monitor and limit O_3 pollution effects on human health, natural ecosystems and agrosystems. These protocols involve actions to lessen the emission of O_3 precursors and limit the pollutant to avoid harmful effects.

4.1 Air Convention of the United Nations

The Air Convention, also known as the Convention on Long-Range Transboundary Air Pollution (CLRTAP), was signed in 1979 under the United Nations Economic Commission for Europe (UNECE) to foster collaboration among nations to identify priorities and negotiate commitments to mitigate air pollution, including tropospheric O_3. With 56 member states, including the EU and Spain, its primary objective is to limit emissions and minimize transboundary air pollution to protect both public health and the environment. For Spain, the national focal actor that oversees activities, negotiations, and protocol compliance is the Ministry for Ecological Transition and Demographic Challenge. It should be noted that though these protocols initially focused on uniformly reducing emissions across countries, these targets were later adapted based on the gravity of the environmental impacts and regional differences (González Fernández et al., 2023).

In the case of tropospheric O_3, the expert Air Convention cooperative panel ICP-Vegetation collectively analyzes databases on the O_3 response of various crops and species that are either representative or particularly sensitive within the main European ecosystems and agrosystems. This analysis aims to identify specific risk indicators, i.e. values that should not be surpassed to safeguard crops and ecosystems. All risk indicator data and the methodology for their calculation and implementation are documented in the technical manual “Manual for Modelling and Mapping Critical Loads & Levels”, and are specifically outlined in Chapter 3 “Mapping Critical Levels for Vegetation” (CLRTAP, 2017). This chapter encompasses all the aspects involved in quantifying the O3 response of crops and natural vegetation, and establishing threshold protection values, known as Critical Levels (CL). This manual is continuously updated to reflect advancements in scientific understanding and is pivotal for conducting precise risk assessments in both present circumstances and future Global Change scenarios. These assessments are essential to identify sensitive areas or those facing heightened risks due to elevated O_3 levels, or due to the susceptibility of their crops or plant communities. Moreover, they play a crucial role in shaping environmental policies aimed at mitigating precursor emissions.

4.2 Ozone indices based on ozone concentration and dose

There are two main types of O_3 indicators to assess O_3 risk in sensitive crops and vegetation, the indices based on O_3 concentration in the air, and those based on O_3-dose absorbed by the plant, both currently in use in different environmental policy frameworks of the UNECE and EU.

The most commonly used O_3-concentration-based index is the Accumulated Ozone over the Threshold of 40 nL L^{-1} (AOT40), which is calculated as the hourly cumulative O_3 levels above the plant-sensitivity threshold of 40 nL L^{-1} during daylight hours for the considered period (Mills et al., 2007). Therefore, this index analyzes the exposure to O_3, that exceeds a threshold, to which the plant was exposed, to understand the damage suffered due to the accumulated exposure. This index has shown a robust linear relationship with the O_3-induced effects on yield for different crops (Mills et al., 2007; González-Fernández et al., 2014, 2016; Sanz et al., 2014; Anav et al., 2016). In the case of wheat, the CL based on AOT40 from experimental studies are also robust (Mills et al., 2007; Pleijel et al., 2019); however, the response functions and CLs are based in wheat cultivars of Central and Northern European area. There is, therefore, a need for consistent knowledge about the O_3-sensitivity of cultivars from the Mediterranean area, which is discussed in Chapter 1.

Risk assessments based on surpassing AOT40-based CLs present a problem, as O_3 damage could be overestimated, especially in water-limited areas (CLRTAP, 2017). These indices quantify the amount of O_3 in the environment, without considering that, depending on the environmental conditions (temperature, light, humidity, water availability in the soil) and the plant (age, phenology), more or less O_3 present in the atmosphere will be absorbed by the plant; i.e., the application of the AOT40 index in the Iberian Peninsula for rainfed wheat causes an important overestimation in the estimated yield loss (González-Fernández et al., 2013).

The second type, the O3-based index, is the Phototoxic Ozone Dose (POD), which is considered an improvement over AOT40 and is currently recommended by the Air Convention (Pleijel et al., 2022). This index considers the real absorbed O_3 dose penetrating the vegetative tissues, specifically calculated for the different species based on a standardized methodology of the Air Convention (CLRTAP, 2017). This calculation is based on the stomatal conductance-based DO3SE model (Deposition of Ozone for Stomatal Exchange), which includes various abiotic factors that limit stomatal conductance and growth conditions that affect stomatal opening.

The DO3SE equation is:

$g_s = g_{max} * [\min(f_{phen}, f_{O3}] * f_{light} \max((f_{min}, (f_{temp} * f_{VPD} * f_{SW}))$ unit:

where g_s (mmol O_3 m^{-2} PLA s^{-1}) is the actual stomatal conductance, g_{max} is the species-specific maximum stomatal conductance, and all of the f parameters (f_{phen} - phenology, f_{O3} – O_3 concentration, f_{light} - light, f_{min}- relative minimum stomatal conductance experienced during daylight hours, f_{temp} - temperature, f_{VPD} – vapor pressure deficit, f_{SW} - soil moisture) are expressed in relative terms as a proportion of g_{max} (ranging from 0 to 1). More specifically for wheat, hourly POD values, based on DO3SE, are aggregated above the 6 mmol m^{-2} PLA s^{-1} threshold as per the CLRTAP methodology, which is why for wheat the indicator is known as POD6 (Grünhage et al., 2012; CLRTAP, 2017). Besides from the aggregated thresholds, each crop, forest tree of vegetation calculated with this model has specific parametrizations that are also included in the manual. f_{O3} was not used in this experiment because, currently, there is no crop species parameterization POD6SPEC for Mediterranean wheat (CLRTAP, 2017), but more experimental information could help calculate one.

The POD index is more complex to calculate and requires extensive fieldwork to achieve robust specific parameterization of the DO_3SE for different species. However, it has more biological meaning and yields more precise results than the AOT40, especially when plants are grown under stress conditions such as water deficit. This prevents the overestimation of O_3-damages, as caused by the AOT40 index (González-Fernández et al., 2013; Pleijel et al., 2022). Moreover, PODs allow for the O_3-risk assessment of crops and ecosystems under future scenarios and different environmental strategies (Karlsson et al., 2017; Guarin et al., 2024). This is why significant effort has been made in the past decade to improve POD calculations for deriving CLs based on the O_3 absorbed by different crops and natural species (Watanabe, Izumi and Matsuyama, 2016; Fernandes and Moura, 2021; Marzuoli et al., 2024).

However, up until now, O_3-risk analyses based on these indices have not considered the interaction of the pollutant with other factors such as nitrogen (N) fertilization, a key component of agricultural production that can modulate the O_3 response (Mills et al., 2018a; Feng et al., 2022). Therefore, it is essential to address this complex interaction to minimize the knowledge gaps in understanding O_3 mechanisms and its effects on plant physiology. These results can then be transposed into the optimization of O_3-risk assessments based on agricultural management. These ideas are developed in Chapter 2.

4.3 Ozone threshold values for plant protection in EU and UNECE

Various legislative frameworks regulate or recommend the control of atmospheric pollutants to prevent their effects. The Directive 2008/50/EC is a comprehensive framework within the European Union that aims to manage tropospheric O_3 levels, among other air pollutants, including some O_3 precursors, to protect human health and the environment. By merging and refining prior legislation, it establishes a strong mechanism to regulate, assess, and improve air quality standards. It establishes a specific O_3 concentration target objective based on an accumulated 3-month AOT40 index (9000 nL L^{-1} =18 000 µg m^3 s^{-1}), and a long-term objective (3000 nL L^{-1} =6000 µg m^3 s^{-1}) to protect vegetation. Spain integrated this directive into its legislation under Royal Decree 102/2011 to improve air quality. In response to evolving scientific understanding, the European Commission proposed revisions of Directive 2008/50/EC to establish updated air quality objectives for 2030 to align with the World Health Organization standards and the 2050 EU "zero pollution" objective. This proposal maintains the AOT40 indicator for both short-term and long-term vegetation objectives and suggests incorporating indicators from Directive (EU) 2016/2284 to assess the ecological impacts of air pollution, particularly tropospheric O_3 based on POD indices.

The UNECE Air Convention recommends using POD indicators to conduct O_3 risk assessments at regional and continental scales. Following this recommendation, the National Emissions Ceilings Directive EU 2016/2284 (NEC Directive) has incorporated the POD-based risk methodology into the NEC network, to monitor atmospheric pollutant-caused damage on EU ecosystems and agrosystems. Currently, there are specific POD-based CLs that can be used to assess the potential effects of O_3 on biomass and grain production or quality in different horticultural and cereal crops (such as tomato, potato and wheat), perennial and annual pastures, and different types of forest (CLRTAP, 2017). Specifically for wheat, the established CLs to avoid a 5% reduction are 1.3, 1.5 and 2 mmol m^{-2} PLA for yield, 1000 grain weight and Protein yield respectively (CLRTAP, 2017).

5. *Ozone levels in Spain and effects on crops*

In the Mediterranean basin, tropospheric O_3 levels surpass the protection thresholds for vegetation of the EU 2008/50 Directive (EEA, 2019, 2023; Massagué et al., 2024). This situation includes the Iberian Peninsula, where despite an observed decrease in O_3 peak values during summer months since 2000, O_3 mean values remain a cause for alarm (EEA, 2023). According to the annual reports of the Spanish Ministry for Ecological Transition and the Demographic Challenge, the objectives of the EU Directive, which also include health indicators based on the World Health Organization Levels, are exceeded every year in most of the country except the northern Atlantic and the Canary Islands (Ceballos, 2023). However, though these thresholds have been surpassed constantly since 2012, frequencies are lower than in the years before the pandemic, where exceedances of legal and WHO standards have decreased by 29% and 18% respectively compared to the average of those recorded in the 2012-2019 period (Ceballos, 2023). These exceedances are particularly high in the warm episodes of late June, July, and August, and pose a particular threat in the Mediterranean region due to its meteorologically stable conditions, high temperatures, and radiation, which facilitate O_3 formation (Fernández-Fernández et al., 2011; EEA, 2018, 2019b; Massagué et al., 2024).

The O_3 CLs established by the Air Convention to protect the different crops and vegetation types are also surpassed systematically in Spain (Fernández-Fernández et al., 2011; Elvira et al., 2016). O_3 levels in Spain are high enough to cause damage to sensitive horticultural species such as tomato (González-Fernández et al., 2014), watermelon (Gimeno et al., 1999), or leafy crops (González-Fernández et al., 2016); and to annual herbaceous communities valuable for their forage quality and high biodiversity richness (Sanz et al., 2014; Calvete-Sogo et al., 2016).

There are no specific studies in Spain that analyze wheat sensitivity to O_3 . Modelling exercises have monetized wheat losses due to O_3 at €58,151,000, calculated for Spain for the year 2019, even taking into account the drought of this year, which reduced O_3 absorption (Colette et al., 2018; Schucht et al., 2021). Schucht et al., (2021) even emphasized the high POD6 values found in the central areas of the Iberian Peninsula, which reach 6 mmol m^{-2} PLA.

The available information suggests that this pollutant could have a harmful impact on Spanish agriculture. There is a need for more experimental research to understand how different crops respond to the pollutant, considering the specific species, cultivars, and conditions of the Mediterranean area. It is also important to consider how management factors such as fertilization and irrigation interact with the pollutant.

6. Wheat production under an ozone-polluted environment

6.1 Wheat production

Wheat was domesticated around 10,000 years ago, and is now one of the global staple crops with 217 million ha being cultivated annually (producing 752 M Mg) (Erenstein et al., 2022). As the population is expected to reach 9.7 billion by 2050, wheat production would need to increase by 132 M Mg of wheat annually by that year. It is consumed in 173 countries, particularly in West/Central Asia, Northern Africa, and Europe, and represents 37% of all annual cereal consumption, being second only to rice. Specifically 34% (255.6 M Mg) of all World wheat production happens in Europe, where its cultivation covers an area of 61.7 M ha. And while 36% of European wheat is used for animal feed, 25.7% of the protein consumed by humans comes from wheat. This is even higher in West/Central Asia and Northern Africa at 38% (Erenstein et al., 2022). Specifically for Spain, wheat covers the largest crop area at 4.5 M ha (MAPA, 2021).

Given the importance of wheat both at a productive level and as a source of protein, the study of its response to a stress factor, such as the high levels of tropospheric O_3, becomes apparent. Furthermore, the importance of research on wheat sensitivity to O_3 is accentuated considering that this crop is the most O_3-sensitive among the main staple crops on a global scale (Pleijel et al., 2019; Broberg et al., 2020). For this reason, wheat was selected as a representative crop for O_3 risk assessment at regional and continental scales, to evaluate potential O_3 pollution effects and the efficiency of environmental policies (CLRTAP, 2017). Therefore, it is crucial to understand the mechanisms underlying O_3 damage, considering cultivars from different agroclimatic zones, and exploring potential mitigation strategies such as fertilization management; these aspects are covered in Chapters 1, 2 and 3 of this thesis.

6.2 Wheat response to ozone

As mentioned in Section 3, O_3 has various effects on the plant. Specifically for wheat, this leads to various responses, including feedback mechanisms on stomatal conductance (depicted in Figure 1, arrows labeled a-c), and defense mechanisms (Figure 1, arrows labeled 1-3). Arrow 1) shows the effect of the balance between ROS and antioxidant activity. Wheat plants increase the production of antioxidant enzymes to detoxify the O_3-formed ROSs caused when O_3 dissolves in the apoplast (Figure 1 under arrow a), like superoxide (O_2^-) and hydrogen peroxide (H2O2) (Feng et al., 2016; Li et al., 2021). During this process, the apoplastic NADPH oxidase plays an important role in triggering the production of antioxidant enzymes (Laloi, Apel and Danon, 2004; Krasensky et al., 2017). Studies indicate that this ability to increase the antioxidant enzymatic defense underlies the tolerance of wheat cultivars to O_3 (Feng et al., 2016).

Meanwhile O_3-uptake can be controlled by decreasing stomatal conductance. The burst of ROS activates stomatal closure (Figure 1, arrow c) due to the alteration of Ca^{2+} homeostasis and the guard cell K^+ channels, along with the production of the ABA photohormone (Hoshika et al., 2020b), preventing absorption of the pollutant (González-Fernández et al., 2013; Emberson et al., 2018; Osborne et al., 2019). In fact, the increased sensitivity of modern wheat cultivars has been attributed to a selection for a higher Harvest Index linked with a higher photosynthetic activity, which is usually related to a higher stomatal conductance to favor gas exchange (Barnes et al., 1990; Pleijel et al., 2006). However, this also favors O_3 flux inside the plant. Chapter 1 of the thesis analyzes whether the sensitivity of Mediterranean varieties and landraces is related to a greater specific stomatal conductance. Antioxidant production and stomatal conductance both define the 'effective O_3 flux' (Musselman et al., 2006), which is the balance between O_3 uptake and the capability of the plant to detoxify.

Another mechanism is shown with arrow 2). As disruptions to the plasma membrane and apoplast due to O_3 cause plasma membrane dysfunction, altering signal transduction proteins and causing changes in the components of the cell wall to act as antioxidants (Castillo and Heath, 1990). Finally, arrow 3) shows how a decreased photosynthesis can lead to an increase in internal CO_2 concentration and therefore a lower stomatal conductance (Reich and Amundson, 1985).

Although these physiological and biochemical mechanisms to defend plants from O_3 stress can improve tolerance to the pollutant, they can also decrease photosynthetic activity due to a lower CO_2 absorption and nutrient availability. Moreover, the pollutant also increases metabolic costs for maintenance and repair (Manderscheid, Jäger and Kress, 1992), such as by redirecting photosynthates towards repair mechanisms to the detriment of flag leaf photosynthesis and starch concentrations in the grain (Singh et al., 2018b; Fatima et al., 2019), or protection of the photosynthetic machinery (Joffe et al., 2022). This could decrease the general growth of the plant, accelerate senesce and decrease nutrient remobilization towards the grain, and impact both grain quality and yield (Mills et al., 2007; Pleijel, 2011; Ma et al., 2022) (Figure 1, arrow b), particularly in the more modern and O_3-sensitive varieties (Biswas et al., 2009).

Various parameters can be measured to analyze the effects of O_3 on plant physiology, and have been considered in the present study. To study the impact of O_3 on carbon intake, photosynthesis and stomatal conductance, discrete gas exchange measurements can be taken during the crop season (Feng et al., 2008). These measurements were perfomed using the portable photosynthetic system Li-Cor 6400 (Li-Cor, Lincoln, NE, USA) calibrated by the company before starting the assay. Additionally, foliar pigment and polyphenol content were measured with the single-photon avalanche diode SPAD (Chlorophyll Meter, Konica Minolta, Japan) and the optical sensor Dualex© (Force-A, Orsay, France) to understand the effect of O_3 on photosynthesis, plant phenology and senescence (Reichenauer, 1998).

Analyzing nutrient content in both leaf and grain can show the effects of O_3 on nitrogen use efficiency (Broberg et al., 2017; Peng et al., 2020) and general nutrient assimilation and remobilization (Pleijel et al., 1999). This thesis also focused on the use of isotopic signatures, as they have been considered very good indicators of chronic stress (Pausch et al., 1996; Jäggi et al., 2005). Nitrogen (Tcherkez, 2011a) and carbon (Tcherkez et al., 2011b) isotope fractionation can be an excellent tool to analyze the effect of O_3 on carbon and nitrogen absorption, assimilation, and remobilization. Section 8 will delve more into the use of isotopic signatures.

6.3 The effects of ozone on wheat affect Global Food Security

Ample studies have been performed on the effects of O_3 on wheat yield and quality due to O_3-induced biochemical and physiological impacts. Meta-analyses performed on 33-57 experiments have shown global yield decreases induced by the pollutant ranging from 7-22% (Mills et al., 2007; Feng et al., 2008; Pleijel and Uddling, 2012; Broberg et al., 2015; Mills et al., 2018b). The strongest effects on wheat yield due to O_3 levels have been observed in India and China (Mills et al., 2018a), with Indian varieties experiencing up to 15% yield losses, and grain nitrogen yield (GNY) losses ranging from 3.9-5.6% (Pandey et al., 2018).

Specifically for Western wheat, a historical analysis by Schauberger et al., (2019) estimated yield losses of up to 27% between 2008-2010. An analysis by Pleijel et al., (2004) of experiments in Belgium, Finland, Sweden, and Northern Italy demonstrated a 20% drop with increased O_3 levels. A widely grown UK variety showed decreases in the range of 6 tons per ha^{-1} (Harmens et al., 2018). Meanwhile, Pleijel et al., (2018) calculated an average yield loss of 0.38% per nL L^{-1} of O_3, a value that remained stable over the experimental years (1982-2010). Finally, Protein yield reductions ranged from 6.2-16% (Pleijel and Uddling, 2012; Broberg et al., 2015; Pleijel et al., 2018), with a 0.44% protein yield loss per nL L^{-1} of O3 (Broberg et al., 2020).

All of this is alarming in terms of current and future food security. More than 20% of crop production in Europe faces the threat of being harmed by O_3 pollution, posing a significant risk to food security (Mills et al., 2007). The O_3-induced yield reductions have significant economic impacts, particularly for the modern wheat varieties which are more O_3 sensitive (Mills et al., 2007; Pleijel et al., 2007, 2018). Calculations for average global reductions in wheat yield by the year 2019 showed losses ranging from 0 to 4% in Northern Europe, to 4 or more than 8% in the Mediterranean (EEA, 2022b), reaching levels of up to 9% in Portugal, Greece, Cyprus, Albania and Czechia, and up to 8% in Spain (Schucht, Tognet and Létinois, 2021). In the Mediterranean region, up until now, only one experiment has been performed with the more tolerant durum wheat species (Monga et al., 2015). This experiment showed an O_3-response of Mediterranean wheat with a yield decrease of 16%. There is no information about the sensitivity of Mediterranean bread wheat varieties; it is therefore necessary to study of O_3 sensitivity of Mediterranean wheat varieties. Chapter 1 adds novel information to this knowledge gap.

Many experimental assays analyzing the wheat response to O_3 emphasize the difference in sensitivity of wheat varieties. Modern wheat cultivars were bred for a higher HI and yield. This is linked to a higher photosynthetic activity through a higher stomatal conductance, which favors CO_2 exchange, but also allows for a high O_3 flux into the plant (Barnes et al., 1990; Pleijel et al., 2006). As breeding for higher yields continues, this poses an increasing threat to wheat. The wheat breeding process has led to O_3-induced reductions of 7-10% in modern cultivars in the Northern Hemisphere compared to older varieties (Brewster, Hayes and Fenner, 2019). Moreover, there is a genetic tolerance/sensitivity provided by the cultivar. When compared to North America and Asia, wheat varieties grown in Europe showed differences in their O_3 response, where the grain yield of both European and Asian genotypes were more affected than North American wheat (Pleijel et al., 2019).

According to Pleijel et al., (2006), older wheat varieties typically store more carbohydrates in the stems, when compared to modern cultivars which usually store them in the flag leaf to then reallocate them to the grains. This pre-grain filling storage in older cultivars has also been seen by Sanchez-Bragado et al., (2014a), who noted that in landraces the ear had a higher contribution for grain filling than in modern cultivars, particularly under water stress, which can also be related to their differences in O_3-response. Consequently, modern cultivars rely more heavily on photosynthesis during grain filling, leading to notable shifts in phenology and distribution patterns that impact their response to O_3 exposure.

Regarding Mediterranean cultivars, there is no information on their response to O_3 or on the differences between differently aged varieties; this topic is addressed in this thesis. Table 1 shows the varieties analyzed in Chapter 1 for their O_3 sensitivity, which include landraces and old and modern varieties. The modern varieties are among those most widely used in Spain, based on the sale of certified seeds, particularly Artur Nick and Nogal (GENVCE, 2023).

Table 1. Wheat genotypes grown under Mediterranean conditions, showing Genotype Age, Growth Habit, and Year of Release. The dashed line represents pre and post Green Revolution release dates.

Genotype Age	Genotype	Growth Habit	Year of Release
Modern	Nogal	Winter	2006
Modern	Artur Nick	Spring	2002
Modern	Berdún	Winter	1998
Modern	Califa Sur	Spring	1999
Old	Marius*	Winter	1976
Old	Yécora	Spring	1972
Old	Ablaca	Spring	1982
Old	Pane 247	Spring	1955
Landrace	Aragón	Winter	<1940
Landrace	Chamorro	Winter	<1940
Landrace	Mocho Rojo	Spring	<1940
Landrace	Candeal de Vellisca	Spring	<1950

7. *Experimental design to analyze the effects of ozone on wheat*

Various methodologies have been developed to assess the sensitivity of plant species to air pollutants. These methodologies encompass experimental conditions ranging from natural to semi-natural and controlled environments, depending on the analysis objective. However, Open Top Chambers (OTCs) (Heagle et al., 1989) are the exposure facility most commonly used for studying O_3 effects on crops, including experimental studies performed with wheat (Pleijel, 2011; Hu et al., 2018; Mills et al., 2018a; Pandey et al., 2018; Fatima et al., 2019). They resemble small open-topped circular greenhouses and provide a controlled atmosphere to expose plants to programmed levels of O_3 while maintaining a semi-natural environment. The advantage of using OTCs is that they strike a balance between natural and controlled settings, which allows for reproducible experiments while closely mimicking environmental conditions. Parameters related to O_3 exposure, such as duration, timing, profile or concentration, are manipulated to understand the extent of O_3-induced damage and plant responses. Meanwhile, other factors such as fertilization regime, irrigation regime or inoculation of plant pathogens, can be manipulated to study their interaction with O_3.

8. *Carbon and nitrogen isotopic signatures to study O_3 stress*

Isotopic signatures refer to the ratio of stable or unstable isotopes of elements present in a studied sample. These isotopes differ in their number of neutrons, which in turn affects their atomic mass without altering their chemical properties. Due to their variations in mass, these isotopes separate because of their different kinetic behaviors. For example, in plants, as lighter isotopes require less energy for physical and chemical processes, the lighter isotope is usually preferred from the source material, while the heavier isotope is discriminated against.

Due to this mass difference, samples can be analyzed for isotopic composition, which is often expressed as the deviation (δ) from a standard reference material, using mass spectrometry:

$\delta = (R_{sample}/R_{standard}) - 1$ (unit: per thousand (‰)) (Farquhar, 1989a)

Some examples of isotopic signatures are carbon and nitrogen isotopes. $\delta^{13}C$ refers to the ratio of the stable isotope carbon-13 (^{13}C) to carbon-12 (^{12}C) in a sample, which exists in nature in a proportion of 1.1% to 98.9% respectively (Karhu, 1998). When under non-stressful situations, as the plant absorbs and assimilates CO_2 into the vegetative tissue, the heavier ^{13}C isotope is discriminated against, which results in more ^{12}C being found in the examined plant parts. This discrimination is driven by the ratio between the intracellular CO_2 partial pressure and the atmospheric CO_2 partial pressure, which is in turn driven by net photosynthesis and stomatal conductance, through which CO_2 demand for the photosynthetic machinery and stomatal opening can alter discrimination (Farquhar and Richards, 1984). This means that any stress that alters either process would alter the isotopic signature, providing information about carbon assimilation, (and indirectly) remobilization, and metabolism in plants (Farquhar et al., 1989b).

The same occurs for $\delta^{15}N$, which is the ratio of nitrogen-15 (^{15}N) to nitrogen-14 (^{14}N) in a sample in deviation from a standard reference material. These are found in the proportion of 99.6% for ^{14}N and 0.4% for ^{15}N in nature (Ostrom and Ostrom, 1998). For $\delta^{15}N$, the source of N needs to be considered because they can differ in their isotopic signature (Bateman and Kelly, 2007), and in their fractionation, as nitrate (NO_3^-) is assimilated by nitrate reductase, and ammonium (NH_4^+) uptake is driven by the glutamine synthetase–glutamate synthase (GS–GOGAT) pathway (Evans, 2001). Another factor that needs to be considered is the complexity of the N absorption, assimilation of inorganic to organic N and remobilization processes (Evans, 2001), as well as its metabolism in plants (Cui et al., 2020; Effah et al., 2022), as the heavier ^{15}N, making $\delta^{15}N$ a complex isotope to study as an indicator of stress.

Both isotopes have been amply used to measure stresses such as drought (Araus et al., 2013; Sanchez-Bragado et al., 2014b) or salinity (Yousfi et al., 2009). However, the analysis of isotopic signatures to study O_3 stress is less known (Jäggi et al., 2005; Cocozza et al., 2020), especially for wheat (Saurer, Fuhrer and Siegenthaler, 1991; Ma et al., 2022), and there is no information about Mediterranean soft wheat cultivars.

Usually gas exchange measurements realized in the field to study O_3 effects offer snapshots of plant physiology and are time-consuming. On the contrary, carbon and nitrogen isotopic signature can provide an integrated plant responses to the pollutant reflecting changes in overall ecophysiological traits, particularly photosynthetic carbon and N assimilation processes (Ma et al., 2022). Leaf isotopic signatures sampled around anthesis integrate the reaction of the plant to O_3 stress by that phenological stage, whereas grain samples taken after harvest provide a perspective on the entire life of the plant and the remobilization that occurred during grain filling (Sanchez-Bragado et al., 2014a; Brewster, Fenner and Hayes, 2024). Stable carbon and nitrogen isotope compositions in grains represent a better-integrated measure and less intrusive analysis of plant gas exchange and assimilation (Sanchez-Bragado et al., 2014a; Araus et al., 2021).

8.1 Carbon isotope and ozone

Stomatal closure is a common response to high O_3 levels for many species, as it prevents O_3 from entering the plant and affecting vegetative tissues, but this closure also limits other gases from entering, such as CO_2. This, in turn, decreases the intracellular over the atmospheric CO_2 concentration ratio (C_i/C_a), as well as the discrimination capacity of the plant against the heavier isotope ^{13}C, therefore affecting the $^{12}C/^{13}C$ ratio and usually (as response to the stomatal closure) increasing the $\delta^{13}C$ of the plant (Farquhar and Richards, 1984; Farquhar et al., 1989b; Jäggi et al., 2005). As this discrimination also happens during Rubisco carboxylation, where O_3 impairs rubisco activity, besides to inducing stomatal closure (Cocozza et al., 2020), $\delta^{13}C$ can help describe the balance between the stomatal conductance of the plant and its net photosynthetic assimilation (A) (Farquhar, 1989a; Tcherkez et al., 2011b; Serret et al., 2018; Ma et al., 2022). It can also help explain the reduction in photosynthetic carbon assimilation caused by stomatal closure (Cocozza et al., 2020), and suggest whether photosynthetic limitations are caused by CO_2 diffusion or carboxylation (Saurer, Fuhrer and Siegenthaler, 1991).

In Chapters 1 and 3, the carbon isotope composition has been used to explore the effects of O_3 in-depth, while the grain carbon isotope is proposed as an integrated indicator of chronic O_3 stress throughout the entire exposure period. New possibilities for the use of isotopic analyses for long-term monitoring of plant responses to O_3 pollution are proposed.

8.2 Nitrogen isotope and ozone

As mentioned before, N is the most important nutrient for plant growth, productivity, and grain quality, however, a great part volatilizes or is leached into the groundwater (Effah et al., 2022). During crop growth, the timing of N remobilization in plants varies depending on fertilizer levels, with lower N fertilization prompting earlier remobilization compared to higher levels (Aranjuelo et al., 2013). This process involves the transfer of N from vegetative tissues like stems and leaves to reproductive organs such as panicles and grains, crucial for grain yield and quality (Sanchez-Bragado et al., 2017; Broberg et al., 2020). Moreover, in Effah et al., (2022), the most significant differences in N excess due to different fertilization levels were seen during the grain-filling stage and maturity, noting the importance of N fertilization during grain-filling, the most susceptible stage for the plant in terms of O_3 exposure.

N uptake and mobilization in plants involve several processes, including absorption from the soil, assimilation into organic compounds, redistribution within the plant, and remobilization to developing tissues (Cui et al., 2020). N is initially taken up by plant roots in the form of nitrate (NO_3^-) or ammonium (NH_4^+). This uptake is facilitated by specific transport proteins in the root cells, and usually more N uptake occurs pre-anthesis than after (Perez, 1989). Once inside the plant, N undergoes assimilation, where it is incorporated into amino acids, proteins, enzymes, and other N-containing compounds.

N isotope ratios, particularly $\delta^{15}N$, can provide insights into N uptake and mobilization processes in plants (Sanchez-Bragado et al., 2017). When N is absorbed by the roots, it undergoes fractionation, with the lighter isotope (^{14}N) being preferentially taken up compared to the heavier isotope (^{15}N). Therefore, N isotopes can be used to trace the movement of N within the plant and between different plant tissues. Changes in $\delta^{15}N$ values can indicate shifts in N allocation and redistribution caused by various factors, including environmental stresses like O_3 pollution (Tcherkez, 2011a; Yousfi et al., 2012; Cui et al., 2020).

O_3 exposure affects N metabolism in plants, which can in turn influence δ15N values. For example, oxidative stress accelerates N metabolism, as significant alterations were seen in the N isotope ratios of non-protein N, followed by differences in soluble protein and structural protein (Ma et al., 2022). O_3 stress can lead to alterations in N absorption (Broberg et al., 2017), alter the N remobilization efficiency between anthesis and harvest (Brewster et al., 2024), and increase the demand for N-containing compounds involved in antioxidant defense mechanisms and repair (Peng et al., 2019). O_3 also increases the N concentration in the wheat grain, but due to the induced reduction in grain size, which ultimately causes a decrease in the protein yield proportional to the yield loss due to the pollutant (Broberg et al., 2020).

N isotope ratios have been considered in Chapter 1, and to a larger extent in Chapter 3 to analyze nutrient assimilation and remobilization as affected by the O_3 x N interaction.

9. Ozone interactions with other factors

Conducting experimental studies involving the interaction of O_3 with other factors, such as water stress, N fertilization, and the presence of pathogens, can be complicated, both due to the experimental management of additional factors together with the O3-exposure. Therefore, few studies delve into these interactions, but advancing this knowledge is crucial because it approaches the real-field situation. Two factors of the utmost interest in this field are N fertilization and common crop diseases, as they are crucial for crop production and its economic viability.

9.1 O_3 x N Interaction: N-modulation of the O_3-response

N fertilization use has increased significantly in the last 40 years to boost productivity. However, as only 30-50% of the N applied is taken by the plant (Broberg et al., 2017;

Hawkesford and Riche, 2020), a significant amount is leached into the environment, causing environmental and economic losses. This is why it is crucial to analyze the impact of factors that may reduce Nitrogen Use Efficiency, such as tropospheric ozone.

As O_3 can decrease root growth (Calvete-Sogo et al., 2016), altering the shoot and root C/N rate (Sanz et al., 2005; Ghosh, Agrawal and Agrawal, 2020), it can decrease the N assimilation of the plant. Similarly, O_3 decreases the nitrogen use efficiency (NUE) of the plant by decreasing remobilization to the grains, which has been seen in wheat (Broberg et al., 2017; Brewster, Fenner and Hayes, 2024), as well as in rice and maize (Peng et al., 2019, 2020), which means that the N supplied would remain more in the vegetative part of the plant than in the reproductive structures, something not optimal for production (Peng et al., 2020).

Nonetheless, the O3 x N is a very complex interaction that requires further study (Pilbeam, 2018), as the effectiveness of N fertilization in mitigating O_3 damage can depend on various factors, including the dose of O_3 and N, as well as the specific physiological processes affected by O_3 stress (Calvete-Sogo et al., 2016). Studies that have delved into this interaction have been inconclusive, and have shown that N can have an ameliorative effect on O_3 (Pandey et al., 2018; Peng et al., 2019) due to an increased resource pool for growth, defense and repair (Manderscheid et al., 1992; Pandey et al., 2018), though not under the highest O_3 levels (Sanz et al., 2011; Broberg et al., 2017). However other studies have also shown that additional N may favor the effect of O_3 (Calvete-Sogo et al., 2016).

Chapters 2 and 3 delve into experimental assays that analyze the O_3 x N interaction and its effects on a modern Mediterranean variety that is commonly used in commercial fields of the Iberian Peninsula despite its sensitivity to the pollutant.

9.2 O_3 x pathogen Interaction: O_3 effect on common fungal infection

There is little experimental work involving O_3-interactive experiments with pathogens. Pathogens tend to require specific conditions to grow, which is why most assays are developed in controlled chambers, in laboratories, where plants are inoculated during the best growth period for the pathogen (Tiedemann et al., 1990). This has not only led to scarce experiments, but as they differ from real-field conditions, they are hard to extrapolate to field measurements.

Fungal infections are among the most important diseases affecting wheat. The high oxidant capacity of O_3 has been seen to limit fungal spore formation and growth (Tzortzakis et al., 2008), and acute post-harvest ozonation of the grains has shown effectiveness against fungal infections and mycotoxin production (Heagle and Key, 1973) without affecting the quality of the wheat grains. This has also been seen in other crops like barley (Piacentini, Savi and Scussel, 2017) or rice (Savi et al., 2020).

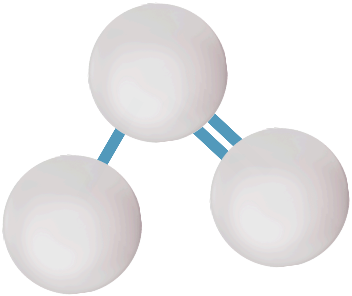
However, the effects of chronic tropospheric O_3 levels on fungal diseases throughout the crop-growing cycle have been studied less and are therefore less understood (Tiedemann and Firsching, 2000). Studies have indicated mixed results, with some showing increased fungal diseases under elevated O_3 levels (Mina, Fuloria and Aggarwal, 2016) while others show reductions (Tiedemann and Firsching, 2000; Olbrich et al., 2010), although most of them considered short but acute O_3 exposures which differ from field conditions. Moreover, if the combination of O_3 and N fertilization is considered, results point to inconclusive results. Some studies have shown that N fertilization can either reduce or facilitate fungal infection under elevated O3, either by boosting the plant defense or by making the plant softer for fungal infection (Almacellas, 2010; Devadas et al., 2014; Liu et al., 2017).

Yellow rust, caused by the fungal pathogen *Puccinia striiformis*, is a common plant disease that can lead to severe wheat yield losses of 24-39%, and even plant death, particularly in Central Asia (Hao et al., 2016; Sharma et al., 2017; Bouvet et al., 2022), and Europe, where it is one of the most significant diseases affecting wheat (Willocquet et al., 2021). Due to its genetic variation, and because it can disperse for hundreds of kilometers with wind, and can germinate at low temperatures, it is highly infectious (Hovmøller, Justesen and Brown, 2002; Hovmøller et al., 2016).

Moreover, even though Spanish wheat has shown great genetic variability in its resistance against yellow rust (Martínez-Moreno et al., 2022), and as the Mediterranean climate was too hot for fungal infections up until the early 2000s, it was not a great concern Central Iberian Peninsula. However, the new fungal races have adapted to germinate in the warmer conditions of the Mediterranean, allowing them to spread further than the older ones in the region, having important effects on production (Aparicio et al., 2014; Vergara-Diaz et al., 2015).

Due to the conflicting information on the O_3 x pathogen, and its important effect on wheat worldwide, studying the impact of the tropospheric O_3 levels on common wheat diseases like rust is crucial. Chapter 4 describes the study performed on the effect of O_3 on yellow rust infection in wheat.

OBJECTIVES



Objectives

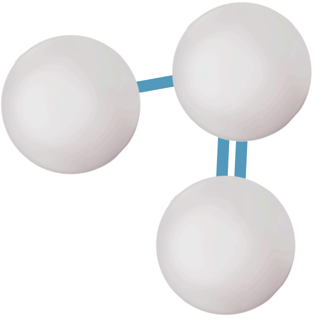
General objective:

This research aims to analyze how O_3 pollution interacts with other factors such as wheat genotype type, fertilization regimen and pathogens to maintain sustainable wheat production in a changing environment.

Main objectives:

- To improve the knowledge of the O_3 sensitivity of Mediterranean wheat genotypes, considering cultivars and landraces (Chapter 1)
- To gain better insight into the plant mechanisms acting against O_3 pollution through indicators like the C and N isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) which integrate the stress throughout the complete O_3 exposure (Chapters 1 and 3)
- To analyze the effect of pre-anthesis O_3-exposure on Mediterranean wheat in contrast with the most frequent assays which use post-anthesis exposure for Central European wheat (Chapters 2 and 3)
- To analyze how N-fertilization modulates the O_3-response of a modern O_3-sensitive Mediterranean cultivar and how the pollutant affects fertilizer efficiency (Chapters 2 and 3)
- To improve the understanding of the O_3-fungal disease interaction on wheat while maintaining close-to-field conditions and considering the modulation of N-fertilization on the response (Chapter 4).
- O_3 response quantification based on exposure-based (AOT40) and dose–based (POD6) indexes, considering different N fertilization regimes (Chapters 2 and 3)
- Improve regional and continental O_3 risk assessments by considering cultivars from the agroclimatic Mediterranean region; as well as N-management in the O_3 response (Chapters 2, 3 and 4).

METHODOLOGICAL APPROACH



Methodological Approach

The experimental work of this study is presented in four different chapters, and was carried out in the CIEMAT Open Top Chamber (OTC) experimental facility of the Ecotoxicology of Environmental Pollution Unit of the Environmental Department, currently the only OTC facility in Spain. It is located in the La Higueruela/MNCN-CSIC Agricultural Research Station in Santa Olalla, Toledo (40°3'N, 4°26'W) and is dedicated to agronomic research on rainfed crops. The location characterizes the continental Mediterranean climate of the Iberian Peninsula.

The experimental setup comprised 12 NCLAN-type chambers (Heagle et al., 1989) arranged randomly in three blocks, each exposed to four different O_3 treatments. Additionally, a chamber-less plot (AA) has been added to each block to evaluate the chamber effects (Figure 2a). These chambers, standing at a height and diameter of 3 meters, feature an open conic top (frustum) to prevent wind inflow.

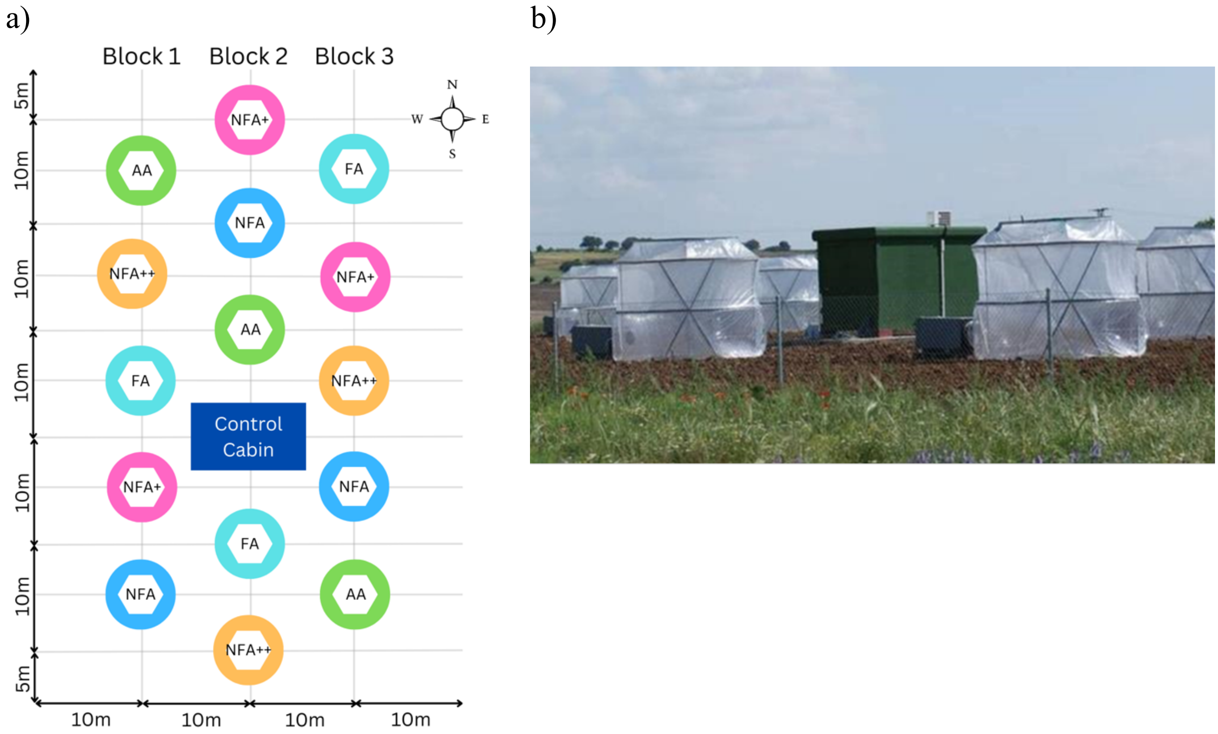


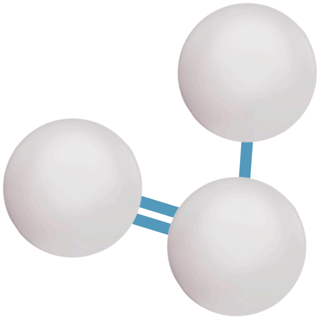
Figure 2. a) Distribution of NCLAN-type chambers in The CIEMAT OTC field site located in *La Higueruela*/MNCN-CSIC Agricultural Research Station (Toledo), with chamberless plots (AA), OTC-chambers with filtered air (FA), with non-filtered air (NFA), and with added O_3 (NFA+ and NFA++); b) Photograph of the Open Top Chambers and the control cabin.

The O_3 levels employed in the OTCs include 3-replicated varying O_3 concentrations: FA (Filtered Air), NFA (Non-Filtered Air; as experienced in the farm), NFA+ (Non-Filtered Air + 20 nL L^{-1} of added O3), and NFA++ (Non-Filtered Air + 40 nL L^{-1} of added O_3). O_3 was added to the NFA+ and NFA++ chambers from a control cabin (Figure 1b), that processed pure O_2 via an O_3 generator system for eight hours per day (6:00 to 14:00 GMT), seven days a week. Monitoring equipment within the OTCs recorded O_3 (ML® 9810B, Teledyne Monitor Labs, Englewood, CO, USA) and nitrogen oxide (NO_2 and NO; ML®9841, Teledyne Monitor Labs, Englewood, CO, USA) levels at canopy height using a timesharing system that sampled every plot for 10 minutes. All machinery and monitors were calibrated following company recommendations at the beginning of each experiment. Meanwhile, measurements of air relative humidity, temperature (HOBO® Pro v2, Onset, Bourne, MA, USA), and photosynthetic active radiation (PAR; OSO-SUN HOBO®, Onset, Bourne, MA, USA) were conducted within six OTCs at canopy height.

Most previous wheat assays on the pollutant effect of O_3 for wheat plants were fumigated from anthesis (Pleijel et al., 2007; Piikki et al., 2008; Effah et al., 2022; Broberg et al., 2023; Brewster, Fenner and Hayes, 2024), since in the growing conditions of Central Europe, this period was determined as the most sensitive to O_3 (Pleijel et al., 1998). However, the situation might be different when wheat is grown under Mediterranean conditions.

In the Mediterranean basin, due to its warm climate and high solar radiation (Proietti et al., 2016) O_3 levels during the pre-anthesis period are high compared to the rest of Europe, continuously surpassing the EU threshold levels (EEA, 2022a). Coupled with the fact that the pre-anthesis period also coincides with the time when the plant does not experience water stress, conditions are favorable for O_3 absorption. Meanwhile, during the post-anthesis period, temperatures are higher and water deficit decreases O_3 absorption. The O3-exposure assays described in Chapters 2-4 consider O_3 exposure from tillering until harvest to mimic the field conditions of O_3 pollution in the area.

REPORT OF THE THESIS DIRECTORS



REPORT OF THE THESIS DIRECTORS

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Dr. Victoria Bermejo-Bermejo and Dr. José Luis Araus, directors of the thesis entitled ***"The effects of ozone on Mediterranean wheat in a changing environment",*** developed by the doctoral student Melissa Carolina Chang Espino, report below on the impact factor and the participation of the doctoral student in the published articles that have been used as the chapters of this doctoral thesis.

Chapter 1.
This chapter encompasses the published scientific article "*The Effect of Increased Ozone Levels on the Stable Carbon and Nitrogen Isotopic Signature of Wheat Cultivars and Landraces*" published in Atmosphere in 2021 with an impact factor of 2.9 and a CiteScore of 4.1. To this date, this article has been cited 9 times (Researchgate, reviewed on May 16th 2024). This article explored the effect of ozone on eight wheat cultivars and four landraces through gas exchange measurements, yield measurements and isotopic signature. It reinforced the effectiveness of the carbon isotopic signature in grain as an indicator of abiotic stress, in this case due to ozone, and noted its ability to show differences that were not detected by gas exchange measurements, such as the behavior or pre and post-green revolution cultivars. Melissa Chang performed the laboratory analysis of the grain samples for isotopic signature, processed and analyzed the data, and conceptualized and led the writing of the manuscript.

Chapter 2.
This chapter is titled "*Nitrogen modulates the ozone response of Mediterranean wheat: considerations for O3-risk assessments".* This article delved into the interactive effect of ozone and nitrogen fertilization on growth and production measurements, noting that the beneficial effects of nitrogen on the plant can be counteracted when exposed to high levels of the pollutant. To further delve into this offset, the critical levels of the most susceptible parameters measured (including grain yield and protein yield) were calculated following the methodology of the Air Convention, upon which the higher nitrogen content was found to cause lower critical levels, indicating a higher susceptibility to the pollutant. This is then discussed in the article due to its implication for O3-risk assessments. Melissa Chang participated in field measurements, processed and analyzed the information and led the writing of the manuscript. This manuscript has been submitted to Science of the Total Environment, which has an impact factor of 9.8 and a CiteScore of 16.8, and being in the first quartile, Q1, in its research fields

Chapter 3.
This chapter is titled "*Assessing the nitrogen modulation of response of wheat to ozone through carbon and nitrogen isotopic signatures" and* analyzes the mechanisms through which additional nitrogen fertilization may aid wheat plants in their response to ozone fumigation. It was found that while additional nitrogen did aid in counteracting the effects of the pollutant through a remobilization of resources towards repair and possibly defense, this reaction could not be maintained after prolonged exposure. The article suggests considering these mechanisms in breeding programs due to the implications it can have for fertilizer use. Melissa Chang collected the samples for isotopic and microscopy analysis and performed said analyses at the University of Barcelona Centres Científics i Tecnològics. She then processed and analyzed the information and conceptualized and led the writing of the manuscript. This manuscript has been prepared for submission to Environmental Pollution, which has an impact factor of 8.9 and CiteScore of 14.9, and is in the first quartile, Q1, in its research fields.

Chapter 4.

This chapter is titled *"Current ambient ozone levels mitigate the effect of Puccinia striiformis on wheat: Is Mediterranean wheat ready for pre-industrial background ozone levels?" and* was published in Science of the Total Environment in 2023, having an impact factor of 9.8 and a CiteScore of 16.8, and being in the first quartile, Q1, in its research fields. This article explored the effect ozone fumigation can have on a pathogen that is very detrimental to wheat health. The results indicated that current levels of ozone can help mitigate the impact of the pathogen on the plant, and suggests that returning to pre-industrial ozone levels, as is suggested in policy-making, could have detrimental effects on the plant. It does state that further increases in ozone levels should not be allowed, as they too cause harmful effects on wheat. Melissa Chang participated in the data processing and analysis, as well as in the conceptualization of the manuscript, and led writing of the article.

It should be noted that none of the coauthors of these articles have used this information for their doctoral thesis, implicitly or explicitly.

Signed for the corresponding purposes,

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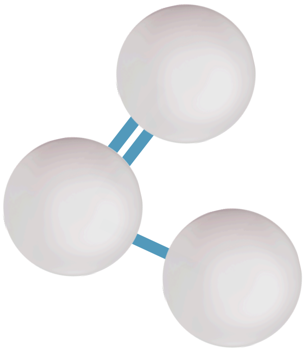
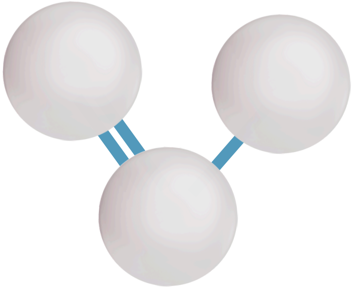
Dr. Victoria Bermejo Bermejo

Dr. Jose Luis Araus Ortega

RESULTS



Chapter 1



Resumen del Capítulo 1

Varios estudios han resaltado los efectos negativos del ozono en el crecimiento y la productividad del trigo. Estos efectos son medidos a través de cambios en el intercambio gaseoso y metabolismo fotosintético del carbono y nitrógeno, los cuales son difíciles de evaluar y toman mucho tiempo, por lo cual solo se miden esporádicamente a lo largo del ciclo de la planta. Las medidas de isótopos estables en los granos ayudan a integrar los efectos de la exposición crónica al ozono durante la vida de la planta.

Este estudio se centró en la medida que la conductancia estomática y la productividad del trigo mediterráneo se relacionan con las señales isotópicas de carbono y nitrógeno bajo exposición crónica al ozono. Se diseñó un experimento en cámaras de techo descubierto para analizar los efectos del contaminante en 12 genotipos de trigo españoles, incluyendo cultivares modernos, antiguos y variedades autóctonas. Se consideraron cuatro tratamientos de ozono. Se realizaron mediciones de conductancia estomática (g_s) durante antesis, y se tomaron parámetros de rendimiento y contenido de nitrógeno en la madurez, al igual que la composición isotópica de carbono ($\delta^{13}C$) y nitrógeno ($\delta^{15}N$), que fue medida en grano.

Los cultivos modernos y antiguos respondieron de manera similar al ozono, ambos fueron sensibles al contaminante en cuanto a los parámetros de rendimiento y g_s, mientras que las variedades autóctonas fueron más tolerantes. El $\delta^{13}C$ en grano tuvo una fuerte correlación negativa con el rendimiento del grano y con la conductancia estomática, particularmente en las variedades más modernas, y esta correlación se intensificó bajo las concentraciones de ozono más altas, mostrando su capacidad para integrar el estrés por ozono a lo largo del ciclo del trigo. Mientras tanto, una mayor concentración de nitrógeno en los granos, junto con granos más pequeños, llevó a una disminución general del rendimiento de nitrógeno en el grano bajo concentraciones más altas de ozono. Este efecto en la concentración de nitrógeno en el grano difirió entre los genotipos criados en diferentes épocas, siguiendo su respectiva sensibilidad al ozono. $\delta^{15}N$ mostró un posible efecto indirecto del ozono en la redistribución de nitrógeno, particularmente bajo la concentración de ozono más alta. Las correlaciones de $\delta^{15}N$ y $\delta^{13}C$ con los efectos usuales del ozono en la planta proponen su potencial como indicadores de exposición crónica al ozono.

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Article

The Effect of Increased Ozone Levels on the Stable Carbon and Nitrogen Isotopic Signature of Wheat Cultivars and Landraces

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Abstract: Several studies have highlighted the negative effects of ozone (O_3) on wheat development and productivity. The negative effects of O_3 are mediated by changes in photosynthetic carbon and nitrogen metabolism, which are difficult and time-consuming to assess and are thus only measured sporadically throughout the plant cycle. Stable isotope measurements in grains can help integrate the effects of chronic O_3 exposure over the lifespan of the plant. This particular study focuses on the extent to which the stomatal conductance and productivity of Mediterranean wheat are related to carbon and nitrogen isotopic signatures under chronic O_3 exposure. An open top chamber experiment was designed to analyse the effects of the pollutant on 12 Spanish wheat genotypes, which included modern cultivars, old cultivars and landraces. Four O_3 treatments were considered. Stomatal conductance (g_s) measurements were carried out during anthesis, and yield and nitrogen content parameters were taken at maturity, along with the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic composition measured in grains. Modern and old cultivars responded similarly to O_3 and were sensitive to the pollutant regarding yield parameters and g_s, while landraces were more O_3-tolerant. Grain $\delta^{13}C$ had a strong negative correlation with grain yield and stomatal conductance across genotypes and O_3 conditions, and increased under higher O_3 concentrations, showing its capacity to integrate O_3 stress throughout the wheat cycle. Meanwhile, a higher nitrogen concentration in grains, coupled with smaller grains, led to an overall decreased grain nitrogen yield under higher O_3 concentrations. This nitrogen concentration effect within the grain differed among genotypes bred at different ages, following their respective O_3-sensitivity. $\delta^{15}N$ showed a possible indirect effect of O_3 on nitrogen redistribution, particularly under the highest O_3 concentration. The correlations of $\delta^{15}N$ and $\delta^{13}C$ to the usual effects of ozone on the plant suggest their potential as indicators of chronic ozone exposure.

Keywords: isotopes; Mediterranean; genotypes; sensitivity; tolerance; breeding

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Citation: 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. https://doi.org/10.3390/atmos12070883

Academic Editors: Alfredo Rocha and Ana Isabel Miranda

Received: 24 May 2021
Accepted: 1 July 2021
Published: 7 July 2021

1. Introduction

Even though tropospheric ozone (O_3) is both a naturally occurring and anthropogenic gas, current global levels are mainly caused by photochemical reactions, due to precursors from industrial and urban emissions [1,2]. Local and regional air masses can transport precursors throughout long distances before leading to ozone production. When high ozone precursors and O_3-favorable meteorological conditions for atmospheric chemistry coincide, pollutant formation increases, causing chronic and extensive O_3-pollution problems in rural and forested areas, far away from the source of emissions [3]. This, along with the complexity of atmospheric O_3 photochemistry, coupled with high NO_x levels, explains the difficulty in maintaining O_3 levels under safe values for crops and ecosystems [2,4]. In Europe, several EU Directives have prompted the reduction of O_3-precursor emissions

(2008/50/EU and 2016/2284/EU), which caused the high O_3 peaks that were of maximum concern during the eighties. Nonetheless, background O_3 levels still do not present a clear decreasing trend [5,6], and safe levels are not expected to be reached by the year 2030 [2].

Regarding chronically-high O_3 levels, the Mediterranean basin is the most affected area in Europe [5,7]. Its stable summer conditions, high temperatures and high solar radiation favour photochemical O_3 formation, which causes surface levels during spring and summer that surpass the threshold targets of the EU Directives (2008/50/EU and 2016/2284/EU) and the critical levels of the United Nations Air Convention for plant protection [5,8,9].

Along with the pollution problem, the expected impact of Climate Change is also recognized as a major concern in the Mediterranean area, a transition zone located between the arid North African climate and the temperate central European climate, which is prone to suffer substantial climatic changes [10,11]. Temperature is expected to increase by up to 5 °C by 2100 and, coupled with 25% less precipitation in the Mediterranean basin, these changes are expected to greatly harm crop yields [11] and worsen the O_3 pollution problem [6,12].

O_3 affects the plant by entering through the leaf stomata and producing reactive oxygen species (ROS), which react with the cell membranes and walls [13]. This early O_3-induced oxidative burst drives cellular chain effects that alter physiological processes such as carbon assimilation by affecting stomatal conductance (g_s), inducing partial closure or less efficient stomatal control [14], reducing foliar pigment content [15], affecting the electron transport chain [16], reducing the efficiency of Calvin cycle enzymes such as Rubisco [17], reducing photosynthates made available for redistribution [18] and increasing respiration rates [19]. As a final result, yield and grain quality are reduced [20,21]. Over 20% of all crop production in Europe is currently considered at risk of damage by O_3 pollution, as O_3-sensitive and moderately sensitive crops are grown in around 16% and 7%, respectively, of the agricultural land in Europe [21,22].

Wheat, one of the most important staple crops on a global scale, is also one of the most O_3-sensitive crops [21,23,24]. Studies from the last decades have shown that this pollutant can induce important wheat yield losses and reduce grain quality (i.e., starch content, seed size), while also affecting N use efficiency and protein yield [25]. O_3-induced wheat yield reduction is important both for its economic impact and its effect on global food security. In terms of wheat yield, modelling exercises have shown that O_3 pollution can account for an average global reduction of 7.1% [26], ranging between 4.1–12.1% for Europe [19]. Moreover, data worsens when coupled with other constraints such as climate change and pressures to maintain natural resources [27].

Breeding for higher grain yield (GY) in wheat and other herbaceous crops has been coupled frequently with an enhancement of stomatal conductance (g_s), which favours higher photosynthetic rates and cools down plants [28–31]. However, the higher g_s of modern wheat cultivars can also potentially increase their O_3-sensitivity, as they absorb the pollutant more readily. This has led to a clear trend in the genotypic variation regarding how wheat cultivars respond to O_3. In general, modern cultivars are more O_3-sensitive than older ones [28,32]. This also applies to wheat bred for Mediterranean conditions, although their adaptation to water-limited environments might have also made them more O_3-tolerant (Bermejo-Bermejo et al., in press).

Ozone-induced effects in plants are usually studied through discrete gas exchange measurements during the crop season [28,33,34]. However, as these measures are very time consuming, they are usually only performed a small number of times throughout the growth cycle [26,35,36]. Usually, what is observed in these instantaneous measurements is extrapolated to the entire cycle, even though O_3 responses can differ depending on the plant growth stage, with grain filling appearing to be the most susceptible stage [37,38]. A better-integrated response of plant gas exchange and assimilation can be analysed through the study of the stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope compositions [39–42], particularly when analysed in grain after harvest.

Carbon fractionation is driven by the discrimination of stable carbon isotopes, favouring the uptake and further assimilation from the surrounding air of the lighter isotope, ^{12}C, against ^{13}C [39,43]. It should, however, be noted that frequently, rather than expressing the isotope signature as a composition (denoted as δ) which is the deviation of the $^{13}C/^{12}C$ molar abundance ratio between the analysed substance and a standard, values are transformed to discrimination (denoted as $\Delta^{13}C$) of the ^{13}C from the atmosphere surrounding the plants [43]. From a physiological point of view, this discrimination depends on the ratio of intercellular to atmospheric CO_2 concentration (Ci/Ca) [39,43]. If this ratio is integrated over time, it shows the balance between the g_s and the net photosynthetic assimilation of the plant during the considered period [43–45]. Thus, $\delta^{13}C$ discrimination occurs due to the diffusion of CO_2 through the stomata and by Rubisco carboxylation [43,46]; therefore, if the stomata close, for example as a response to water stress, g_s decreases, restricting the CO_2 supply to the carboxylation sites, and overall decreasing the Ci/Ca and consequently $\Delta^{13}C$, while increasing $\delta^{13}C$ [39,40,43].

Meanwhile, $\delta^{15}N$ values are driven by discrimination of ^{14}N against ^{15}N, which depends largely on the capacity of the plant to assimilate the nitrogen available in the soil. Thus, $\delta^{15}N$ in the plant may reflect $\delta^{15}N$ of the nitrogen available in the soil, as well as metabolism, including recycling, transport through the plant, including the grains and even volatilization during senescence [38,47,48].

In that sense, changes in g_s may affect the transpiration stream and, subsequently, nitrogen uptake from the soil, its transport to the leaves and volatilization, thus affecting $\delta^{15}N$ [49]. Similarly, retranslocation of nitrogen through the plant via phloem, as well as photorespiration, can cause $\delta^{15}N$ to increase in the upper organs, whereas roots tend to have lower $\delta^{15}N$ than the stems and leaves, and these have less $\delta^{15}N$ than the ears [47]. Nitrogen uptake after anthesis is quite low [50], therefore, as O_3-effect is more predominant post-anthesis [37], N redistribution could be expected to be affected more than uptake by the pollutant.

In the present study, the effects of O_3 exposure on the stable carbon and nitrogen isotope composition and its relation to yield parameters, as well as g_s, were studied, considering a set of Mediterranean bread wheat genotypes that included landraces, along with old and modern commercial varieties, of which all except for one were developed after the so-called Green Revolution, which took place in the middle of the last century. The objective of the study was to assess the performance of grain $\delta^{13}C$ and $\delta^{15}N$ as an integrated tool to monitor chronic O_3 effects on C assimilation and N metabolism in Mediterranean wheat genotypes. Both $\delta^{13}C$ and $\delta^{15}N$ have been shown to perform as useful indicators in wheat under other types of stresses, such as water-stress and salinity [39,42,51].

The starting hypotheses were that higher O_3-stress will decrease g_s, increasing $\delta^{13}C$ (or lowering carbon isotope discrimination, $\Delta^{13}C$) particularly on the more O_3-sensitive genotypes, in comparison to the tolerant genotypes. We postulate that this would cause a negative correlation between $\delta^{13}C$ and yield parameters or g_s due to the effect of O_3, differing among wheat genotypes developed at different times. Even though the mechanisms explaining $\delta^{15}N$ are less established than those of $\delta^{13}C$, since N absorption, metabolism and redistribution affect the nitrogen isotope signature, continuous O_3-exposure is also expected to affect $\delta^{15}N$ of mature grains.

2. Materials and Methods

2.1. Field Site

The experiment was developed in the CIEMAT Open Top Chambers (OTCs) facility located at "La Higueruela" MNCN/CSIC Research Farm (Santa Olalla, Toledo) in central Spain (450 m.a.s.l.; 40°3′ N, 4°26′ W) (Figure 1), which was specifically designed to study the effects of increasing O_3 levels on crop plants. According to the Köppen–Geiger climate classification, this area experiences a hot-summer Mediterranean Climate (Csa) [52] and is used for rainfed cereals, characteristic of the agriculture of the central Iberian Peninsula.

Figure 1. Location of the CIEMAT OTC facility at the "La Higueruela" MNCN/CSIC Research Farm (Santa Olalla municipality, Toledo, Spain).

2.2. *Open Top Chamber Facility*

The experiment employed fifteen NCLAN-type OTCs, which are circular-like greenhouses (3 m high × 3 m diameter) that are open at the top through a conic structure (*frustrum*) to prevent wind from entering the chambers [53].

The experimental design of the facility included a complete randomized 3-block design with four O_3 treatments replicated three times: thus, 3 OTCs per O_3 treatment: Charcoal Filtered Air (FA), Non-filtered air (NFA) to reproduce ambient levels, Non-filtered air + 20 nL L^{-1} of O_3 (NFA+) and Non-filtered air + 40 nL L^{-1} of O_3 (NFA++); where the last two reproduce O_3 levels that are expected along this decade, as well as three chamberless plots (ambient air—AA) to check for chamber effect. More detailed information on the OTC facility can be found in Calvete-Sogo et al., 2016 [54].

2.3. *Instrumentation OTC Facility*

An O_3 generator (A2Z Ozone Systems Inc., Louisville, KY, USA) system, located in a control cabin and fed with pure O_2, was used to apply O_3 to the NFA+ and NFA++ OTCs for 8 h day^{-1} (6:00 to 14:00 GTM), seven days a week. Air pollution was monitored continuously above the canopy inside each of the OTCs and AA plots by employing a timesharing system, with a sampling period of 10 min per plot. The air pollutants measured were: Ozone (ML® 9810B, Teledyne Monitor Labs, Englewood, CO, USA) and nitrogen oxides (NO_2 and NO; ML®9841, Teledyne Monitor Labs, Englewood, CO, USA). Monitors were calibrated at the beginning of the experiment following a standard protocol.

Meteorological parameters were also monitored within six of the OTCs and the three AA plots: air relative humidity (RH), temperature (HOBO® Pro v2, Onset, Bourne, MA, USA) and photosynthetic active radiation (PAR; OSO-SUN HOBO®, Onset, Bourne, MA, USA).

2.4. *Ozone Exposure Indices*

Several ozone exposure indices were calculated to describe ozone exposure during the fumigation period: the accumulated AOT40 index was calculated as the sum of exceedances of O_3-hourly mean values over 40 nL L^{-1} during daylight hours through the O_3-exposure period [55]. In addition, 24 h-mean, 7 h mean and maximum hourly O_3 values were also calculated.

2.5. Plant Material

Twelve Spanish wheat genotypes were selected for the assay: 4 modern commercial varieties (CVs), 4 old CVs and 4 landraces (Table 1). Pane 247 and the landraces were released pre-green revolution. The Plant Breeding Unit of the Agronomic Engineer School of Madrid (UPM) provided the seeds which were germinated in a plant nursery greenhouse. Seedlings were transported to the OTC field for transplanting into pots (18 cm^3) using a mixture of turf (60%), perlite (20%) and vermiculite (20%) on January 26–28 of the year 2015. Four plants per pot were considered as well as four pots per cultivar and OTC (totalling 12 pots per CVs and O_3 treatment). Pots were fertilized following the commercial management of wheat fields in the area, with 120 kg N ha^{-1} applied in two doses, on April 14th and 30th. Regarding irrigation, plants were watered manually based on the plant needs at the time (e.g., climatic conditions) to maintain water saturation at field capacity, approximately every two days, until the beginning of grain hardening towards the end of May, at which point watering stopped to allow for grain maturity.

Table 1. Wheat genotype grown under Mediterranean conditions, Genotype Age, Harvest Date, Growth Habit, Year of Release and References. The dashed line represents pre and post green revolution release dates.

Genotype Age	Genotype	Harvest Date	Growth Habit	Year of Release	References
Modern	Nogal	June 9	Winter	2006	Grupo para la Evaluación de Nuevas Variedades de Cultivos Extensivos en España (GENVCE) database [a]
Modern	Arthur Nick	June 3	Spring	2002	
Modern	Berdún	June 10	Winter	1998	
Modern	Califa Sur	June 3	Spring	1999	
Old	Marius *	June 10	Winter	1976	Genetic Resources Information and Analytical System (GRIS) database [b]
Old	Yécora	June 9	Spring	1972	
Old	Ablaca	June 18	Spring	1982	
Old	Pane 247	June 17	Spring	1955	
Landrace	Aragón	June 18	Winter	<1940	Spanish National Plant Genetic Resources Centre (CRF) database [c]
Landrace	Chamorro	June 23	Winter	<1940	
Landrace	Mocho Rojo	June 17	Spring	<1940	
Landrace	Candeal de Vellisca	June 23	Spring	<1950	

[a] GENVCE. Fichas archivos. 2021. https://genvce.org/productos/fichas/ (accessed on 4 July 2021). [b] GRIS. Genetic Resources Information System for Wheat and Triticale. 2021. http://wheatpedigree.net (accessed on 4 July 2021). [c] CRF. National Plant Genetic Resources Centre (CRF) Databases. 2021. http://wwwsp.inia.es/en-us/Investigacion/centros/crf/databases/Paginas/inicioDatabases.aspx (accessed on 4 July 2021). * Marius is a reference CV for Central Spain.

Plant O_3 exposure began on April 14th, 77 days after transplant to pot (DaT), when plants were close to the anthesis stage. Fumigation lasted for 51–71 days, depending on the CV, until plants reached full grain maturity and were harvested.

2.6. Physiological and Yield Parameters

Stomatal conductance (g_s) measurements were performed using a portable gas exchange system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA) between 9:00–13:00 h GMT from May 4–14 (depending on the cultivar) after 20–30 days of exposure to the different O_3-treatments and, therefore, around 100 DaT. Two plants per OTC for two blocks were randomly selected for the measurements, with one flag leaf per plant. The standardized conditions during gas exchange measurements for all the cultivars were: PAR 1000 μmol m^2 s^{-1}, RH 55% and a temperature range of 20–22 °C.

Yield, agronomical yield components and nitrogen content parameters were taken at grain maturity from June 3 to June 23 depending on each genotype (Table 1). After harvesting plants from the base, they were divided into straw and spikes, dried at 60 °C for 48 h and weighted to obtain the dry weight of the different plant parts (DW pot^{-1}), totalling 48 pots per CV and 12 pots per CV and O_3 treatment. Spikes were manually shelled to calculate the grain yield. Harvest Index (HI) was calculated as the ratio between grain and the total weight of the aerial plant (straw plus grain husks). Individual grain weight was then calculated and further grain nitrogen content analysed as described below. The number of grains per ear was calculated by dividing the total number of grains over the total number of ears for each pot.

2.7. Nitrogen Content and Stable Carbon and Nitrogen Composition

After measuring harvest parameters, the whole grain production from each genotype and OTC was pooled for isotopic analysis (15 samples per genotype and 3 samples per O_3 treatment and genotype). Pooled grain per OTC and genotype was ground and analysed for total nitrogen content and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ signature using an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan, Bremen, Germany) paired with an elemental analyser (Flash 1112 EA, ThermoFinnigan, Bremen, Germany). One-mg samples of the ground wheat, and reference materials, were weighed, placed in tin capsules, sealed and loaded onto an automatic sampler (ThermoFinnigan, Bremen, Germany) for EA-IRMS analysis. These measurements were carried out at the CCiT (Centres Científics i Tecnológics) at the University of Barcelona. Total nitrogen content was expressed as percentage (%) of dry matter, while the isotope $^{13}C/^{12}C$ ratio was expressed in δ notation [46]:

$$\delta^{13}C\ (‰) = (^{13}C/^{12}C)\text{sample}/(^{13}C/^{12}C)\text{standard} - 1$$

where "sample" refers to plant material and "standard" to the international secondary standards of known $^{13}C/^{12}C$ ratios (IAEA CH7 polyethylene foil, IAEA CH6 sucrose, and USGS 40 L-glutamic acid) calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB). Analyses were performed with an analytical precision (SD) of 0.10‰. The $^{15}N/^{14}N$ ratio is also expressed in δ notation ($\delta^{15}N$); however, using international secondary standards of known $^{15}N/^{14}N$ ratios (IAEA N1 and IAEA N2 ammonium sulphate and IAEA NO3 potassium nitrate) calibrated against N_2 in the air, with an analytical precision of 0.18‰. Nitrogen as a concentration (N%) was converted into Nitrogen Grain Yield (NGY) following the function: NGY = (N% × Grain Yield)/100.

2.8. Statistical Analysis

Effects of O_3 and genotype age-type factors on the different variables were analysed via two-way analysis of variance (ANOVA) with IBM® SPSS® Statistics 20 (Chicago, IL, USA) using block as a random factor. Significant differences among levels of the factors ($p < 0.05$) were assessed with the Tukey Honestly Significant Difference test (HSD). The two-way ANOVA data were previously checked to fulfil the ANOVA requirements of normality and homoscedasticity through Shapiro–Wilk and Levene's tests, respectively, using the SPSS Explore Descriptive Statistics function, with Plots. The same procedure was then followed with a one-way ANOVA analysis of each genotype, using the post-hoc Tukey's HSD test. The old CV Pane 247 was excluded when the grouped analysis of the modern and old CVs was done, due to its proximity to the landrace group and its behaviour as an outlier within these age-groups.

Pearson correlations between $\delta^{13}C$ and $\delta^{15}N$ and stomatal conductance and yield parameters were analysed. The significance of the regressions was assessed by p-value ($p < 0.05$) and the goodness of fit was judged by the determination coefficient (R^2).

Finally, a Principal Component Analysis, without rotation and with Keiser Normalization, was performed. The standardized data, excluding Pane 247, were checked for Kaiser–Meyer–Olkin Measure of Sampling Adequacy and Bartlett's Test of Sphericity for the usefulness of the factor analysis. Then, the components with an Eigenvalue higher than 1 were selected (leading to two components) and verified with the scree plot to check for a clean drop in the Percentage of Variance after PC2. Variables selected for each component presented a Component Matrix value higher than 0.5. This was repeated for the data in general, and then the data were divided by genotype group. The PCA images were acquired with R, version 3.6.3 (29 February 2020) to include the ellipses.

Table 2 summarizes the methodology and instrumentation for each experimental stage.

Table 2. Summary of the experimental conditions and instrumentation used in the experiment per stage.

Experimental Stage	Methodology	Instrumentation
Stage 1. Planting	January 26–28th: Seedlings transplanted into pots in the open top chambers (OTC)—four plants per pot—with four pots per CV and OTC, totalling 12 pots per CVs and O_3 treatment. April 14th and 30th: Pots fertilized with 120 kg N ha^{-1} in two doses Manually irrigated to maintain water saturation at field capacity (approx. every 2 days) until grain hardening in May.	12 cultivars—See Table 3 (3 Modern, 3 Old, 3 Landraces) 17,671.5 cm^3 pots Mix of turf (60%), perlite (20%) and vermiculite (20%)
Stage 2. Ozone Fumigation	Randomized design with 3 chamberless plots and four O_3 treatments in 3 blocks: • AA: Ambient Air (chamberless plots) • FA: Charcoal Filtered Air • NFA: Non-filtered Air • NFA+: Non-filtered Air + 20 nL L^{-1} • NFA++: Non-filtered Air + 40 nL L^{-1}	Fifteen 3 m × 3 m NCLAN-type OTC with a frustrum.
	O_3 application to the NFA+ and NFA++ OTCs for 8 h day^{-1} (6:00 to 14:00 GTM), 7 days a week. April 14th: fumigation begins 77 days after transplantation, lasting 51–71 days (depending on the CV) until plants reached full grain maturity.	A2Z Ozone Systems Inc., Louisvilla, KY, USA O_3 generator system, located in a control cabin and fed with pure O_2, with system monitors calibrated at the beginning of the experiment.
	Monitoring of ozone and nitrogen oxide concentrations inside every OTC and AA plot (above canopy) every 10 min.	Ozone monitoring (ML® 9810B, Teledyne Monitor Labs, Englewood, CO, USA) Nitrogen oxides monitoring (NO_2 and NO; ML®9841, Teledyne Monitor Labs, Englewood, CO, USA).
	Monitoring of the air relative humidity (RH), temperature and photosynthetic active radiation (PAR) in 6 of the OTCs and all 3 AA plots.	RH and temperature monitoring: HOBO® Pro v2, Onset, Bourne, MA, USA. PAR monitoring: OSO-SUN HOBO®, Onset, Bourne, MA, USA.
Stage 3. Measurements	May 4–14th: Stomatal conductance (g_s) measurements taken between 9:00–13:00 h GMT after 20–30 days (depending on the cultivar) of O_3 exposure. Two plants randomly selected per OTC for 2 blocks, measured on the flag leaf.	Li-Cor 6400; Li-Cor, Lincoln, NE, USA, with the standardized conditions: PAR 1000 µmol m^2 s^{-1} RH 55% Temperature range 20–22 °C
	June 3rd: Yield, agronomical yield components, taken after grain maturity and harvest • Dry weight of straw, spikes and grains • Number of grains • Spikes shelled manually to calculate GY. • HI, individual grain weight and number of grains per ear calculated per pot.	Harvested plants divided into straw and spikes, which were later shelled, dried at 60 °C for 48 h and weighed.
	Nitrogen content parameters and isotopic signalling (15 samples per genotype and 3 samples per O_3 treatment and genotype)	EA-IRMS analysis: Elemental analyser (EA): Flash 1112 EA, ThermoFinnigan, Bremen, Germany. Ratio mass spectrometer: Delta C IRMS, Thermo Finnigan, Bremen, Germany.
Stage 4. Statistical Analysis	Analysis of variance (ANOVA) with Tukey Honestly Significant Difference test (HSD) Pearson correlations between $\delta^{13}C$ and $\delta^{15}N$ and gs with yield parameters	IBM® SPSS® Statistics 20 (Chicago, IL, USA)
	Principal Component Analysis without rotation and with Keiser Normalization	R, version 3.6.3, factoMineR package *

* For further information, refer to: Lê, S., Josse, J. & Husson, F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Softw.* **2008**, *25.1*, 1–18.

3. Results

3.1. Climatic Variables and Ozone Exposure

Figure 2a shows the mean daily O_3 profiles for the different O_3 treatments during the months of the experiment. Hourly mean FA values ranged between 20–35 nL L^{-1}, well reproducing the natural background levels currently found in the area during the winter and early fall seasons. NFA represents the common chronic high spring-summer levels of rural areas in the Central Iberian Peninsula, with values at the central hours of the day ranging between 50–60 nL L^{-1}. Fumigated treatments (NFA+ and NFA++) increased the maximum hourly values to around 65 and 90 nL L^{-1}, respectively. NFA values increased from April to June, following the O_3-seasonal variability in the area, while the NFA+ and NFA++ values in June decreased and even matched NFA, following the fumigation shutdown on June 8th, when the plants were dry and the grain mature. April average values for the fumigated treatments were also lower due to the start of the fumigation on April 14th; before this date plants were grown under O_3-ambient levels.

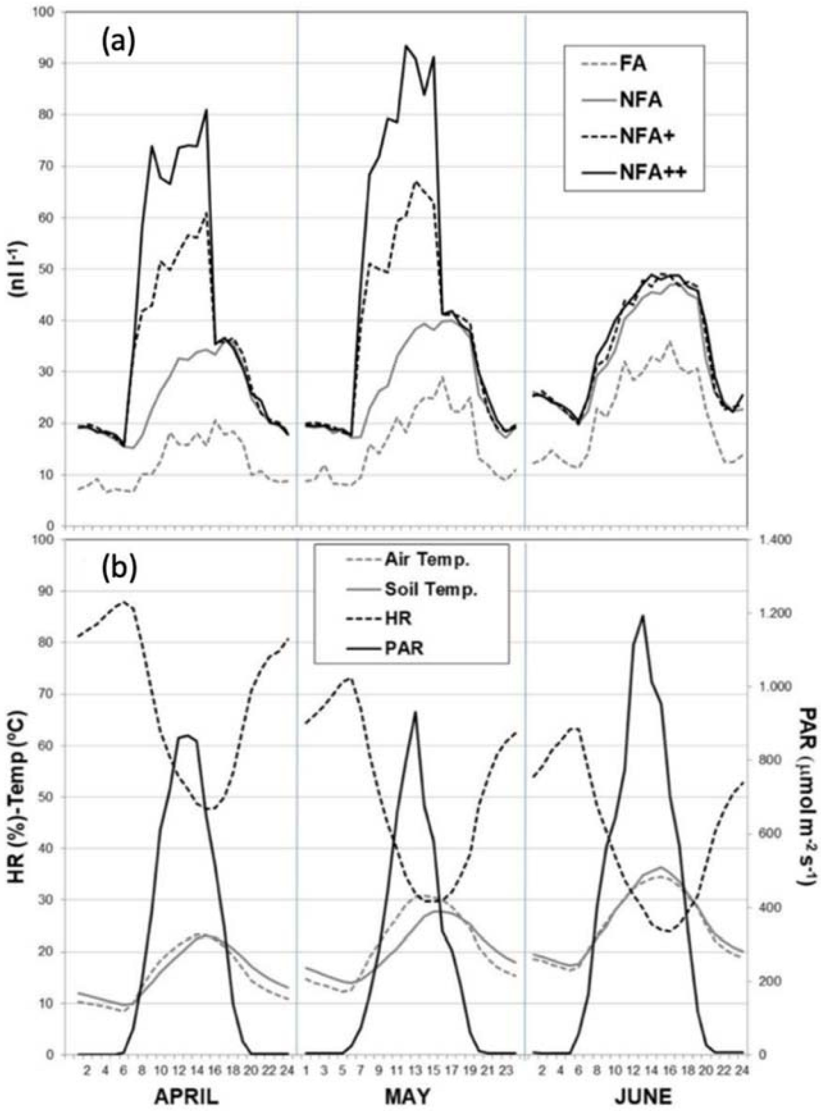

Figure 2. (**a**) Mean daily profiles of O_3 concentration for the different O_3-treatments during the experimental period: FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3; (**b**) Mean daily profiles of meteorological parameters during the exposure period inside the OTCs: HR (%,), air and soil temperature (°C) and photosynthetic active radiation (PAR µmol m^{-2} s^{-1}).

Mean values for the meteorological parameters inside the chambers during the exposure period are represented in Figure 2b. All the parameters followed the seasonal trend of the climatic area at the OTC site. Air and soil temperature increased from April to June, with maximum mean daily values ranging from 22–36 °C for air, as the growth cycle progressed, and 22–34 °C for soil. On the contrary, RH values dropped from 48% to 24% between April and June, reaching the minimum mean daily values at midday (minimum hourly mean values around 24% in June). Photosynthetic active radiation (PAR) also increased throughout the cycle, ranging between 850–1200 µmol m^{-2} s^{-1} inside the OTCs, and peaking in June at the end of the experiment.

3.2. Genotype Age-Type Effects

The age-type genotype factor caused clear significant differences in the means of all the parameters assayed: GY and GNY, g_s and stable isotopic composition (Table 3, Age-type factor). In general, no significant differences existed between the modern and old CVs, which, in turn, differed from the landraces significantly. More specifically, breeding caused an improvement in the GY of both categories of CVs, which increased by 34% (mean across O_3 treatments and cultivars) when compared to the landraces. Regarding HI, it increased by 37% in CVs compared to the landraces. Cultivars exhibited a higher number of grains per ear (by 54%) than the landraces, but at the expense of lower grain size. Thus, single seed size was 14% lower in CVs than landraces; also, breeding caused a decreased straw yield (SY) (38%) and total aboveground biomass (3%) (Appendix A: Table A1).

Table 3. Ozone and age type effects on yield parameters, g_s and isotopic signals: GY, grain yield (g pot^{-1}); HI, Harvest Index; GNY, grain nitrogen yield (g pot^{-1}); g_s, stomatal conductance (mmol H_2O s^{-1}); $\delta^{13}C$, stable carbon isotope composition (‰) of grains; N, nitrogen concentration (% DW); $\delta^{15}N$, stable nitrogen isotope composition (‰) of grains. FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl L^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3; Mean values ± SE. Different letters indicate statistically significant differences in the mean ($p < 0.05$).

		GY	**HI**	**GNY**	**g_s**	**$\delta^{13}C_{grain}$**	**N%**	**$\delta^{15}N_{grain}$**
Age-Type								
Modern CVs.		$30.51^{b} \pm 0.71$	$0.47^{b} \pm 0.00$	$0.81^{b} \pm 0.02$	$0.43^{b} \pm 0.03$	$-26.55^{a} \pm 0.07$	$2.67^{a} \pm 0.07$	$4.30^{a} \pm 0.03$
Old CVs.		$30.98^{b} \pm 0.53$	$0.45^{b} \pm 0.01$	$0.84^{b} \pm 0.02$	$0.34^{ab} \pm 0.03$	$-25.79^{b} \pm 0.09$	$2.70^{ab} \pm 0.04$	$4.59^{a} \pm 0.09$
Landraces		$20.21^{a} \pm 0.75$	$0.29^{a} \pm 0.01$	$0.56^{a} \pm 0.02$	$0.28^{a} \pm 0.02$	$-24.42^{c} \pm 0.06$	$2.84^{b} \pm 0.04$	$5.26^{b} \pm 0.17$
p-value		**<0.0001**	**<0.0001**	**<0.0001**	**0.002**	**<0.0001**	**0.039**	**<0.0001**
Ozone								
FA		$28.80^{b} \pm 1.15$	0.41 ± 0.02	0.77 ± 0.03	0.38 ± 0.04	$-25.88^{a} \pm 0.19$	2.70 ± 0.05	$4.66^{ab} \pm 0.14$
NFA		$27.75^{b} \pm 1.24$	0.40 ± 0.02	0.75 ± 0.03	0.37 ± 0.03	$-25.67^{ab} \pm 0.18$	2.75 ± 0.04	$4.86^{b} \pm 0.16$
NFA+		$27.44^{ab} \pm 1.15$	0.40 ± 0.02	0.73 ± 0.03	0.38 ± 0.04	$-25.53^{bc} \pm 0.17$	2.70 ± 0.09	$4.93^{b} \pm 0.18$
NFA++		$24.94^{a} \pm 0.90$	0.40 ± 0.01	0.69 ± 0.03	0.28 ± 0.02	$-25.27^{c} \pm 0.14$	2.79 ± 0.05	$4.39^{a} \pm 0.07$
p-value		**0.003**	0.646	0.062	0.120	**<0.0001**	0.645	**0.006**
Age× Ozone								
Cultivars *	FA	$32.93^{b} \pm 0.60$	0.48 ± 0.01	$0.86^{b} \pm 0.02$	$0.44^{b} \pm 0.04$	$-26.56^{a} \pm 0.13$	2.63 ± 0.05	$4.35^{ab} \pm 0.07$
	NFA	$32.18^{b} \pm 0.66$	0.47 ± 0.01	$0.86^{b} \pm 0.02$	$0.41^{ab} \pm 0.03$	$-26.28^{ab} \pm 0.14$	2.69 ± 0.05	$4.48^{b} \pm 0.05$
	NFA+	$30.55^{b} \pm 0.97$	0.46 ± 0.01	$0.79^{ab} \pm 0.04$	$0.41^{ab} \pm 0.06$	$-26.07^{bc} \pm 0.15$	2.70 ± 0.05	$4.45^{b} \pm 0.06$
	NFA++	$27.31^{a} \pm 0.80$	0.45 ± 0.01	$0.77^{a} \pm 0.02$	$0.28^{a} \pm 0.03$	$-25.77^{c} \pm 0.11$	2.82 ± 0.06	$4.22^{a} \pm 0.05$
p-value (CVs)		**<0.0001**	0.111	**0.005**	**0.046**	**<0.0001**	0.091	**0.008**
Landraces	FA	20.52 ± 1.32	0.29 ± 0.02	0.57 ± 0.03	0.241 ± 0.042	−24.50 ± 0.10	2.85 ± 0.09	5.05 ± 0.31
	NFA	18.89 ± 1.43	0.27 ± 0.02	0.53 ± 0.03	0.284 ± 0.063	−24.44 ± 0.12	2.88 ± 0.08	5.50 ± 0.38
	NFA+	21.22 ± 1.87	0.30 ± 0.02	0.61 ± 0.04	0.301 ± 0.058	−24.45 ± 0.12	2.92 ± 0.08	5.87 ± 0.44
	NFA++	20.19 ± 1.43	0.30 ± 0.02	0.54 ± 0.03	0.294 ± 0.018	−24.29 ± 0.11	2.73 ± 0.10	4.74 ± 0.12
p-value (Land.)		0.748	0.786	0.375	0.821	0.542	0.443	0.089
p-value (Age × O_3)		**0.018**	0.261	0.133	0.180	0.179	0.065	**0.045**

* Cultivars, grouped Modern & Old CVs due to their very similar behavior. Bold highlights statistical significant differences.

These improvements on yield parameters, caused by the varietal selection process, have been accompanied by an increment in the g_s of the CVs. Post-anthesis measurements of g_s were 27% higher in CVs than in landraces.

This same pattern was observed for $\delta^{13}C$, where landraces showed 2.13 ‰ higher (i.e., less negative) values compared with the modern and old CVs. The breeding process decreased grain N concentration by 6%, while enhancing GNY by 32% for the CVs (modern and old) vs. landraces. δ^{15}N signal decreased by 0.96‰ for the cultivars compared with landraces.

3.3. Ozone Effects

Considering the general response across genotypes, O_3-exposure affected GY, grain nitrogen yield (GNY) and both isotopic signals (Table 3, Ozone factor). When O_3 increased from FA to NFA++, yield parameters decreased: GY by 13% and GNY by 10% (mean values across the three age-type genotypes), with the effect being highly significant for GY ($p = 0.003$) and a trend near significant ($p = 0.062$) for GNY. Grain size ($p < 0.0001$), biomass ($p < 0.0001$) and Straw Yield SY (also a trend near significant—$p = 0.057$) also decreased by 11%, 9% and 7%, respectively, when FA increased to NFA++ (Appendix A: Table A1). Neither HI nor number of grains per ear were affected by the pollutant.

The observed δ^{13}C enrichment induced by the pollutant was small; 0.61‰ in NFA++ compared with FA; however, this response was stable and highly significant ($p < 0.0001$). The significant response of grain δ^{15}N to O_3 exposure was not linear: increasing from FA to NFA+ 0.27‰ to then decrease by 0.54‰ from NFA+ to NFA++. Meanwhile, the O_3-induced reduction observed for g_s was only significant for the CVs.

3.4. Age-Type Genotypes × Ozone Interactions

Modern and old CVs together responded similarly to O_3 and contrasted with landraces (Table 3, Age × Ozone). Cultivars subjected to the breeding process, no matter their age, were O_3-sensitive. Considering yield parameters, the general response was an O_3-induced yield decrease. The percentage of yield loss for the NFA++ treatment compared with FA across CVs were 17% for GY and 11% for GNY. Meanwhile, grain δ^{13}C increased by 0.79‰ (again with a stable and significant response, <0.0001). When only CVs were considered, the O_3 effect on g_s was also significant ($p < 0.05$), inducing a strong (37%) reduction in NFA++ treatment when compared to the FA control. Other parameters affected were grain size and biomass, which decreased by 15% and 11%, respectively. The non-linear O_3-response pattern observed in the general O_3-effect on the grain δ^{15}N signal was softened but maintained within the CVs group: the pollutant first caused an increase from FA to NFA+ by 0.10‰, to then decrease by 0.23‰ from NFA+ to NFA++. CVs also presented a general N% increasing trend of around 7%. Meanwhile, when landraces were analysed alone, they presented no significant response to the O_3 exposure to any of the parameters assayed.

Nonetheless, despite their O_3-sensitivity, CVs subjected to genetic selection still presented an overall 34% higher GY when compared to Landraces (mean value across cultivars and O_3 treatments) and maintained this yield advantage under the highest O_3 treatment (26% higher). This breeding improvement under NFA++ exposure also persists when other parameters are considered, such as GNY (30% higher), HI (34% higher) and grain δ^{13}C (1.48‰ lower; 6% more negative); or other parameters, such as seed size (25% lower), number of grains per ear (54% higher), SY (41% lower) and total biomass (8% lower) (Table A1). On the contrary, for g_s and nitrogen, the pollutant outweighed the benefits of varietal selection and values between CVs and landraces under NFA++ were similar (without significant differences). For grain δ^{15}N, the increased discrimination by breeding also remained even when exposed to the highest O_3 treatment, with CVs values 0.52‰ lower than Landraces.

Intra-variability in the O_3-responses within each of the three age categories of genotypes was lower for the δ^{13}C measurements (Figure 3a) than for the g_s measurements (Figure 3b). However, some genotypes differed from the average response of their own group. All of the δ^{13}C values for the modern and old CVs, except for Marius, showed a significant response to increasing O_3 levels; however, some exhibited a more sensitive response: Berdún, Califa Sur, Arthur Nick and Ablaca suffered the most intense effect under the NFA++ treatment, with increases ranging between 0.87–1.04‰, while Califa Sur, Yecora and Pane 247 were also significantly affected by the lower O_3-exposure of the NFA+ treatment (increments from 0.43–0.81‰). This was followed by Pane 247, Yecora and Nogal, which, although less O_3-sensitive, had a corresponding increased $\delta^{13}C$ signal ranging from 0.56–0.69‰ under NFA++. In contrast, none of the landraces showed significant changes.

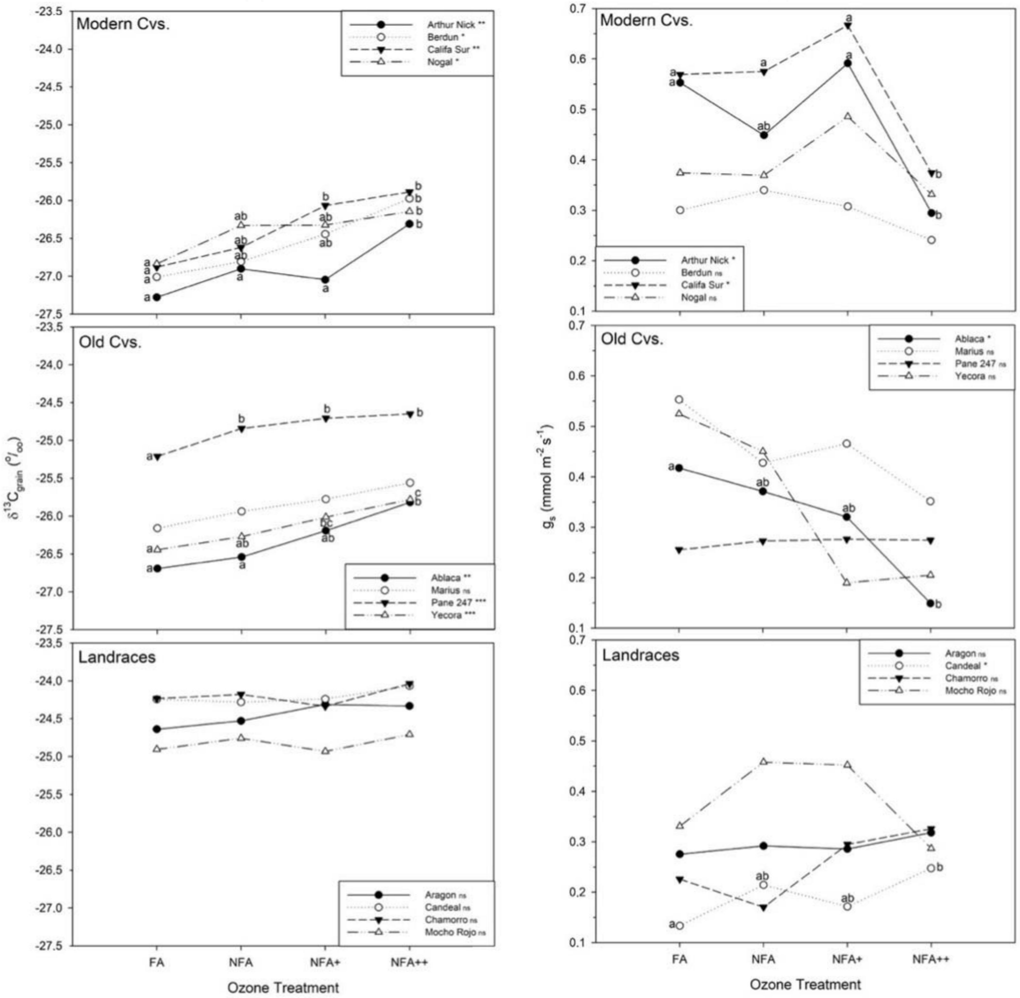
(**a**) (**b**)

Figure 3. (**a**) Stable carbon isotope composition ($\delta^{13}C$; ‰) of mature grains and (**b**) stomatal conductance (mmol H_2O m^{-2} s^{-1}) for the different O_3 treatments: FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3. Different letters indicate statistically significant differences (*** $p < 0.001$, ** $p < 0.01$, $p < 0.05$, ns: non-significant) between O_3 levels for each cultivar under Tukey analysis.

When g_s was considered, Califa Sur, Arthur Nick and Ablaca can be considered as the most sensitive; O_3 induced a g_s decrease in the range 34–64% for these CVs (Figure 2). The other $\delta^{13}C$-affected CVs presented a non-significant gs-reduction pattern. A more surprising behaviour was observed in the landrace group for Candeal de Vellisca, whose g_s strongly increased (by 85%) from FA until NFA++.

The same individual analysis per cultivar was done for grain $\delta^{15}N$ (Figure 4a) and nitrogen concentration (Figure 4b). Individual patterns of the modern and old CVs showed the overall average trend of the CVs group: grain $\delta^{15}N$ presented an increase in NFA+ and a subsequent drop by NFA++. Marius had the only significant differences in O_3, decreasing from NFA to NFA++ by 0.56‰. Landraces showed a higher variability, and clear amplification of the quadratic response, especially for Chamorro and Candeal de Vellisca; meanwhile, Mocho Rojo presented a decreasing trend. Regarding grain N values (Figure 4b), increases were significant for Arthur Nick and Yecora; Aragon showed an increase until NFA+ but then decreased by NFA++.

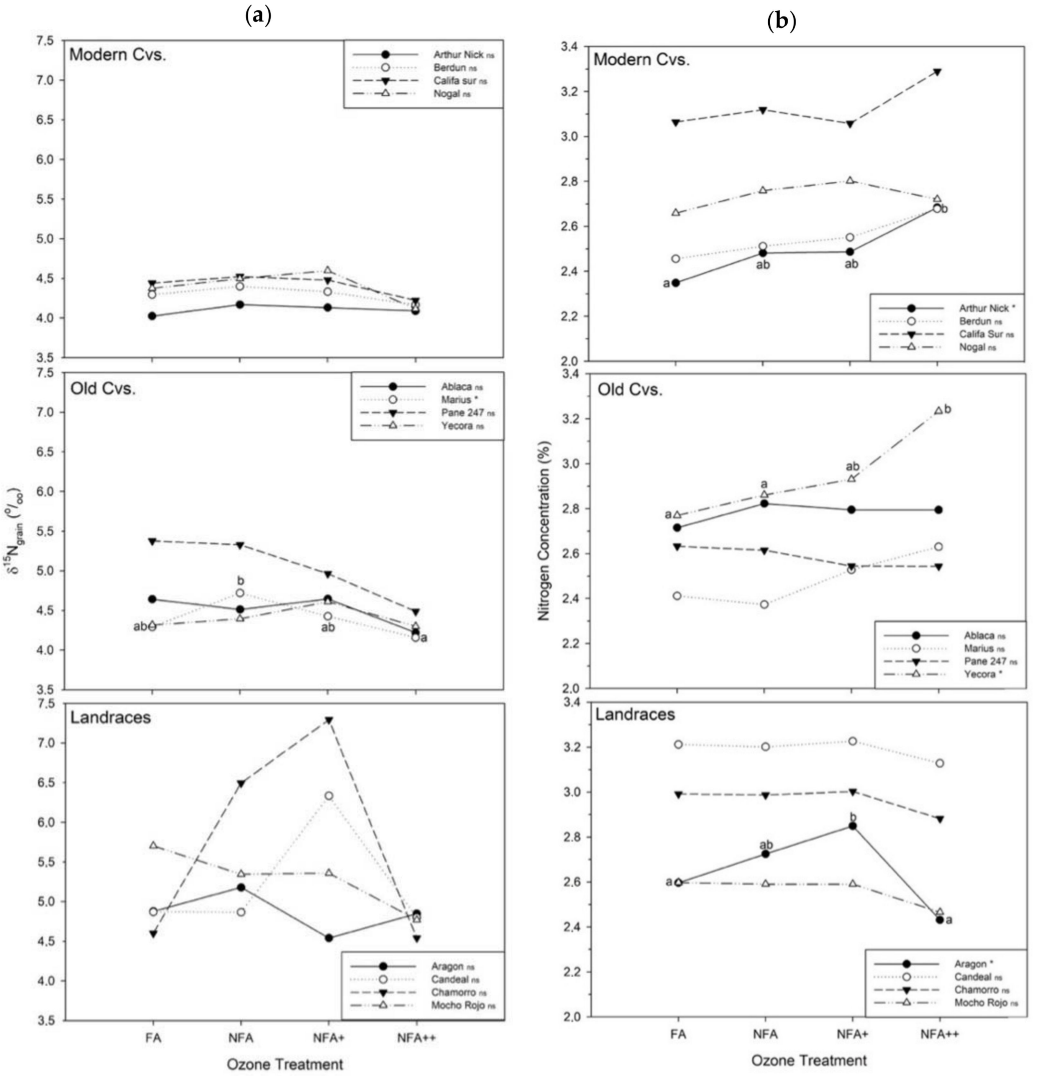

Figure 4. (**a**) δ $^{15}N_{grain}$ stable nitrogen isotope composition (‰) and (**b**) nitrogen concentration (N, % DW) for the different O-treatments: FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3. Different letters indicate statistically significant differences (* $p < 0.05$, ns: non-significant) between O_3 levels for each cultivar under Tukey analysis.

3.5. Yield and Isotopic Signals Correlations

Following the different O_3-sensitivities of the age-type genotypes, grain $\delta^{13}C$ of modern and old CVs together showed highly significant ($p < 0.001$) negative correlations with GY ($R^2 = 0.60$), GNY ($R^2 = 0.35$), total plant biomass ($R^2 = 0.3$) and significant ($p < 0.05$) negative correlations with HI ($R^2 = 0.18$) and g_s ($R^2 = 0.16$) across O_3 levels (Table 4).

Table 4. Regression analysis for grain δ^{13}C (‰) with the assayed yield parameters and gs: GY, grain yield (g pot^{-1}); Total Biomass (straw yield plus ear and grain, g pot^{-1}); HI, Harvest Index; SY, straw yield (g pot^{-1}); Single sw, single seed weight (mg); GNY, grain N yield (g N pot^{-1}); N%, N concentration (% DW); g_s, stomatal conductance (mmol H_2O m^{-2} s^{-1}); grain δ^{15}N (‰). *** Correlation significant at $p \leq 0.001$, ** $p \leq 0.01$ or * $p \leq 0.05$. Pane 247—Old CV, not included.

	Modern & Old CVs			**Landraces**		
δ^{13}C	**R^2**	***p*-Value**	**Function**	**R^2**	***p*-Value**	**Function**
GY	0.5979	***	$y = -6.7319x - 146.98$	0.2705	*	$y = -8.2655x - 181.64$
Total Biomass	0.2899	**	$y = -9.6042x - 173.28$	ns		
HI	0.1769	*	$y = -0.0296x - 0.3083$	0.4434	**	$y = -0.1196x - 2.6314$
SY	ns			0.2876	*	$y = 6.8189x + 215.73$
Single sw	ns			0.2431	*	$y = 2.5208x - 21.953$
GNY	0.3532	***	$y = -0.1221x - 2.3929$	ns		
N%	ns			0.5162	**	$y = 0.6917x + 19.733$
g_s	0.1576	*	$y = -0.1163x - 2.6639$	0.4360	**	$y = -0.2078x - 4.7941$
δ^{15}N	ns			ns		

Meanwhile, landraces did not present a significant correlation for δ^{13}C against total biomass or GNY, but a significant ($p < 0.05$) negative correlation with GY ($R^2 = 0.27$) and highly significant correlation ($p < 0.01$) with g_s ($R^2 = 0.44$). All δ^{15}N and g_s correlations for Modern & Old CVs and Landraces were non-significant.

Regarding other traits, the correlation between grain δ^{13}C and HI, though still negative, was stronger for landraces ($R^2 = 0.44$) than for modern and old CVs ($p < 0.01$; $R^2 = 0.18$); SY, single seed weight ($p < 0.05$) and grain nitrogen ($p < 0.01$) only showed correlations under the landraces but not for the CVs, with R^2 values of 0.29, 0.24 and 0.52, respectively.

Regression functions for δ^{13}C with total biomass, GY, GNY and g_s are shown in Figure 5 specifying the different O_3 treatments with different symbols. It can be observed that the higher O_3 treatments also presented the lowest δ^{13}C, with some exceptions such as for the tolerant CV Pane 247, which behaves closer to landraces and has not been included in the analysis. No significant correlations were found between grain δ^{13}C and δ^{15}N, or between grain δ^{15}N or g_s with any of the assayed parameters for both the CVs and landrace groups.

3.6. Principal Component Analysis

The Kaiser–Meyer–Olkin Measure of Sampling Adequacy and Bartlett's Test of Sphericity deemed the data for each of the three groups of genotypes (general), the modern and old CVs together and the landraces alone as useful for factor analysis (Figure 6a). As harvest index and biomass already correlated strongly with GY and SY, respectively, they were removed to avoid redundancies. Nitrogen concentration was also removed as it had a component matrix under 0.5.

On the general biplot (Figure 6a), both components explained 77.8% of the total variance. Strong differences can be seen between the different age-type genotypes as shown by the separate ellipses. GY, GNY and number of grains per ear were associated positively with g_s, and negatively with grain δ^{13}C, grain δ^{15}N, single seed weight and SY. The ellipse embracing the CVs is associated with higher yields, and, to a lower extent, a higher g_s, while the ellipse embracing the landraces is associated with a higher SY and higher grain δ^{13}C values. Seed weight does not seem to be generally associated with the other variables, only slightly positively with δ^{13}C and SY.

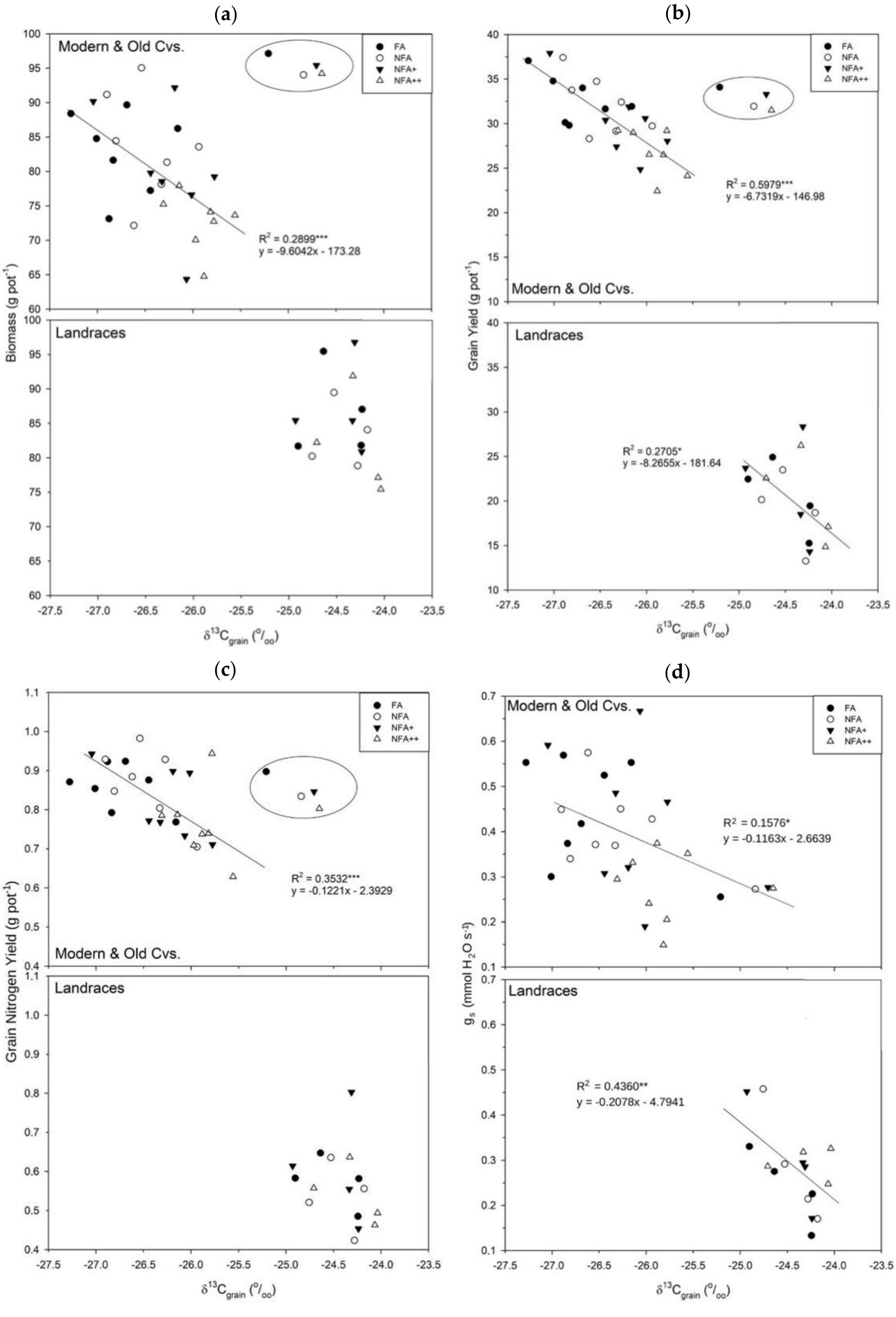
(**a**) (**b**) (**c**) (**d**)

Figure 5. Regression of δ^{13}C (‰) with: (**a**) GY (Grain Yield, g pot^{-1}), (**b**) GNY (Grain N Yield, g pot^{-1}), (**c**) total biomass (g pot^{-1}) and (**d**) g_s (mmol H_2O m^{-2} s^{-1}) for the different O_3-treatments: FA = charcoal filtered air, NFA = non-filtered air, NFA+ = non-filtered air supplemented with 20 nL L^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3. Circle encloses Pane 247—Old CV, not included in correlation analyses. *** significant at $p \leq 0.001$, ** $p \leq 0.01$ or * $p \leq 0.05$.

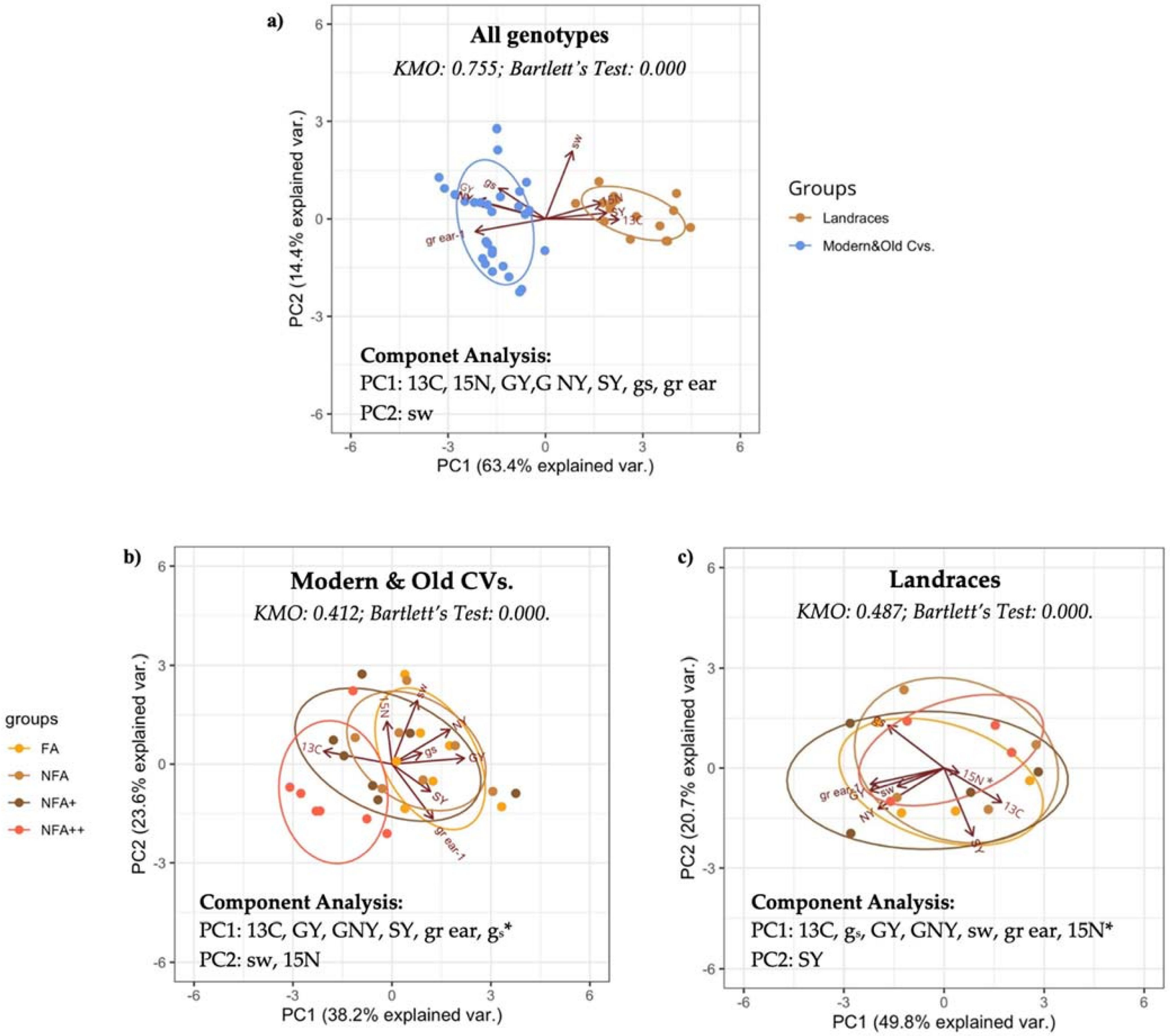

Figure 6. Principal Component Analysis for: (**a**) each age-type genotype based on standardized values of 13C, $\delta^{13}C_{grain}$ (‰); 15N, $\delta^{15}N_{grain}$ (‰); GY, grain yield (g pot^{-1}); GNY, grain N yield (g N pot^{-1}); SY, straw yield (g pot^{-1}); sw, single seed weight (mg); g_s, stomatal conductance (mmol H_2O m^{-2} s^{-1}); and gr ear^{-1}, grains per ear, for eleven (exc. Pane 247) Mediterranean wheat genotypes, (**b**) Modern & Old CVs and (**c**) Landraces separated by ozone treatment. * Low Component Matrix value.

When the response to O_3 was considered in the PCA analysis (Figure 6b), modern and old CVs differed from landraces. Landraces showed a great overlap of the ellipses of the different O_3 treatments, while modern and old CVs together show a deviation under the NFA++ treatment. For the modern and old CVs together, both components explained 61.8% of the total variance. PC1 showed negative associations between grain $\delta^{13}C$, and GY, GNY, SY and number of grains per ear, whereas grain $\delta^{13}C$ was positively associated with increasing ozone concentrations. In PC2, grain $\delta^{15}N$ and single seed weight do not seem to be associated with the latter, but correlate positively to each other, though with a short grain $\delta^{15}N$ vector.

In the landraces, the two components explained 70.5% of the variance. The PC distribution here mirrors the distribution seen in the general analysis, though SY is less correlated to all the other variables, and seed weight is negatively correlated to the isotopic signals.

For both groups of age-type genotypes, the grain $\delta^{15}N$ component matrix values were extremely low, lying almost directly on the component 2 axis and following the lack of correlations seen before.

4. Discussion

Spain has the largest surface dedicated to cereal production in the Mediterranean basin [56]. This mainly rainfed, drought-adapted wheat is located in the middle of the Iberian peninsula, where the recorded O_3 values chronically remain high above the protection thresholds for crops and vegetation defined by the European Directive of Air Quality (2008/50/CE) [57]. As hourly O_3 frequently reaches and exceeds 60 nL L^{-1} during early spring [58], which coincides with the non-water limitation period in the area, it allows for maximum physiological activity and gas exchange in the crop and thus, for maximum O_3 absorption [17,26], increasing the O_3-risk for the wheat despite the characteristic water-deficit later in the season.

During the experiment, the FA O_3 values, in the range 20–30 nL L^{-1}, well reproduced the natural background levels currently found in the area during the winter and early fall seasons, which are considered as natural O_3 background levels for pre-industrial times [59]; thus, this treatment can be referred to as an adequate control treatment. The NFA treatment represented the daily and seasonal pattern and levels currently registered in the rural areas of the central Iberian Peninsula during spring and summer. It increases from early April until the end of the wheat season, and spans no longer than June, reaching values around 55 nL L^{-1} at the central hours of the day. Similarly, the O_3 levels in the fumigated treatments, NFA and NFA++, reached maximum hourly values of around 90 nL L^{-1} in May, reproducing the maximum hourly values sporadically achieved during the high O_3 episodes in the area [57]. Therefore, the experiment could be considered to adequately represent the poor air quality conditions under which rainfed cereals are growing in the area, and to represent the situation in the Mediterranean basin [60]. This chronic O_3-pollution is enough to experimentally induce negative effects on crops such as tomato [61], leafy crops [62], watermelon [63] or durum wheat [36]. The latter is more O_3-tolerant than the spring bread wheat assayed in this study [64]; in fact, bread wheat is currently considered the most economically important sensitive crop and is used as a reference crop for O_3 risk assessment [9]. Air meteorological conditions during the experiment also resembled the characteristic spring-summer conditions of the Mediterranean wheat fields, with temperatures increasing during the season and reaching maximum monthly mean values of around 35 °C at the central hours of the day in June, towards the end of the assay, along with the lowest HR value of around 25%.

Results showed strong differences between age-type genotypes, where breeding improved both yield and yield components; for CVs, these effects were strongly related to a decreased carbon isotope composition and an increase in g_s. Landraces and CVs differed significantly; however, old and modern CVs behaved similarly. These results follow previous works performed under field conditions [30], which compared 12 Spanish varieties of soft and durum wheat under different tillage, also considering the age-type factor. Here, landraces had the lowest GY and behaved rather steadily throughout the trial seasons, while CVs presented a higher yield but depended more on the environmental conditions of the year and their independent growth habit. Winter genotypes, such as Marius, presented a higher GY in the humid years, and spring genotypes such as Califa Sur and Yecora performed comparatively better in the dry years. In the present study, no differences were seen due to growth habit since water availability was not a limiting condition in the experimental design and the date of sowing and transplantation was homogenized for all genotypes; however, CVs presented a 34% higher GY than landraces (across all O_3 treatments), which is similar to the 32% observed in Ruiz et al., 2019 [30] across seasons. Nonetheless, differences were seen between genotypes released pre- and post-green revolution (Table 1), particularly displaying the different behaviour Pane-247 had from the other CVs due to its pre-green revolution release. In this experiment, its very high $\delta^{13}C$ and low g_s fit much better within the landraces group, which was also released pre-green revolution, causing its removal when all genotypes were joined to analyse the general O_3 response. Actually, strong increases in GY and HI characterized the green revolution; however, further increases in these traits during the following six decades have been comparatively lower, explaining the lack of clear differences between old and modern CVs.

Achieving a higher GY in modern and old CVs was possibly not only due to a higher HI, but also a higher g_s, which favoured photosynthetic activity and caused the more negative $\delta^{13}C$ values in the newer genotypes [31,42,65]. Therefore, when compared to the landraces, the higher g_s of the post-green revolution cultivars (Table 1) allowed for increased discrimination, favouring the lighter 12C isotope and diminishing the $\delta^{13}C$ by 2.13‰. A decrease in the $\delta^{13}C$ (or increase in $\Delta^{13}C$) has been reported before for Spanish and Italian modern durum wheat cultivars when compared with old cultivars and landraces [42,65], where the difference in $\delta^{13}C$ between some modern genotypes and landraces from the Iberian Peninsula was around 1.2‰.

However, it should be noted that the higher $\delta^{13}C$ values seen in the landraces could also be influenced by their longer phenology when compared with the CVs, which exposes them to a more severe terminal (i.e., during grain filling) stress, even under irrigated conditions. The higher g_s observed in the more modern genotypes might seem contradictory for the adaptation process required for the characteristic water limited conditions of Mediterranean rainfed wheat; however, an increase in transpiration would not only aid photosynthesis, but would also limit leaf temperature, which is another important stress that affects plant physiological activity in hot and dry areas [66].

The effect of breeding on increasing $\delta^{15}N$ and GNY would be associated with the higher assimilation of the more modern varieties, while the observed reduction in grain N% would be related to a grain dilution effect, providing N availability is constant for the plant [28]. In essence, as GY increases with breeding, N% is diluted in the grain when paired with a constant N availability.

The PCA for the combined modern plus old CVs group and the landraces group (Figure 6a) reinforced the aforementioned two very different behaviours. The PCA pattern of the analysed parameters, except for seed weight, which did not correlate well with the others, showed the expected behaviours for the two age-groups, where the yield increase (GY and GNY) linked to breeding selection was positively associated with g_s, and negatively associated with the isotopic $\delta^{13}C$ and $\delta^{15}N$ signals and SY. This behaviour is opposite to that of the landraces.

In terms of O_3-response, the effect was age-type dependent; while modern and old CVs presented an O_3-induced reduction in yield parameters and g_s, as well as an alteration of both isotopic signals (less negative $\delta^{13}C$ and lower $\delta^{15}N$), landraces were not affected by O_3 exposure, even under the highest levels of the NFA++ treatment. The similarity in the O_3 response of the post-green revolution CVs (modern and old CVs without Pane 247 as shown in Table 1) was striking and allowed them to be analysed jointly. Exposure to O_3 induced high yield losses, 17% and 11 % for GY and GNY, respectively, as an average value across CVs in the NFA++ treatment. A meta-analysis derived by Feng et al., 2008 [67], considering more than 50 studies, obtained mean GY losses of 29% in modern wheat varieties for O_3-exposures based on Accumulated Ozone Exposure (AOT) functions that were slightly higher than the present NFA++ treatment. Meanwhile, in a review for Europe-level O_3 effects, similar O_3 exposures between 80–100 ppb produced yield reductions that rounded 30% [68]. Compared with these results, the Spanish CVs seem to lie within the O_3-sensitivity range observed for modern wheat CVs from other agronomic regions and for Central and North European levels, which do not have the characteristic water limitation of the Mediterranean region.

The O_3 effects on yield parameters of the sensitive CVs paralleled those of the g_s reduction. The greater sensitivity of modern CVs, compared to traditional genotypes not subjected to a programmed selection process, has been observed in previous works on wheat [28,32]. All of them showed smaller O_3-effects on the lower yielding old cultivars, and associated breeding selection for higher yield with an increased gas exchange via higher g_s, which led to an unintentional greater O_3 absorption and damage. Other studies have defined some of the O_3-damage mechanisms that affect modern varieties, which relate the O_3 induced lower photosynthetic rates to non-stomatal factors, such as a decreased carboxylation or electronic transport and lower capability of the antioxidative system [69].

Previous studies have concluded that, for wheat, the most O_3-sensitive period is anthesis, as O_3-effects on previous stages were not statistically significant [37]. Therefore, the present experiment was designed to start fumigation after spike emergence, close to anthesis. Accordingly, the overall negative O_3-effects were detected more in grain parameters than in biomass parameters, which follow the observed decrease in HI induced by the pollutant. The GY reduction was caused mainly by smaller grains while maintaining the number of grains per ear, also following results found in previous studies [68].

While this experiment followed many of the yield related O_3-effects seen in previous studies, few studies have delved into the effects O_3 has on the isotopic signature of wheat [25,40], and none have sought this for Mediterranean wheat. In experimental assays, gas exchange and carbon fixation factors affected by O_3 are often measured sporadically, and the results are extrapolated to the entire exposure period; even though these parameters usually present daily and seasonal patterns depending on the plant development stage [28], which might lead to a misinterpretation of the O_3 response. As $\delta^{13}C$ in plant dry matter depends on both stomata and Rubisco carboxylation [46], if the stomata close or their conductance decreases due to any stress, such as high O_3, stomatal discrimination decreases, restricting the CO_2 supply to the carboxylation sites and overall increasing $\delta^{13}C$ (or decreasing $\Delta^{13}C$) [39,40]. The integrated effect on the physiological activity of the plant throughout its lifespan, for whichever metabolic route the pollutant has affected, can be assessed by considering the isotopic parameters [43].

Results of the present work confirm the starting hypothesis that the $\delta^{13}C$ of mature grains becomes less negative (increases) under O_3 stress for O_3-sensitive CVs, due to the g_s reduction induced by the pollutant, which both decreases the capacity of the plant for carbon discrimination and negatively affects photosynthesis, decreasing GY. On the contrary, the landraces O_3-tolerance correlates with the non-effect seen in $\delta^{13}C$ of mature grains, along with a decrease in g_s and yield parameters. This also follows the postulate that, under stress conditions, cultivars use their current photosynthetic activities to produce carbohydrates for grain filling, while landraces rely more on storing carbohydrates in the stem to then redistribute them to the grain [28,29]. This could suggest that if the landraces had been exposed to ozone earlier, when they were storing carbohydrates in the stem, they could have shown a more sensitive behavior in their GY, which could be analyzed with an experiment with chronic ozone exposure beginning before anthesis.

Both the regression functions and the PCA analysis pointed to these results. It should be noted that a negative correlation between $\delta^{13}C$ of mature grains and g_s was observed within CVs and landraces; however, for the latter there was no direct relationship with the pollutant. The observed results of the PCA for each of the O_3 treatments (Figure 6b) reiterate the commented O_3-tolerance of the landraces, and the sensitivity of the modern and old CVs, particularly under the NFA++ treatment related to the decreased g_s, and hence, in ^{13}C discrimination (and thus higher $\delta^{13}C$), and lower GY, GNY, SY and number of grains per ear. Meanwhile, for both CVs and landraces, this effect is not shown in single seed weight, nor grain $\delta^{15}N$, most possibly due to its more complex isotopic fractionation processes [47].

The general response pattern of the grain N concentration, which increases under increasing O_3 levels, was more evident under the sensitive CVs, presenting a 7% increase due to the pollutant. This aligned with previous studies that analysed wheat grown under

O_3 stress [28,70], and has also been seen with drought [71]. As senescence is accelerated by O_3-stress, it would favour N translocation to the reproductive parts, enriching the grain more with protein than starch [72]; moreover, as the individual grain becomes smaller, it further increases the concentration [19]; however, when coupled with a decreasing GY, it actually leads to an overall decrease in GNY.

In general, previous studies on wheat indicate that when there is a positive O_3-effect on N grain concentration, there is an approximately equally negative effect on the yield [73]. Therefore, even though grain quality improves, the amount of protein accumulated per unit area, related to GNY, decreases, which can lead to food security and economic problems. In the present study, the effect on GNY was softer than the one observed in yield loss, with a GNY reduction of 11% compared to the 17% found for GY. This could be because O_3 exposure began close to anthesis, and even though this period is the most O_3-sensitive for wheat [37], it does not match the main period for N absorption, which usually decreases greatly after anthesis and during grain filling [50]. At anthesis, wheat focuses more on N redistribution from the vegetative tissues (e.g., flag leaf) for grain filling [47]. Therefore, a reason for a lack of stronger differences regarding the N-related parameters could be because N uptake from the soil had greatly decreased before the O_3 treatment began. Nonetheless, the landraces are characterized by a later phenology, in the sense that heading and anthesis occur later than in CVs; however, in the present study, all the genotypes were homogenized for growing so differences caused by their growth habit would not apply here.

As landraces rely more on ears, which are generally less affected by stress, and on protein degradation from other plant organs during grain filling stages [49,74], the O_3-effect on assimilation and redistribution could be particularly detrimental to the sensitive CVs when under stress; since they rely more on the flag leaf for N translocation for grain filling which senesces faster under O_3 exposure. At the same time, as active nutrient uptake by the roots occurs at early spring, future experiments designed to begin O_3 fumigation at the pre-anthesis stage are needed to take O_3 effects on N into consideration, especially for rainfed wheat.

Concerning the $\delta^{15}N$ of mature grains, as the plants were grown in pots with artificial substrate and had the same N fertilizer source (with a given $\delta^{15}N$), the starting pattern of assimilation and further fractionation is expected to be very similar for all the plants. $\delta^{15}N$ values are largely driven by nitrogen metabolism, including nitrogen assimilation, recycling and redistribution [38,47–49]. When N moves from one organ to another, the $\delta^{15}N$ signal in the organ increases as it builds up moving upwards towards the grain [47]. Therefore, alterations in plant metabolism and growth can modify $\delta^{15}N$. However, in our study, the assimilated N was accumulated in the flag leaf before the O_3 fumigation treatment began [49]. Due to the low levels of N absorption after anthesis [73], this suggests that only processes related to the N translocation from the leaves to the grains during grain filling were responsible for the effect of O_3 on $\delta^{15}N$, relating the O_3 response of the N parameters, such as $\delta^{15}N$, more with N redistribution.

The general response of $\delta^{15}N$ of the grain to the pollutant followed a non-linear pattern, upon which it increased from the control to the NFA+ treatment, to then being decreased by NFA++. Here, the effect of O_3 on $\delta^{15}N$ of grains was significantly driven by the CVs as opposed to the landraces and could again be related to the higher O_3-sensitivity of the CVs as well as on their dependence on N redistribution from organs that are highly affected by O_3, such as the flag leaf. Meanwhile, as mentioned, N redistribution from the ears, which is more associated to landraces, works well both with and without stress [49], giving landraces an advantage in redistribution under O_3-stress. The negative correlation between $\delta^{15}N$ and GY has been observed before [42], although it was not significant in the present assay. An alternative explanation for the decrease in $\delta^{15}N$ of the CVs as a response to the more severe O_3 treatment might be related to the decrease in g_s, which would limit volatilization of the lighter ^{14}N isotope with relation to the heavier ^{15}N [47,49,75], in turn leading to a lower $\delta^{15}N$ of mature grains.

It should also be noted that the landraces displayed a large heterogeneity in the $\delta^{15}N$ signal compared to the CVs, which could be due to their higher genetic variability [76] coupled with the aforementioned N redistribution from the ears and protein degradation of other plant parts [49]. The importance of the N redistribution mechanisms in the grain N should be considered for future plant breeding programs.

5. Conclusions

Modern and old CVs behaved very similarly under O_3 exposure, showing a high O_3-sensitivity based on the significant impacts seen on yield parameters, such as GY or GNY, and biomass, and, accordingly, on g_s and $\delta^{13}C$ and $\delta^{15}N$ values. On the contrary, landraces showed a stable and tolerant behaviour under the increasing O_3 exposures.

A different behavior was found between genotypes depending on their release date, where pre-green revolution genotypes presented very high $\delta^{13}C$ and low $g_{s.}$

$\delta^{13}C$ grain correlated strongly with both stomatal conductance and yield factors such as GY, GNY or total biomass; therefore, $\delta^{13}C_{grain}$, which can congregate all the intrinsic mechanisms by which the plant reacts to chronic O_3 exposure, and which hinder its yield, could be used as a good indicator of the cumulative effects of O_3 at the end of the wheat plant lifespan. Moreover, $\delta^{13}C$ values presented a more homogeneous response within each age-type group than other physiological response parameters such as g_s, backing its potential use as an indicator for accumulated O_3 effects.

Since the O_3 exposure period started post-anthesis, $\delta^{15}N_{grain}$ response was more indicative of alterations caused by the pollutant on N redistribution and N volatilization, and requires a more integrated future analysis due to its dependence on other N-related factors, such as absorption, metabolism and redistribution.

In the present study, the benefits of modern breeding through varietal selection mostly counterbalanced the negative O_3 effects on the modern and old CVs; however, the strong differences found in O_3-sensitivity between CVs and landraces indicate that there is still a broad genetic base for improving O_3-tolerance to enhance productivity and profits in Mediterranean agriculture, and to better adapt Mediterranean wheat to global change factors such as chronic high O_3 levels.

Author Contributions: Conceptualization, M.C.-E., I.G.-F., J.L.A. and V.B.-B.; Data Curation, M.C.-E., R.A., I.G.-F. and V.B.-B.; Formal Analysis, M.C.-E.; Funding Acquisition, M.C.-E., J.L.A. and V.B.-B.; Investigation, M.C.-E., I.G.-F., R.A. and V.B.-B.; Methodology, M.C.-E., I.G.-F. and V.B.-B.; Project Administration, I.G.-F. and V.B.-B.; Resources, J.L.A. and V.B.-B.; Supervision, J.L.A. and V.B.-B.; Validation, M.C.-E. and V.B.-B.; Visualization, M.C.-E. and V.B.-B.; Writing—Original Draft, M.C.-E.; Writing—Review & Editing, M.C-.E., J.L.A. and V.B.-B. All authors have read and agreed to the published version of the manuscript.

Funding: Funding was provided by Comunidad de Madrid (Spain) projects AGRISOST-CM (S2018/BAA-4330) and Grupo Operativo OZOCAM (PDR Comunidad de Madrid 2014-2020m MAPAMA/FEADER) and the EU project ERANET/SUSCROP SUSCAP (PCI2019–103521/AEI). M.C-E. and J.L.A. acknowledge the support from PID2019-106650RB-C21, from MICIN, Spain. J.L.A. thanks the support from the ICREA Academia program, Generalitat de Catalunya, (Spain). Finally, the isotopic analyses were supported, in part, by the l'Institut de Recerca de l'Aigua (IdRA) of the University of Barcelona.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Information for the cultivars mentioned in Table 1 can be found at https://genvce.org/productos/fichas/ (accessed on 4 July 2021).

Acknowledgments: The OTC experimental facility located at La Higueruela Research Farm is supported by an agreement between MNCN (CSIC) and CIEMAT. The farm personnel, and especially the valuable work of Jose María Gómez Camacho on plant care and OTC facility maintenance are greatly acknowledged. Thanks are given to S. Elvira, H. Calvete and J. Sanz for their help on the fieldwork.

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

Appendix A

Table A1. Ozone and age type effects on yield parameters: SY, straw yield (g pot^{-1}); Single sw., single seed weight (mg); # grains ear^{-1}, number of grains per ear per pot; Biomass, added value of the straw yield and the ears with grains (g pot^{-1}). FA= charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nL L^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nl L^{-1} of O_3; Mean values ± SE. Different letters indicate statistically significant differences ($p < 0.05$).

		SY (g)	Single sw. (mg)	# Grains Ear^{-1}	Biomass (g)
Age Type					
Modern CVs.		33.61 ^{a} ± 0.66	31.57 ^{a} ± 0.66	44.18 ^{c} ± 1.07	78.42 ^{a} ± 1.24
Old CVs.		37.77 ^{b} ± 1.08	38.08 ^{b} ± 0.90	36.66 ^{b} ± 1.08	85.16 ^{b} ± 1.40
Landraces		49.22 ^{c} ± 0.63	39.61 ^{b} ± 0.33	18.61 ^{a} ± 0.93	84.61 ^{b} ± 1.05
p-value		**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Ozone					
FA		40.94 ± 1.49	37.85 ^{b} ± 0.93	33.55 ± 2.18	85.36 ^{b} ± 1.31
NFA		40.97 ± 1.34	37.18 ^{b} ± 0.83	32.98 ± 2.19	84.38 ^{b} ± 1.27
NFA+		40.96 ± 1.56	36.95 ^{b} ± 0.90	33.25 ± 2.14	83.74 ^{b} ± 1.61
NFA++		37.93 ± 1.38	33.69 ^{a} ± 1.06	32.81 ± 2.15	77.44 ^{a} ± 1.52
p-value		0.057	**<0.0001**	0.975	**<0.0001**
Age × Ozone					
Cultivars *	FA	36.18 ± 1.26	36.71 ^{b} ± 1.33	41.18 ± 1.64	84.79 ^{b} ± 1.67
	NFA	36.80 ± 1.22	36.02 ^{b} ± 1.13	40.76 ± 1.57	84.99 ^{b} ± 1.66
	NFA+	36.41 ± 1.58	35.47 ^{ab} ± 1.22	39.76 ± 1.85	82.04 ^{ab} ± 2.14
	NFA++	33.38 ± 1.19	31.10 ^{a} ± 1.25	39.96 ± 1.79	75.34 ^{a} ± 1.89
p-value (CVs)		0.248	**0.008**	0.929	**0.001**
Landraces	FA	50.45 ± 1.51	40.13 ± 0.48	18.31 ± 1.56	86.50 ± 2.13
	NFA	49.32 ± 1.17	39.52 ± 0.73	17.41 ± 1.57	83.15 ± 1.93
	NFA+	50.06 ± 1.25	39.90 ± 0.61	20.23 ± 2.56	87.13 ± 1.98
	NFA++	47.04 ± 0.94	38.87± 0.79	18.51 ± 1.75	81.66 ± 2.17
p-value (Land.)		0.219	0.558	0.764	0.193
p-value (Age × O_3)		0.946	0.289	0.767	0.218

* Cultivars, grouped Modern & Old CVs due to their very similar behavior. Bold highlights statistical significant differences.

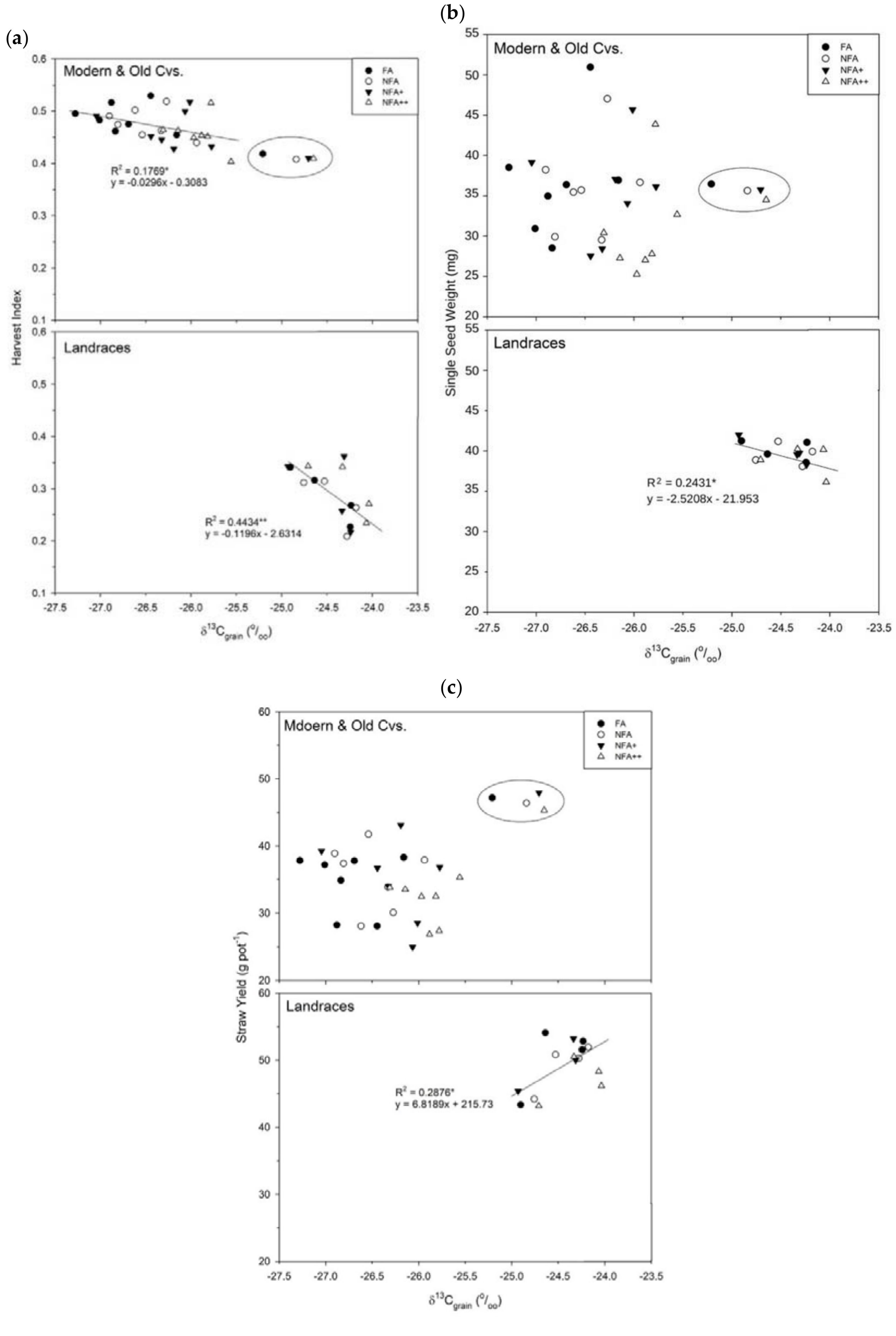

Figure A1. Regression functions of δ^{13}C (stable C isotope composition, ‰) with: (**a**) Harvest Index, (**b**) Single seed weight (mg) and (**c**) Straw yield (g pot^{-1}) for the different O_3-treatments: FA= charcoal filtered air, NFA = non-filtered air, NFA+ = non-filtered air supplemented with 20 nL L^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nL L^{-1} of O_3. Circle encloses Pane 247—Old CV, not considered for correlation analyses. Correlation is significant at ** $p \leq 0.01$ or * $p \leq 0.05$.

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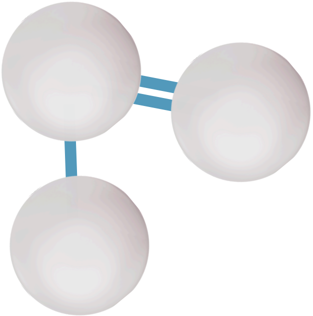
Chapter 2



Chapter 2

The manuscript used for Chapter 2 has been submitted to Science of the Total Environment, which has an impact factor of 9.8 and a CiteScore of 16.8, and being in the first quartile, Q1, in its research fields

Resumen del Capítulo 2

El ensayo se realizó en una instalación de Cámaras de Techo Descubierto (OTC) situada en la cuenca mediterránea para investigar cómo la fertilización con nitrógeno (N) afecta la respuesta del trigo a la exposición al ozono (O_3). El estudio examinó la respuesta de Artur Nick, una variedad de trigo moderno comúnmente utilizado en la zona, a tres niveles de exposición al O_3 (ambiente y ambiente +20 y +40 nL L^{-1}) y dos dosis de fertilización con nitrógeno (100 y 200 kg ha^{-1}). Se midieron el intercambio gaseoso fotosintético y transpirativo, el contenido de clorofila en las hojas, el contenido de N en las hojas y granos, parámetros de crecimiento y rendimiento de las plantas. Se estudiaron y cuantificaron los efectos interactivos del ozono y el nitrógeno basados en concentraciones acumuladas de O_3 por encima de un umbral de 40 nL L^{-1} (AOT40) y el índice de dosis fitotóxica de O_3 (POD), que se utilizan en las evaluaciones de riesgo de O_3, y a partir de los cuales se derivaron niveles críticos (CL).

Los resultados mostraron que los impactos del O_3 en los parámetros de crecimiento y rendimiento analizados, fueron más fuertes bajo la dosis más alta de fertilización con N. Esto llevó a que los CL de O_3 fueran hasta 3 o 4 veces menores para el rendimiento de grano bajo el tratamiento de N alto en comparación al tratamiento de N bajo. El O_3 limitó el estímulo del fertilizante, reduciendo fuertemente la eficiencia del uso del N sobre el rendimiento de grano y la eficiencia agronómica del N sobre el rendimiento de proteínas. Otro aspecto importante fue que el 71% del POD se acumuló antes de la antesis, acentuando la potencial importancia de la exposición al O_3 durante la fase vegetativa del trigo en condiciones mediterráneas, el cual es generalmente considerado como menos importante que la exposición post-antésis.

Este estudio sugiere considerar la gestión del N en los cultivos cuando se deriven los CL de O_3, debido a su efecto en las relaciones dosis-respuesta utilizadas para la derivación de los CL y para incluir los potenciales efectos del O_3 en la eficiencia del uso del N, la cual actualmente no se considera en la evaluación de riesgos en el marco de las políticas de reducción de la contaminación del aire.

Nitrogen modulates the ozone response of Mediterranean wheat: considerations for ozone risk assessment

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Abstract

The assay was conducted in an Open Top Chamber facility located in the Mediterranean basin to investigate how nitrogen (N) fertilization affects the response of wheat to ozone (O_3) exposure. The study considered the response of Artur Nick, a modern wheat cultivar commonly used in the area, to three O_3 exposure levels (ambient and elevated ambient, +20 and +40 nL L^{-1} O_3), and two nitrogen (N) fertilization doses (100 and 200 kg ha^{-1}). Measurements included photosynthetic and transpirative gas exchange, leaf chlorophyll content, leaf and grain N content, plant growth and yield parameters. Ozone x N interactive effects were studied and quantified based on accumulated O_3 concentrations above a 40 nL L^{-1} threshold (AOT40) and phytotoxic O_3 dose (POD) indexes, which are used in O_3-risk assessments, from which critical levels (CL) for a 5% effect were derived.

Results revealed that O_3 impacts on growth and yield parameters analysed were stronger under the highest N fertilization dose. In consequence, O_3 CL where as much as 3-4 times lower for grain yield in the high-N compared to the low-N treatment. Interestingly, O_3 limited the fertilizer stimulus, strongly reducing the N use efficiency for grain yield and the agronomic efficiency of N for protein yield. Another important aspect was that 71% of the POD was accumulated before anthesis, stressing the potential importance of O_3 exposure during the vegetative phase of wheat under Mediterranean conditions, which is usually considered less important than post-anthesis exposure.

In conclusion, this study suggests the need to consider crop N management in the derivation of O_3 CL, due to its effect on dose-response relationships used for CL derivation and for including potential O_3 effects in N use efficiency that are currently not considered in risk assessment exercises in the framework of negotiations of air pollution abatement policies.

Keywords: ozone x nitrogen interaction, fertilization, nitrogen use efficiency, phytotoxic ozone dose, AOT40, ozone critical levels

1. **Introduction**

Tropospheric ozone (O_3) levels have been increasing since the beginning of industrialization, especially in the Northern Hemisphere (Ainsworth et al., 2012; Schultz et al., 2017). Its deposition process and effects have been abundantly studied regarding health impacts, agricultural production, and ecosystem biodiversity (CLRTAP, 2020; Fleming et al., 2018; Lefohn et al., 2018; Mills et al., 2018a). Wheat, a global staple crop, has been amply studied and, despite its genotypic variability, generally exhibits a high sensitivity to the pollutant across different agroclimatic zones (Chang-Espino et al., 2021; Feng et al., 2022; Pleijel et al., 2019; Zhao et al., 2020). In fact, risk assessment methodologies for O_3 impacts on cereal crops adopted by the United Nations Air Convention framework rely on the response of wheat grain yield and quality to this pollutant (CLRTAP, 2017; EEA, 2022). Ozone risk assessment is currently considered in policymaking to assess air quality impacts on crops amid present and future scenarios for air pollution emission abatement negotiations (CLRTAP, 2017; EEA, 2019a; Pleijel et al., 2019), and to monitor O_3-risk trends for crops and ecosystems under the European air quality legislation (Directives 2008/50/EC and EU 2016/2284).

Ozone enters the plant through stomatal pores, producing an oxidative burst within the vegetative tissues (Wilkinson et al., 2012). This causes early physiological disruptions related to carbon fixation, such as stomatal closure (Hoshika et al., 2015), decreased carboxylation capacity (Rubisco activity) and electron transport rates (Feng et al., 2016) as well as loss of foliar chlorophyll content (Emberson et al., 2018; Feng et al., 2011, 2019). In wheat, these alterations reduce carbon fixation, shorten the duration of photosynthesis, increase metabolic costs for detoxification and repair, and decrease leaf longevity (Osborne et al., 2019; Pleijel et al., 2007). Nutrient remobilization and leaf-to-grain translocation are also affected and the grain-filling period is shortened (Broberg et al., 2015; Kaur et al., 2017), causing yield and quality reductions (Schauberger et al., 2019; Zhao et al., 2020). These effects have been substantiated by exposing wheat to elevated O_3-levels post-anthesis, the period of maximum O_3-sensitivity in temperate climates (Pleijel et al., 1998). Nevertheless, pre-anthesis O_3 exposure might play a crucial role in climates like the Mediterranean, where considerable resources are allocated to grain-filling reserves before anthesis, to be remobilized later in the season, especially when water stress decreases photosynthesis during grain filling (Sanchez-Bragado et al., 2014).

Modern wheat varieties are more O_3-sensitive than older ones or landraces (Barnes et al., 1990; Harmens et al., 2018). This has been related to the greater photosynthetic activity sought by varietal selection to improve crop yields, leading to higher stomatal conductance (g_s) and causing higher air pollutant absorption. This effect has been particularly seen in Mediterranean varieties (Chang-Espino et al., 2021). Direct O_3 effects on the main modern-bred wheat and other staple crops pose a growing threat to global food security, even under optimistic scenarios. Some predictions state that by the year 2030, the pollutant could decrease global wheat production by 2.6 to 5.4% (Avnery, 2013). Hourly mean values in the range of 41-60 nL L^{-1}, which already occur at the anthesis stage, might cause an estimated reduction in European wheat production of 4.1-12.1% (Ainsworth, 2017). Even though future O_3 levels would depend on the policy restrictions on the emission of O_3 precursors, some scenarios anticipate that global warming will exacerbate O_3 production by 0.2-2 ppbv $^{o}C^{-1}$ in areas affected by anthropogenic O_3-precursors (Zanis et al., 2022), causing a food security alert (Lefohn et al., 2018; Mills et al., 2018a; Pleijel et al., 2018). In the Mediterranean basin, O_3 values are generally the highest in Europe (EEA, 2019b) due to its characteristic hot and sunny climate and atmospheric stability, which favors photochemical O_3 production (Proietti et al., 2016), deriving in O_3 concentrations that continually exceed the plant protection thresholds established in the air quality EU Directives (EEA, 2022) throughout the entire cereal crop cycle.

Crop productivity is greatly influenced by N-fertilization. Its use has risen significantly in the past 40 years to produce greater yields, but this has also affected the environment as only 30-50% of the applied fertilizer is assimilated by the plant (Broberg et al., 2017; Hawkesford & Riche, 2020). Significant amounts of nitrate or ammonium fertilizer leach onto the soil water systems, while gas N-compounds such as N_2O or NH_3 are emitted into the atmosphere (Kaur et al., 2017). Additionally, higher N supplies tend to contribute more resources towards vegetative growth than to the economically relevant reproductive structures (Peng et al., 2020), prompting the search for the optimum point of N Use Efficiency (NUE), where most of the N supplied to agrosystems derives into plant yield.

Ozone can affect NUE by altering plant N metabolism through different plant-mediated mechanisms like decreasing root growth (Calvete-Sogo et al., 2016; Sanz et al., 2005), unbalancing C/N rate between shoot and root (Sanz et al., 2005), or altering root exudates and rhizosphere composition (Hu et al., 2018)). These changes directly impact N absorption and metabolism, and the N mobilization routes within the plant (Broberg et al., 2017). However, studies delving into experimental analysis of the O_3 and N interaction are still scarce and non-generalizable patterns have yet been determined. Some of the studies have shown a certain reduction of the damage caused by the pollutant due to a larger N availability for the plant (Broberg et al., 2017; Pandey et al., 2018; J. Peng et al., 2020; Schauberger et al., 2019). However, the beneficial effect of the fertilizer in limiting O_3-induced plant senescence or biomass loss in wheat and other grass species does not occur under high levels of the pollutant (Broberg et al., 2017; Feng et al., 2019; Sanz et al., 2011). The fertilizer may even favor O_3 impacts depending on the O_3 level and N dose and the specific plant parameter considered (Calvete-Sogo et al., 2016). These findings show the complexity of the O_3 x N interaction and the need for further experimental research to find consistent response patterns.

Despite the available evidence of N modulation on wheat O_3-response, current O_3 risk assessment methodologies employed in the framework of the Air Convention (CLRTAP, 2017) do not consider the modulation effect of the fertilizer on the O_3-induced wheat yield loss. However, to achieve more consistent O_3-impacts and improve risk analysis, it is crucial to consider and quantify the N modulation of the O_3-response. This quantification is also decisive for modeling exercises studying agricultural production and sustainable management under future Global Change scenarios (Feng et al., 2022; Mills et al., 2018b).

Within this context, the present study focuses on experimentally analyzing how N fertilization modulates the O_3-response of a modern O_3-sensitive wheat cultivar exposed to the pollutant, from the tillering stage, similar to plants growing in the field in the central Iberian Peninsula, and to quantify these effects based on both O_3-exposure-based (AOT40) and O_3-absorbed dose based (POD) indexes. The starting hypotheses, based on previous studies were: 1.- that O_3-exposure during the pre-anthesis stage will intensify the effects of the pollutant; 2.- that the fertilizer would counteract O_3-effects but only until a certain level of the pollutant; 3.- the fertilizer will affect O_3-response functions and the derived critical levels (CLs) for plant protection, introducing an uncertainty in the O_3 risk assessments.

2. Material and Methods

2.1 OTC Field Site and Experimental Design

The assay was performed in the Open Top Chamber (OTC) facility of CIEMAT which is specifically placed to study the effects of O_3 under Mediterranean conditions. The OTC facility is located at 450 m.a.s.l. in the Central Iberian Peninsula (40º3’ N, 4º26’ W) in the “La Higueruela/MNCN-CSIC” Research Farm (Santa Olalla, Toledo, Spain), reproducing the continental Mediterranean climate of the main rainfed cereal production areas in Spain (MAPA, 2022).

Three blocks, each with 3 randomly distributed NCLAN-type OTCs (Heagle et al., 1989) were used to analyze the effects of the O_3 treatments: NFA (Non-Filtered air), reproducing ambient levels at the farm, NFA+ (Non-Filtered Air + 20 nL L^{-1} of added O_3) and NFA++ (Non-Filtered Air + 40 nL L^{-1} of added O_3) reproducing expected O_3 levels in the area. Nonetheless, the maximum hourly values of the highest treatments have already been registered during high-O_3 episodes in the area (Massagué et al., 2022; MITECO, 2022). The OTCs were 3 m high × 3 m diameter with a frustum to prevent wind inflow. The NFA+ and NFA++ OTCs were fed from a control cabin, which processed pure O_2, through an O_3 generator system (A2Z Ozone Systems Inc., Louisville, KY, USA), for 8 h day^{-1} (6:00 to 14:00 GMT), seven days a week. Ozone (ML® 9810B, Teledyne Monitor Labs, Englewood, CO, USA) and nitrogen oxides (NO_2 and NO; ML®9841, Teledyne Monitor Labs, Englewood, CO, USA) levels were monitored at canopy height, within each OTC through a timesharing system with a sampling period of 10 minutes per plot. Air pollution monitors and flowmeters of the fumigation system were calibrated at the beginning of the experiment following company recommendations.

Air relative humidity (RH), air temperature (HOBO® Pro v2, Onset, Bourne, MA, USA), and photosynthetic active radiation (PAR; OSO-SUN HOBO®, Onset, Bourne, MA, USA) were measured within six of the OTCs at canopy height. More information on the OTC facility can be found in the literature (Calvete-Sogo et al., 2016).

2.2 Plant material and nitrogen treatments

The variety employed in the assay was the commercial spring bread wheat (*Triticum aestivum* L.) cultivar Artur Nick, which was registered in the year 2002. This variety is extensively used in the Iberian Peninsula and currently is the 3rd cultivar in importance based on certified seed sales in Spain (MAPA, 2023).

Agronomical field assays with this variety have shown a medium grain weight and a high yield potential in this region, being one of the reference cultivars for testing new cultivars in the area (GENVCE, 2003). Previous studies indicated that Artur Nick is an O_3-sensitive variety (Chang-Espino et al., 2021). Due to its wide use and sensitivity to the pollutant, this variety is a good option for studying the O_3 x N interactive effects. After sowing in a greenhouse, two wheat seedlings per pot were transplanted into 20 cm diameter pots filled with 60/20/20 peat/perlite/vermiculite substrate. The whole O_3-fumigation period comprised 77 days from the start of fumigation on April 24 (Days after the beginning of the Exposure period, DaE 0), when wheat was at the tillering stage (stage 23 according to Meier, 2018), until harvest on July 10 (DaE 77). Pot nets were set at the start of grain development to avoid ant and bird damage on the grains. Different crop management events and sampling dates throughout the wheat cycle are shown in Figure 1S.

Two top N fertilization treatments consisting of a total of 100 kg N ha^{-1} (Low-N) and 200 kg N ha^{-1} (High-N) were applied in two partial doses, from dissolved ammonium nitrate (26%), 18 days apart (Fig. 1S). The Low-N dose represents the usual fertilization of the commercial rainfed wheat fields in the area. Four pots were allocated to each of the N treatments per OTC (12 pots per O_3 and N treatments).

2.3 Gas exchange and chlorophyll content measurements

Chlorophyll content (□g cm^{-2}) of the flag leaf (FL) was evaluated using the optical sensors DUALEX on June 5 (DaE 42), and SPAD on June 14 (DaE 51). SPAD units were converted to chlorophyll content (□g cm^{-2}) using the function of Uddling et al., (2007) for wheat ($y = 0.0599 * e^{0.0493x}$; $r^2=0.89$). Non-visibly damaged FLs of secondary stems were selected for these measurements.

Gas exchange parameters, net photosynthetic activity (A), stomatal conductance (g_s), transpiration (Tr) and intercellular concentration (Ci) were measured employing a portable gas exchange system (Li-Cor 6400; Li-Cor, Lincoln, NE, USA). Measurements were taken between 07:00-12:00 h GMT on May 31 and June 1, (DaE 37-38) in fully expanded FLs. Measurements were taken under standardized conditions (PAR 1000 µmol m^2 s^{-1}, RH 55%, and temperature 20–22 °C). The rate of A/Ci, associated with the efficiency of maximum carboxylation (Bernacchi et al., 2013), was also calculated.

Plant photosynthesis provided by the total FL surface (A_{FLsurf}) of the plant was calculated based on the total FL dry weight per pot (g DW) at harvest, and considering the Specific Leaf Area (SLA) of Artur Nick variety: A_{FLsurf} =A*SLA*FL dry weight (□mol CO_2 s^{-1}). To calculate specific Artur Nick SLA, 12 fully developed FLs, from pots not included in the OTC assay but grown under OTC conditions, were collected at anthesis, scanned to calculate the area (ImageJ2 V2.14.0/1.54f), and dried to constant weight to calculate dry weight (g DW). Mean values (± SE) for single leaf blade area were 20.13±2.38 cm^2, which corresponded with 0.103±0.014 g DW, thus giving a mean SLA value of 19.98±1.22 m^2 $kgDW^{-1}$ for Artur Nick variety. This SLA value was in the range measured for other wheat varieties (Giunta et al., 2008).

2.4 Biomass and Yield Parameters

On July 10th (DaE 77) plants were harvested at the base and divided among ears, FLs and straw. Mature and immature ears were counted and then manually shelled. The non-grain harvested parts were dried for 48 h at 60 °C and weighed. The number and weight of immature and mature ears, FL blade weight, hulled spikelet weight (HS weight), straw weight (SW) and total aboveground biomass per pot were recorded. Grain yield parameters measured were grain weight per pot (GY), grain weight ear^{-1} and Harvest Index (HI), as the ratio between grain and total aboveground weight. Potential maximum yield ($GY_{potential}$) was calculated by adding the potential yield of immature ears (number of immature ears pot^{-1} * mean grain weight ear^{-1}) to the GY.

2.5 Carbon and Nitrogen Allocation

Carbon and N content were measured on FLs (C_L and N_L respectively), collected at anthesis (DaE 42) and Carbon and N content of mature grains, (C_G and N_G, respectively), were measured at harvest (DaE 77). Full FLs of the main stem were taken per pot, pooled per OTC and grounded (n=3 per O_3 and N treatment). Grain samples were analyzed per pot (n=12 per O_3 and N treatment). FL samples were analyzed in the CCiT (Centres Científics i Tecnològics) at the University of Barcelona by combustion using an elemental analyzer (Flash 1112 EA, ThermoFinnigan, Bremen, Germany). Grain samples were analyzed by combustion in the CIEMAT lab of the Chemistry Division using a LECO TruSpec CHN elemental analyzer (Leco Instruments S.L., Michigan, USA).

The N allocated to the harvested part of the plant was calculated from N_G (%) as Grain N Yield: GNY = (N_G × GY)/100, gN pot^{-1}. Protein content in the grain (Protein Yield, PY) was converted as GNY * 5.83 (g pot^{-1}), using the specific Jones factor to convert N to protein content in wheat (FAO, 2003).

2.6 Nitrogen use efficiency

Nitrogen use efficiency (NUE) was calculated per pot to analyze how the additional amount of added N-fertilizer aided in increasing grain yield; NUE = (GY$_{\text{Nhigh}}$-GY$_{\text{Nlow}}$)/(N-applied$_{\text{Nhigh}}$-N-applied$_{\text{Nlow}}$) (gDW gN^{-1}). The Agronomic Efficiency of Nitrogen (AEN) delved into the amount of N applied that actually went into N in the grain, was calculated as: (GNY$_{\text{Nhigh}}$-GNY$_{\text{Nlow}}$)/N-applied$_{\text{Nhigh}}$-N-applied$_{\text{Nlow}}$ (gN$_{\text{grain}}$ gN$_{\text{applied}}$$^{-1}$) (Adeoluwa et al., 2022; Moll et al., 1982) where GY$_{\text{Nhigh}}$ and GY$_{\text{Nlow}}$ refer to GY for High-N and Low-N respectively; and GNY$_{\text{Nhigh}}$ and GNY$_{\text{Nlow}}$ refer to GNY for High-N and Low-N respectively. N-applied$_{\text{Nhigh}}$ and N-applied$_{\text{Nlow}}$ refer to the amount of fertilizer applied to High-N and Low-N respectively calculated as grams of N supplemented per pot.

Two ratios were also considered to evaluate the N$_L$ use in the photosynthetic-related process under the different O$_3$ levels. The rate Chl to N$_L$ (ChlN) and the Photosynthetic Nitrogen Use Efficiency (PNUE), are both calculated based on the specific SLA for Artur Nick. The foliar chlorophyll content provided by the leaf N concentration (ChlN), was calculated as: ChlN= Chl/ N$_L$ * SLA (□mol Chl gN^{-1}). PNUE was calculated to determine the photosynthetic capacity per unit of leaf N as the A to N$_L$ concentration ratio: PNUE = A / N$_L$ * SLA (□molCO$_2$ gN^{-1} s^{-1}).

2.7 Ozone exposure indices AOT40 and POD and response functions

Two currently employed O$_3$-exposure indices, which are based on the concentration of the pollutant in the air (AOT40), and the O$_3$ dose absorbed by the plant (POD6), were calculated to quantify the exposure and dose thresholds associated with a 5% effect on different growth and yield parameters, following the methodology of the United Nations Air Convention (UNECE) for establishing O$_3$ critical levels (CLs) for plant and crops protection (CLRTAP, 2017). Considering that, in the present assay, the O$_3$-exposure period began at tillering, O$_3$ indexes were also calculated for both pre-anthesis and post-anthesis periods, where anthesis began at DaE 31 (Figure 1S).

The Accumulated hourly Ozone concentration over a Threshold of 40 nL L^{-1} index (AOT40), was calculated by adding the O$_3$-hourly concentration values over 40 nL L^{-1} during daylight hours (PAR>100 □mol m^{-2} s^{-1}) for the O$_3$-exposure period, from the beginning of exposure (April 24, DaE 0) till the final harvest (July 10, 77 DaE), expressed as nL L^{-1} h (Fuhrer et al., 1997). This index showed a robust linear relationship between wheat yield losses and O$_3$ levels (CLRTAP, 2017; Pleijel et al., 2004) and is currently considered in the EU Air Quality Directive (2008/50/EU) for the definition of the Objective and the Long-Term target values to protect vegetation (EEA, 2022). The O_3 7-hour mean value (nL L^{-1}), from 09:00-16:00 GMT, was also calculated as an alternative exposure index.

Phytotoxic Ozone Dose (POD) indexes were calculated through the DO3SE (Deposition of Ozone for Stomatal Exchange) model adopted by the Air Convention-UNECE (CLRTAP, 2017). The POD approach was developed more recently than exposure-based indices and comprises the estimation of O_3 uptake by upper canopy sunlit leaves. POD indexes are biologically more meaningful and better related to effects than exposure-based indicators (Pleijel et al., 2022). They are currently recommended within the Air Convention for O_3-risk analysis for crops at the regional and global scale (CLRTAP, 2017) and are also suggested for monitoring O_3-risk for vegetation and crops within the European network established for this purpose in the frame of the EU National Emissions Ceiling Directive (NEC monitoring network) (EU 2016/2284).

The O_3 dose absorbed through the stomata is specifically calculated from O_3 concentration at the surface of the leaf and the stomatal pore opening based on g_s. Ozone concentration at the surface of the leaf is modeled using aerodynamic, boundary layer and surface resistances, affected by wind speed and leaf morphology (CLRTAP, 2017; Emberson et al., 2000; Grünhage et al., 2012). The g_s is estimated considering abiotic factors and plant phenology that modulate a species-specific maximum stomatal conductance (g_{max}) (CLRTAP, 2017; Emberson et al., 2000; Grünhage et al., 2012; Jarvis, 1976). Among the factors considered, some are related to growth conditions like air temperature and humidity measured through the Vapor Pressure deficit (VPD), Photosynthetic Active solar Radiation (PAR) and soil water availability (SW); other factors related to plant physiology, like the plant phenological stage (Phen), are also considered in the g_s calculation. Stomatal conductance due to O_3 was modeled with: $g_s = g_{max} * f_{light} * f_{phen} * MAX((f_{temp} * f_{VPD} * f_{SW}); f_{min})$. In the model g_{max} is the species-specific maximum stomatal conductance. The factors f_{light}, f_{temp}, f_{VPD} and f_{sw} refer to the limiting effect of environmental variables on g_s by PAR, air temperature, vapor pressure deficit and soil water potential, respectively, expressed in relative terms ranging from 0 to 1, proportionally to g_{max}. Finally, f_{phen} refers to the phenology and f_{min} to the relative minimum stomatal conductance happening during daylight hours (CLRTAP, 2017). This methodology has been widely applied to estimate and map O_3 effects on wheat yield and quality (Colette et al., 2018; Gonzalez-Fernandez et al., 2010; Grünhage et al., 2012; Mills et al., 2018c; Pleijel et al., 2007; Pleijel et al., 2022). The DO3SE specific parameterization for Mediterranean bread wheat has also been described (CLRTAP, 2017; González-Fernández et al., 2013). In this experiment, f_{SW} was set to 1 due to the absence of water deficit during the assay; f_{phen} was estimated based on thermal time accumulated from the date of anthesis. The parameters of the g_s model are described in Table 1S. Hourly POD values, based on DO3SE were accumulated over the threshold of 6 mmol m^{-2} PLA s^{-1} to calculate POD6 (mmol m^{-2} PLA s^{-1}) following the CLRTAP methodology (CLRTAP, 2017).

Ozone response functions, adhering to the Air Convention methodology (CLRTAP, 2017), were developed for the AOT40 and POD6 indices, both collectively for all N treatments, and separately. Baseline values corresponding to the different O_3-sensitive parameters under low O_3-exposure (AOT40= 37 nL L^{-1}; POD6=0.8 mmol m^{-2} PLA s^{-1}) were calculated from linear response functions, and these values were used to build the standardized response functions. Ozone Critical Levels (CLs) were calculated as the O_3 exposure or dose resulting in a relative yield or growth loss of 5% according to the Air Convention methodology (CLRTAP, 2017).

2.8. Statistical Analysis

Differences among the O_3 levels and N factors were assessed with a modeled univariate analysis following a split-plot statistical design, where O_3 was the complete randomized factor and N the split-plot factor; the block was also considered as a random factor to integrate the randomization of the OTC field design. Significant differences were described for p-values lower than 0.05; p-values lower or equal to 0.1 were described as trends. The Tukey Honestly Significant Difference test (HSD) analysis was performed to identify differences among treatments when significant differences ($p<0.05$) were observed in the split-plot analysis. All data were first checked for normality and homoscedasticity with Shapiro–Wilk and Levene tests respectively ($p<0.05$) and when data did not fulfil these assumptions, these were log or square root transformed. Outlayers were rejected based on the studentized residuals. Relationships among variables were studied through the significance of the Pearson correlation coefficient. Differences between slopes of the Low-N and High-N functions were tested with ANOVA. Statistical analyses were performed on Statistica v.13 (StatSoft Inc., Tulsa, OK, USA).

3. Results

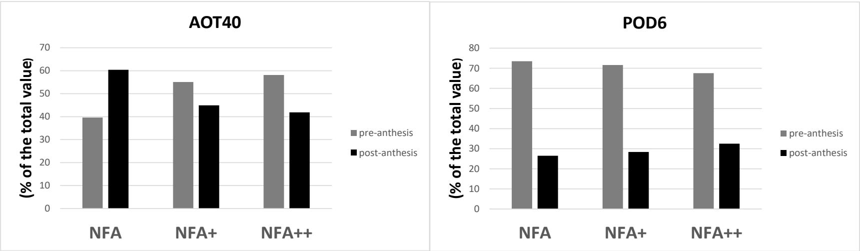
3.1 Experimental growing conditions, ozone exposure and dose

Throughout the O_3-exposure period, the monthly hourly mean temperature inside the OTCs ranged between 8 and 42ºC with a daily pattern that peaked around 14:00 GMT (Fig. 2Sa). Temperatures increased through the season reaching maximum values in July. RH followed the opposite pattern to temperature: the monthly hourly mean RH ranged between 18 and 89%, reaching its lowest point during the central hours of the day around 15:00 GMT, and declined throughout the season, reaching its lowest values in July coinciding with the maximum temperatures. Both parameters were reflected by the VPD, where the daily patterns peaked slightly earlier than for temperature, around 13:00 GMT, and increased through the season, with monthly hourly means ranging between 1.2 KPa in April and 6.8 KPa in July.

Throughout the fumigation period (Fig. 2Sb), the NFA daily O_3 profile reproducing the ambient air quality of the farm, followed similar patterns every month, from April to June, increasing slightly in July to reach a maximum hourly average of 55 nL L^{-1}. The fumigated NFA+ and NFA++ treatments reached maximum means of 65 and 90 nL L^{-1}, respectively. Table 1 shows the corresponding 7h-mean, AOT40 and POD6 indices for the different O_3 treatments during the exposure period. The 7h-mean ranged from 45 nL L^{-1} in NFA to 65 nL L^{-1} in NFA++. AOT40 values were 5374, 10057 and 16720 nL L^{-1} h for NFA, NFA+ and NFA++, respectively. The calculation of the O_3-absorbed dose index POD6 led to an accumulated value of 5.44 mmol m^{-2} PLA for the NFA treatment (Table 1). NFA+ and NFA++ treatments had POD6 values of 8.49 and 12.56 mmol m^{-2} PLA, respectively. Upon separating the O_3 values into the pre-anthesis and post-anthesis periods, a consistent distribution was observed for AOT40, indicating similar O_3 levels in both periods. However, the POD6 index revealed an important difference, with nearly 70% of the O_3 absorption occurring during pre-anthesis for all the O_3 treatments (Table 1).

Table 1. Ozone indices based on O_3-concentration (7h mean and AOT40), and on O_3-absorbed dose (POD6) for the experimental period from the start of the fumigation on April 24 (DAE 0) till the harvest on July 10 (DAE 77). 7-h mean (nL L^{-1}) from 09:00-16:00 h solar hours; AOT40=Accumulated O_3 diurnal hourly values over 40 nL L^{-1}. POD6= Phytotoxic O_3 dose accumulated over 6 mmol m^{-2} PLA (mmol m^{-2} PLA). FA =filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++=non-filtered air + 40 nL L^{-1} of added O_3. Graphs represent the percentage of the O_3-exposure to the total for the pre and post-anthesis periods.

O_3 treatment	**7-hour mean** (nL L^{-1})	**AOT40** (nL L^{-1} h)	**POD6** (mmol m^{-2} PLA)
NFA	45.0	5374	5.44
NFA+	53.9	10 057	8.49
NFA++	65.3	16 720	12.57
Pre-anthesis			
NFA	45.0	2128	4.00
NFA+	60.4	5539	6.08
NFA++	77.8	9720	8.49
Post-anthesis			
NFA	44.9	3246	1.44
NFA+	49.6	4519	2.41
NFA++	57.1	7000	4.08



3.2. Carbon and nitrogen foliar content, chlorophyll and ChlN

Foliar leaf N at 42 DaE showed an increasing trend when exposed to O_3 (p=0.075), especially under High-N. Plants grown under the highest O_3 levels (NFA++) increased N_F by 10% when compared with NFA (Fig. 1a). Regarding C_F, the analysis also revealed a trend in the O_3 response (p=0.083) but modulated by the O_3xN interaction (p=0.10). At the highest O_3 levels, C_F tended to increase slightly under High-N but tended to decrease under Low-N (Fig. 1b). The differences in the C_F and N_F responses, unbalanced the C/N rate in High-N (Fig. 1c), but the variability in the data did not allow a significant effect.

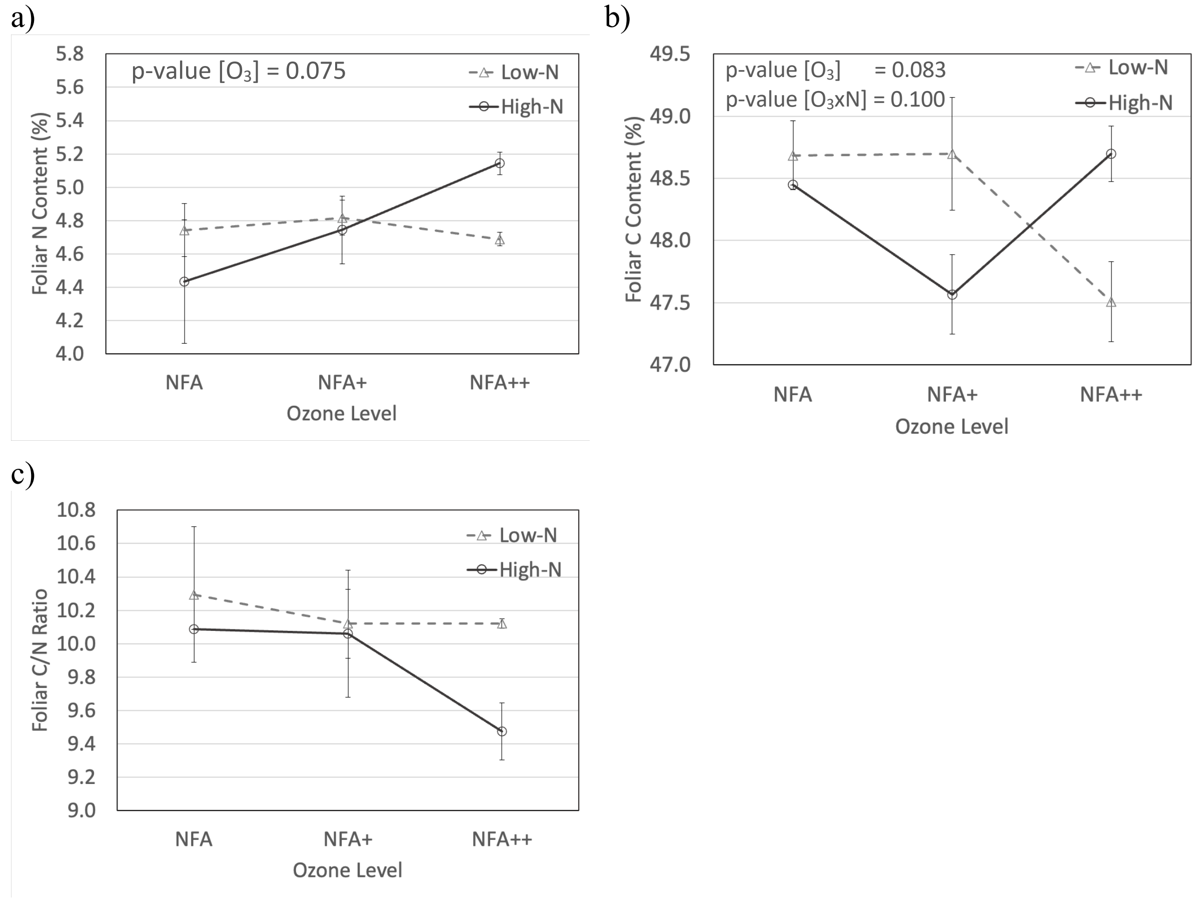


Figure 1. Effects of O_3 and additional N fertilizer on a) foliar nitrogen content (N_F, %); b) carbon foliar content (C_F, %); and c) C/N ratio of the flag leaves. Mean □ SE. FA =filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++ = non-filtered air + 40 nL L^{-1} of added O_3; N-low= total added dose of 100 kg N ha^{-1}, N-high= total added dose of 200 kg N ha^{-1}.

Flag leaf Chl measurements on recently expanded flag leaves (42 DaE) with additional N tended to increase across O_3 levels (p=0.100). A significant interaction between N and O_3 was also found (p=0.025): O_3 increased Chl content by 6% in NFA++ under High-N, but not under Low-N treatment (Fig. 2a). Thus, NFA++ Chl levels in High-N were 26% higher than at

Low-N. Although non-significant, the N-modulation of the O_3-response was perceived in the response pattern of the ChlN rate, which tended to decrease in NFA++ under High-N but was stable through the O_3 levels under the lower dose of the fertilizer (Fig. 2b). When High-N results were analyzed separately, O_3 tended to decrease ChlN ($p=0.09$) by 5.6% in NFA++.

After a longer O_3-exposure of flag leaves at 51 DaE, the pollutant intensified pigment loss in the FLs similarly under both N treatments ($p= 0.033$) (Fig. 2c). The chlorophyll reduction in NFA++ compared with NFA lay within the 22-34% range for High-N and Low-N, showing a comparable FL senescence induced by the exposure to the pollutant despite the fertilizer dose applied.

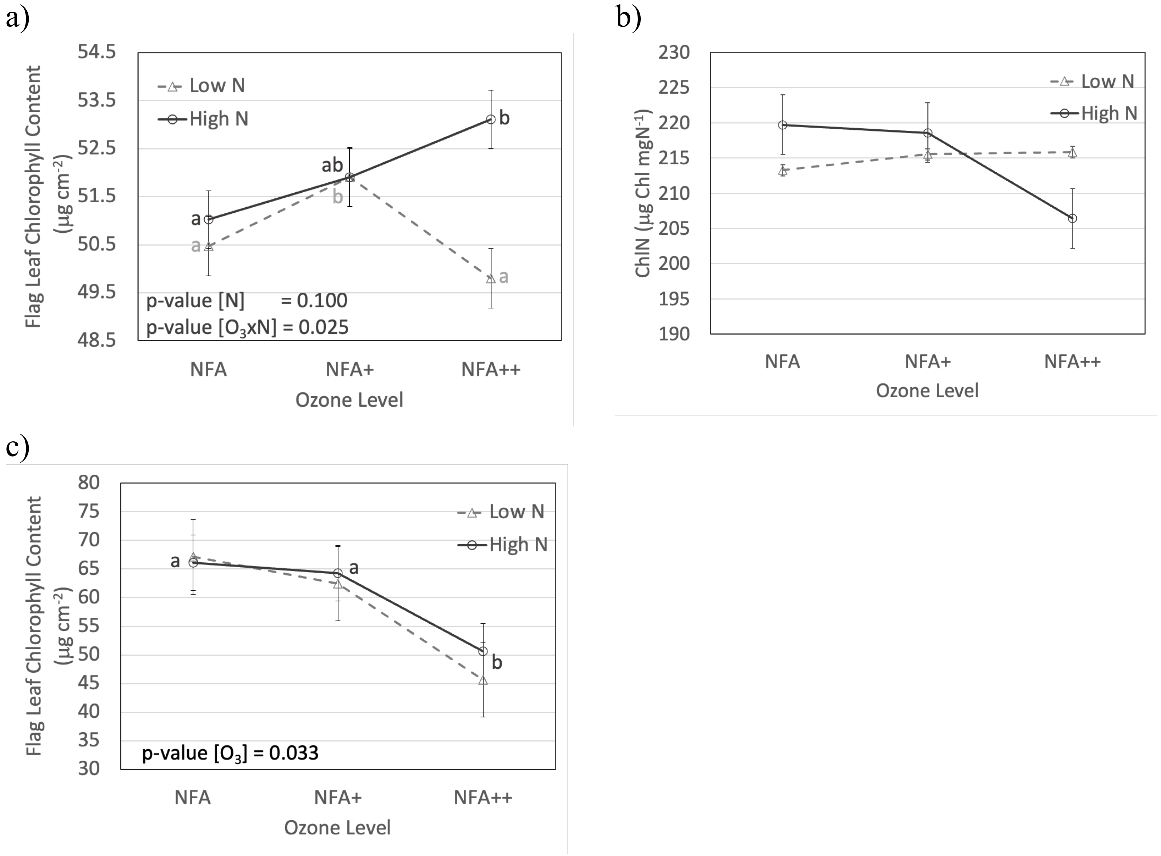


Figure 2. The effects of O_3 and additional N fertilizer on a) FL Chlorophyll content (□g cm^{-2}) at 42 DaE (June 5); b) ChlN (□g Chl mgN^{-1}) at 42 DaE (June 5) and c) FL Chlorophyll content (□g cm^{-2}) at 52 DaE (June 14). Mean □ SE. FA =filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++=non-filtered air + 40 nL L^{-1} of added O_3; N-low= total added dose of 100 kg N ha^{-1}, N-high= total added dose of 200 kg N ha^{-1}. Letters indicate significant differences among O_3 levels

3.3 Gas Exchange and PNUE

Gas exchange parameters analyzed on 37-38 DaE showed non-significant differences in A, g_s or Tr between O_3 levels. However, Ci showed a decreasing pattern with O_3, especially under High-N, which caused an increasing trend in the A/Ci ratio with the pollutant exposure (p=0.09) (Fig. 3a). The highest N dose caused an A/Ci increment of 10% in NFA++ compared with NFA. When A_{FLsurf} was calculated considering photosynthesis of the whole FL surface, the pollutant caused a stronger effect on plant photosynthesis (p=0.03), decreasing by 15% in NFA++ compared with NFA under High-N (Fig. 3b). A_{FLsurf} was also 18% larger under High-N than Low-N (p=0.04) across O_3 levels (Fig. 3b).

Considering PNUE, the pattern caused by the pollutant seems to follow A_{FLsurf}, remaining rather stable across O_3 levels under Low-N but decreasing under High-N as the pollutant increased (Fig. 3c). However, the O_3 effect across N levels was not significant and only a reducing trend was observed with the N factor, due to the lower values found in High-N (p=0.1) (Fig. 3c).

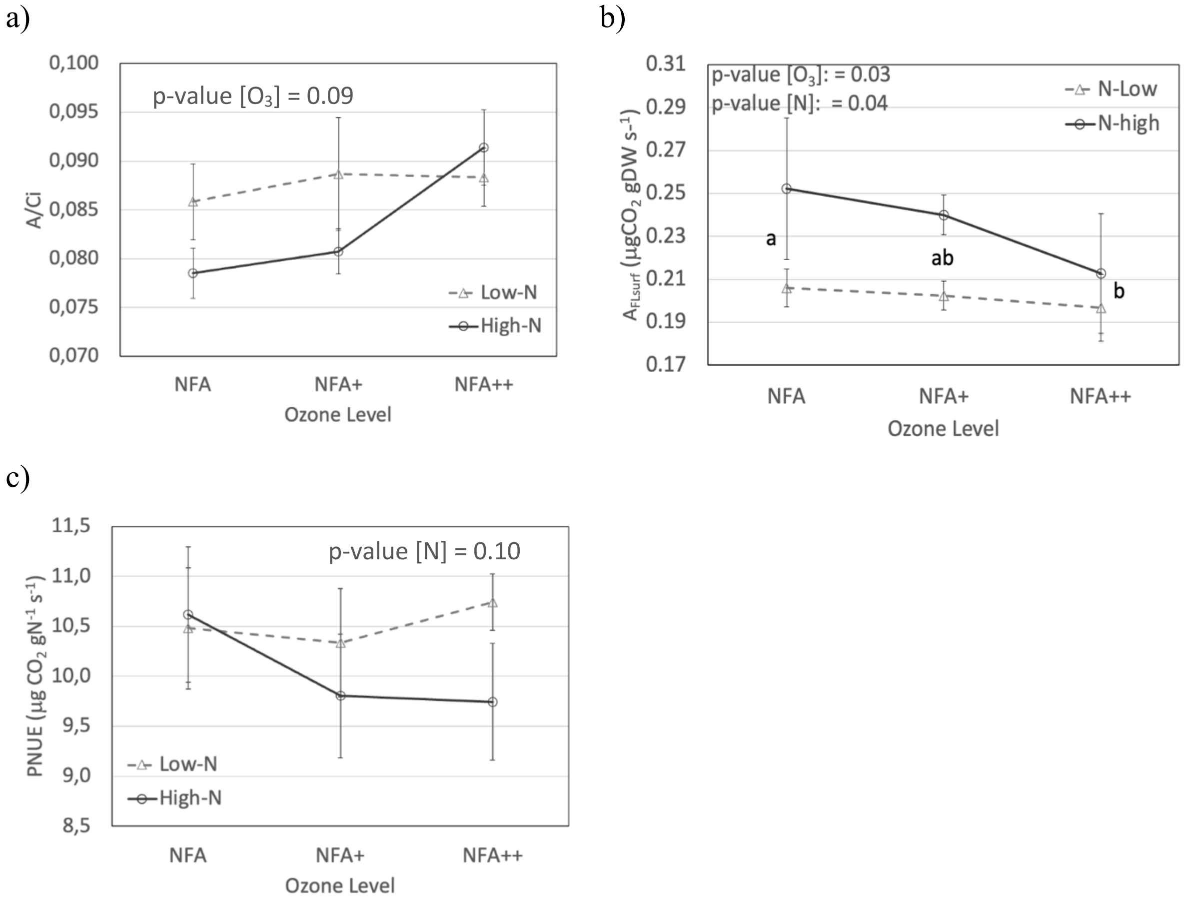


Figure 3. Effects of O_3 and additional N fertilizer on: a) A/Ci rate; b)Photosynthesis of total FL surface (A_{FLsurf}, □mol CO_2 s^{-1}); c) Photosynthetic Nitrogen Use Efficiency (PNUE, □g CO_2 gN^{-1} s^{-1}). Mean □ SE. FA =filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++=non-filtered air + 40 nL L^{-1} of added O_3; N-low= total added dose of 100 kg N ha^{-1}, N-high= total added dose of 200 kg N ha^{-1}. Letters indicate significant differences among O_3 levels.

3.4 Growth and Yield

Increasing N fertilization up to a total dose of 200 kg ha^{-1} led to larger plants and increased most of the growth parameters analyzed (Table 2S). Significant increments in response to N fertilization were found for the number of mature ears (increased by 8% across O_3 treatments), total number of ears (by 9%), HS weight (by 13%), FL weight (by 19%), and total aboveground biomass (by 12%). The number and weight of immature ears and the straw weight (SW) were not affected by the fertilizer across O_3 levels.

A significant (p=0.039) effect of the O_3 factor was observed in the total number of ears, which were reduced by 10% (across N levels) under the highest O_3-levels of the NFA++ treatment (Table 2S, Fig. 4). However, for most of the growth parameters, the significant O_3xN interaction revealed that the impact of the pollutant depended on the fertilizer dose. The O_3 effect was stronger under High-N, limiting the growth stimuli induced by the highest fertilizer dose (Table 2S, Fig. 4).

FL weight experienced a 17% reduction in plants exposed to NFA++ under High-N, compared to both NFA and NFA+. Interestingly, despite the lower FL weight values observed under Low-N compared to High-N, O_3 did not significantly affect FL weight in Low-N plants. Furthermore, the O_3-induced reductions in FL weight counteracted the weight gain induced by the higher fertilization dose. FL weight increases in High-N compared to Low-N reached 22% and 28% in NFA and NFA+, respectively, but differences diminished to a 9% increase under NFA++ (Fig. 4a). Similarly, HS weight presented a close response pattern: under Low-N, HS weight was 9% lower in NFA++ than in NFA. However, this effect was significantly amplified under High-N, where the weight decreased by 28% by NFA++. As a result, even though under NFA and NFA+ treatments, additional nitrogen increased HS by 28% and 17%, respectively, this effect was lost by NFA++ (Fig. 4b). Plant SW, which was 29 and 20% larger under NFA and NFA+ in High-N compared to Low-N, was only 4% higher in NFA++ (Fig. 4c). Total aboveground biomass and total number of ears follow the same pattern: differences between Low-N and High-N ranged from 12-24% in NFA and NFA+ but disappeared in NFA++ (Fig .4d-e).

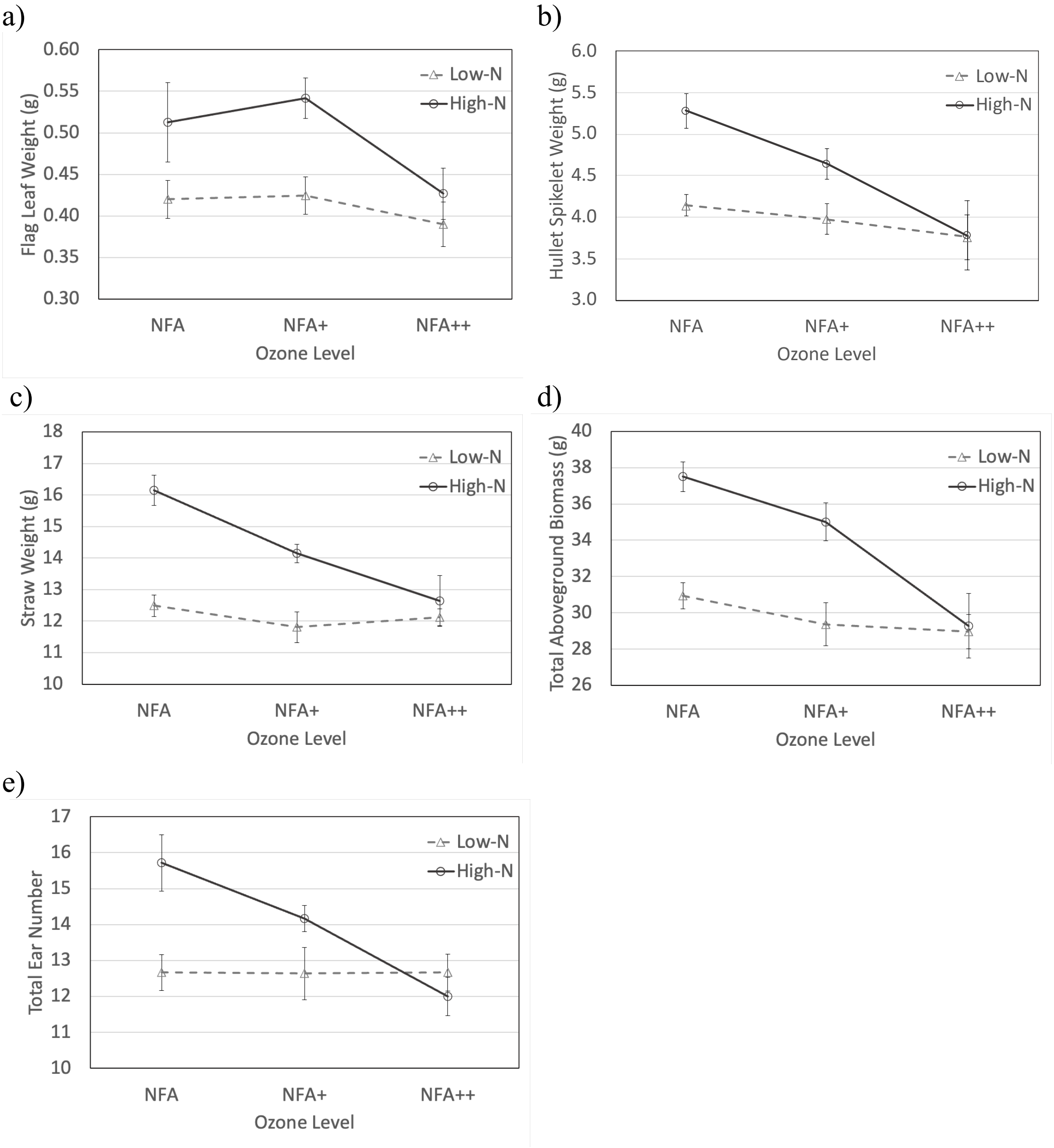


Figure 4. Effects of O_3 and additional N fertilizer on growth parameters (Mean □ SE): a) flag leaf blade weight (gDW pot^{-1}); b) hulled spikelet weight (gDW pot^{-1}); c) straw weight (gDW pot^{-1}); d) total aboveground biomass (gDW pot^{-1}); and e) total number of ears. FA =filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++=non-filtered air + 40 nL L^{-1} of added O_3; N-low= total added dose of 100 kg N ha^{-1}, N-high= total added dose of 200 kg N ha^{-1}.

Yield parameters followed a close pattern to the growth parameters. N-fertilization caused a general increase in mature ears weight, GY, GY per ear, and especially in Protein Y, which grew by 15% under High-N (mean value across O_3 treatments). Nitrogen also reduced HI significantly ($p=0.002$) by 5% (across O_3 levels) (Table 3S, Fig. 5). O_3 effects were only found under the highest fertilizer treatment, and yield gains caused by the fertilizer in High-N were counteracted by O_3 in the NFA++ treatment. Under High-N, the observed losses induced by the pollutant for GY and Potential GY in NFA++ (compared with NFA) were in the range of 21-23%. On the contrary, no significant differences were found for these parameters under Low-N. In consequence, differences in the GY between High-N and Low-N, which were 12% in NFA and NFA+, disappeared in the NFA++ treatment. This effect was even greater when the Potential GY was considered, where the fertilizer improved values by 16% under NFA+, but was completely lost by NFA++. In the case of Protein Y, Low-N did not experience any effect due to the exposure to the pollutant, and grain protein was stable through the O_3 treatments. However, in High-N, losses caused by NFA++ were around 20% compared with NFA. Moreover, while the differences between N levels were 18 and 19% for NFA and NFA+, respectively, they were eliminated by NFA++.

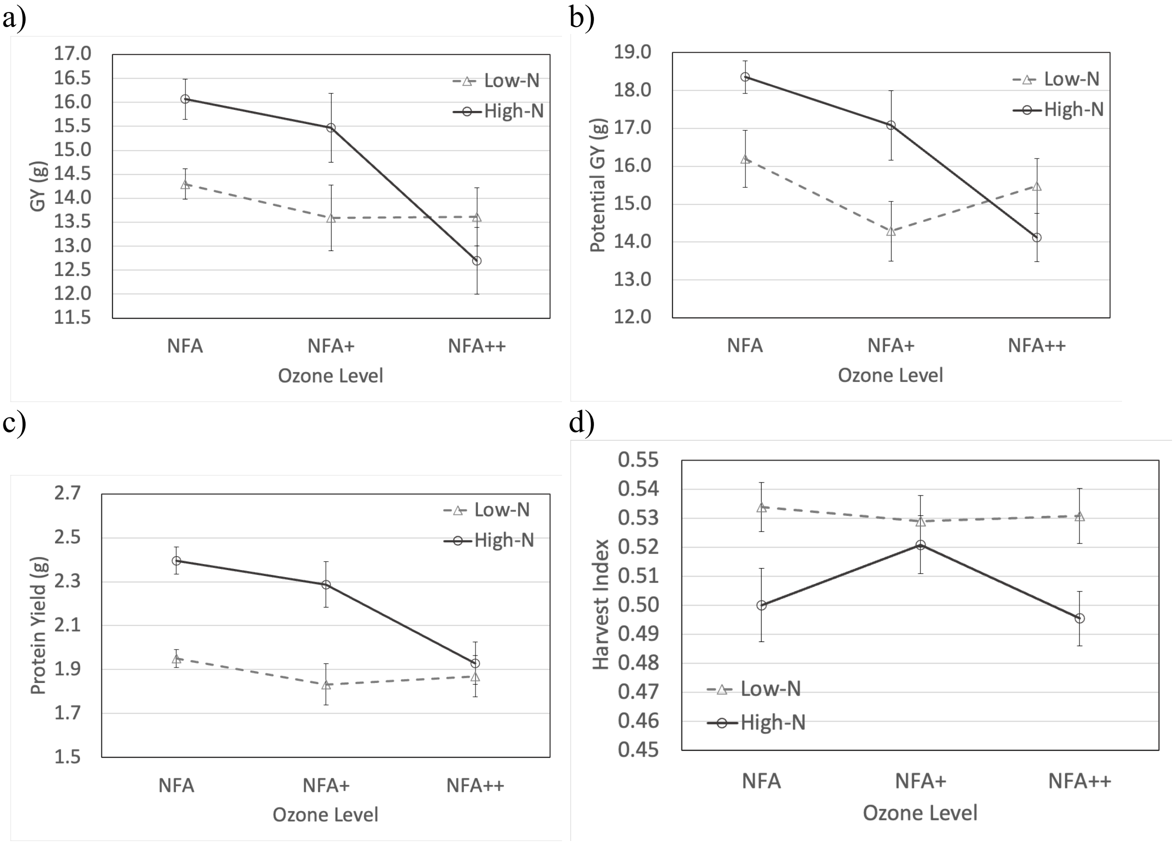

a) b) c) d)

Figure 5. Effects of O_3 and additional N fertilizer on yield parameters (Mean □ SE): a) Grain Yield (GY, g pot^{-1}); b) Potential Grain Yield (Potential GY, g pot^{-1}); c) Protein Yield (g pot^{-1}) and d) Harvest Index. FA =filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++=non-filtered air + 40 nL L^{-1} of added O_3; N-low= total added dose of 100 kg N ha^{-1}, N-high= total added dose of 200 kg N ha^{-1}.

3.5 Nitrogen use efficiency

The pattern seen in growth and yield parameters was reflected by the NUE and AEN indexes (Fig. 6). Additional N maintained high values of both indices up to NFA+, but a pronounced decline was observed under the highest O_3 exposure. AEN dropped by 83% between NFA and NFA++ ($p=0.022$). The NUE index also experienced an intense decline in NFA++ ($p=0.041$), pointing out that differences in yield due to the fertilization increase were lost under the highest exposure to the pollutant.

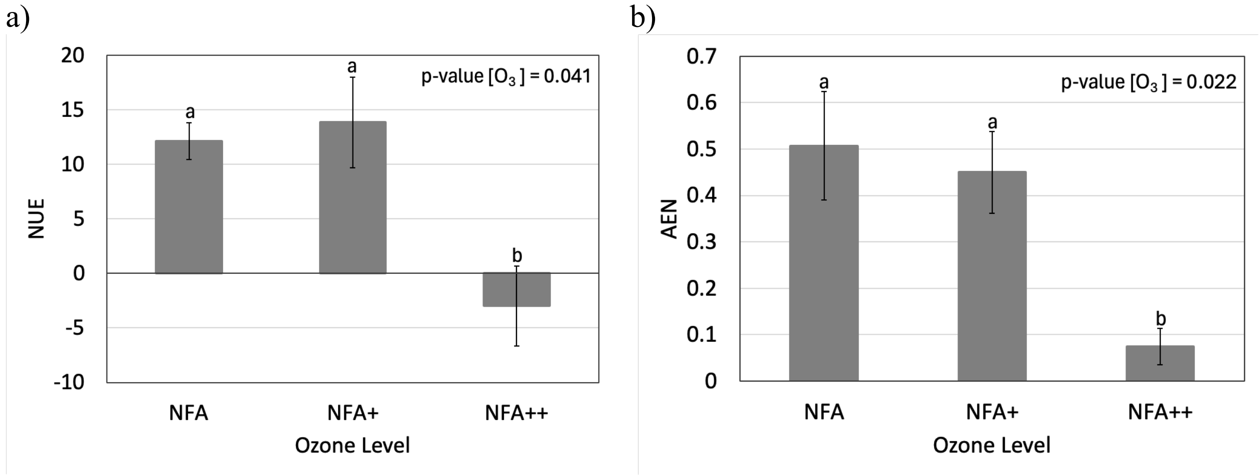


Figure 6. Effect of O_3 and additional N fertilizer on: a) NUE, Nitrogen Use Efficiency, and b) AEN, Agronomic Efficiency of Nitrogen. FA =filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++=non-filtered air + 40 nL L^{-1} of added O_3. Mean values ± SE. Letters indicate significantly different values calculated under Tukey HSD.

3.6 POD and AOT40 response functions and critical levels

Ozone exposure- and dose-response functions were constructed for the O_3-sensitive parameters, considering both AOT40 and POD6 indexes. Table 4S lists these functions relativized to clean background exposure levels according to the Air Convention methodology (CLRTAP, 2017). Functions were constructed joining data from both N treatments, but also for each N treatment separately, on account of the significant O_3xN interactions found in this assay. When a joint analysis was conducted to confirm the statistical differences between the slopes of both N doses for all the O_3-sensitive parameters (Table 4S), results confirmed that High-N slopes were more negative than Low-N slopes considering AOT40 ($F_{1, 18}= 24.79$, $p< 0.001$) and POD6 ($F_{1, 18}= 21.8$, $p< 0.001$) indexes.

In general, most of the growth and yield parameters showed a significant negative linear correlation with O_3 exposure or dose (across N levels). Most functions presented a high R^2 (range 0.91-0.99), except for FL weight, which showed lower values ($R^2=0.46$), being the fit similar for both the AOT40 and POD6 indices. When data was split between N treatments, High-N functions indicated a stronger O_3 effect given their more negative slopes, which more than doubled the Low-N values. Moreover, for some parameters like GY and Protein Y, differences in the slopes were intensified by 4-5 times (Fig. 7, Table 4S). Parameters like SW or Protein Y did not respond to O_3 under low N-dose, but showed a negative strong correlation with the pollutant under higher levels of fertilization. The fitness in High-N functions was slightly better than in Low-N, especially for GY and Protein Y.

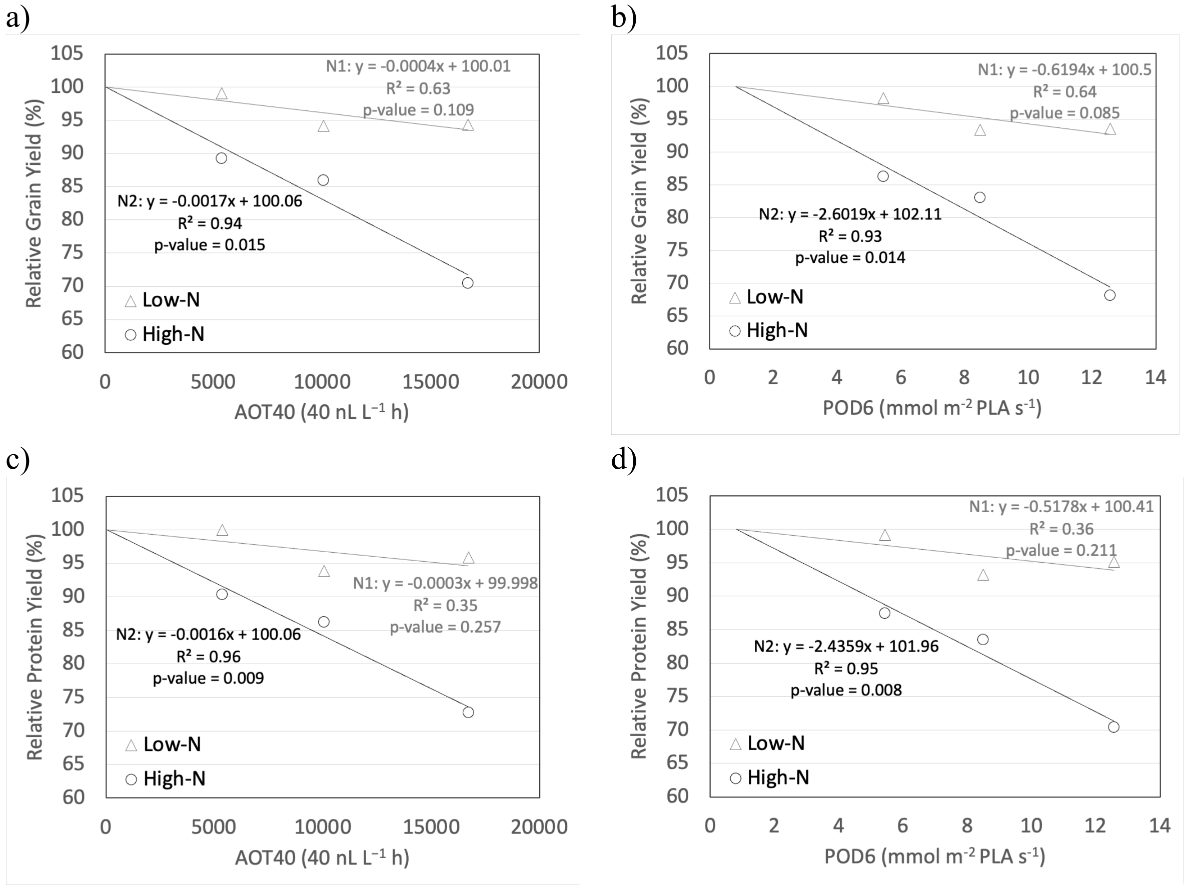


Figure 7. Ozone standardized response functions for AOT40 (40 nL L^{-1} h) and POD6 (mmol m^{-2} PLA s^{-1}): a) Grain Yield (GY) and; b) Protein Yield.

Ozone Critical Levels for a 5% effect on yield, growth and chlorophyll loss were calculated following the Air Convention Methodology from the different exposure- and dose-response functions (Table 4S). The most sensitive parameter was Chl loss showing an AOT40 CL of only 2309 nL L^{-1} and POD6 of 2.3 mmol m^{-2} PLA. The CLs calculated for GY were 5050 nL L^{-1} h and 4.2 mmol m^{-2} PLA for AOT40 and POD6 respectively; most of the studied parameters presented CLs in the range of 5000 nL L^{-1} h and 4 mmol m^{-2} PLA. When functions for each N treatment were considered, CLs greatly differed for the same parameter. In general, CLs based on Low-N functions more than doubled the CLs of the High-N functions. For GY, CLs for Low-N were 12525 nL L^{-1} h for AOT40 and 8.9 mmol m^{-2} PLA for POD6; meanwhile, the target values decreased in High-N to 2976 nL L^{-1} h and 2.7 mmol m^{-2} PLA. Similarly, Protein Y CLs decreased by 5 times in High-N functions compared to Low-N.

4. Discussion

Experimental conditions

The meteorological conditions of the OTC-assay reproduced the continental Mediterranean climate of the central Iberian Peninsula. Temperatures increased and RH decreased throughout the season, causing the most stressful atmospheric conditions, shown by the VPD, in early July, coinciding with the end of the cereal life cycle. Meanwhile, anthesis occurred in late May, under more benign atmospheric conditions, favoring the wheat photosynthetic activity required for the grain filling period (Nasehzadeh & Ellis, 2017; Teng et al., 2023) and allowing high stomatal absorption rates of air pollutants such as O_3 (Pleijel et al., 1994). The reproductive period is considered the most O_3 sensitive period for wheat (Pleijel et al., 1998), and under Mediterranean growing conditions, this stage coincides with the high O_3 values that occur in spring and when environmental conditions (absence of severe water stress causing stomatal closure) still favor its absorption. However, it should be noted that from the tillering stage until anthesis (late April-late May) O_3 values could also be high. In this experiment, the 7-hour mean and AOT40 were similar for both the pre-anthesis and post-anthesis periods under NFA. Nonetheless, the O_3 dose index POD6, which is calculated over the flag leaf lifespan, was higher during the FL development stage, prior to anthesis (Table 1), when the atmospheric conditions were less stressful for the gas exchange process. On average for all the O_3 treatments, 71% of the O_3-dose was absorbed pre-anthesis. This result emphasizes the potential importance of O_3 exposure during the vegetative stage for wheat growth and storage capacity. This is especially important for areas chronically affected by water deficit and high temperatures post-anthesis, like the Mediterranean, where grain filling can depend highly on pre-anthesis stored assimilates (Sanchez-Bragado et al., 2014).

The AOT40 under NFA, which reproduced the ambient levels in the farm, widely exceeded the air quality objective value for plant protection of the EU Directive of 3000 nL L^{-1} h (2008/50/EC). Moreover, this exceedance happened after 77 days of exposure, while the EU Directive establishes a 90-day period for this cumulative index. These levels reproduced the high O_3 values that chronically affect crop productivity in one of the main cereal-producing areas of the Iberian Peninsula, and which frequently exceed the crop protection thresholds of the European Air Quality Directive (MITERD, 2022). The accumulated value of 3000 nL L^{-1} h is also the threshold established by the Air Convention to specifically protect wheat from excessive yield losses (CLRTAP, 2017). However, it should be noted that early spring O_3 concentrations are not considered since the index considers the cumulative period from anthesis to harvest. As discussed earlier, the high O_3 concentrations recorded in early spring, which match the period when water deficit and other atmospheric and phenological factors do not limit O_3 absorption, may cause a greater potential damage to cereal production in Mediterranean wheat than late spring O_3 levels.

Dose-based O_3 indexes, such as POD6, are thought to represent crop responses to the pollutant more accurately than exposure-based indices since they estimate the amount of the pollutant entering the plant and producing damage (CLRTAP, 2017; Mills et al., 2011; Pleijel et al., 2022). Following experimental results from central and Northern Europe used to develop the dose-response relationships (Grünhage et al., 2012), O_3 dose-based critical levels of 1.3, 1.5 and 2 mmol m^{-2} PLA have been established for a 5% effect on grain yield, 1000-grain weight, and protein yield, respectively. In this assay, the post-anthesis POD6 in NFA was 1.44 mmol m^{-2} PLA, slightly exceeding some of the CL for wheat. Cumulative POD6 during pre-anthesis in NFA reached 4.00 mmol m^{-2} PLA, and in the whole period, POD6 was 5.44 mmol m^{-2} PLA, highlighting the problem of tropospheric O_3 on Iberian agriculture. This is of special concern considering that wheat is one of the most extensively cultivated crops in Spain occupying 4.5 M ha (MAPA, 2021). POD6 values spatially calculated for central areas of the Iberian Peninsula for O_3 risk assessment on agricultural production, rate among the highest in Europe. POD values can exceed 6 mmol m^{-2} PLA, even after considering the reduction in the O_3 absorption caused by drought (Colette et al., 2018; Schucht et al., 2021). This suggests that, at present, the calculated O_3-doses for NFA+ and NFA++ may have already been reached during elevated O_3 episodes under irrigated conditions.

Plant physiological responses

At the earlier sampling date (DaE 42), O_3 induced increases in Chl and N_F under High-N, resulting in 26 and 16% greater values, respectively, than Low-N under NFA++. Chlorophyll loss and early senescence have been frequently associated with O_3 exposure for various plant species (Brewster et al., 2024; Héctor Calvete-Sogo et al., 2014; Emberson et al., 2018). However, the results of the present study could be related to the ability of the plant to avoid pigment losses under high levels of the pollutant thanks to the supplementation of extra N. The capacity of preserving foliar pigments under high O_3 levels has been related to plant tolerance to the pollutant (Pleijel et al., 2006). While the Low-N dose would suffice to maintain Chl levels under increasing O_3 concentrations, the additional N could aid in maintenance and better preparation to tolerate sustained stress at the early stages of FL development.

The O_3-induced N_F increase, seemed to unbalance the ChlN ratio (Fig. 2b), indicating that a lower proportion of N would be dedicated to leaf pigments. The joined responses of Chl, N_F and ChlN pointed out that under high O_3 levels, if excessive N is supplied to the plant, N could be used in processes other than foliar pigment maintenance. The pattern seen in C/N would also indicate that the extra N assimilated and translocated to the leaf was not used in the construction of carbonaceous structures, as that would have led to more stable patterns under NFA+ and High-N (Fig. 1c) neither led to an increase in A, due the pattern of PNUE (Fig. 3c). Results, would suggest that in polluted environments there is less use of N_F for photosynthetic activity and that the excess N aids in other metabolic pathways than carbon fixation, related to defense, protective or reparative processes, as has been suggested by previous works

(Calatayud et al., 2002). N-based compounds like antioxidant enzymes or secondary metabolic pathways to provide resistance (i.e. isoprenoids, alkaloids) can give the plant a greater ability to cope with O_3 stress when growing under High-N (Fatima et al., 2018; Mukherjee et al., 2018). In fact, previous works have reported that the O_3 tolerance of wheat cultivars was mostly related to their capacity to activate the antioxidant cellular system, showing a clear increase in the expression of the antioxidant molecules and enzymes under high O_3-levels (Feng et al., 2016; Wang et al., 2014).

Previous studies carried out with wheat showed that anthesis is a crucial phase for O_3 impact on wheat and usually, at this phenological stage, reduction in FL photosynthesis has been observed in sensitive cultivars, frequently related to a decrease in g_s (Feng et al., 2008). This response has been found in previous studies on the Artur Nick variety, positioning it as an O_3-sensitive variety (Chang-Espino et al., 2021). In the present assay, gas exchange measurements were performed on secondary stems that were in anthesis, which were phenologically one week behind the main stem; therefore, these recently expanded FLs were not yet affected by prolonged exposure to O_3. However, some early effects on gas exchange had begun to emerge. Ci presented a decrease pattern with O_3 but without affecting A, causing an increase in the A/Ci ratio with the pollutant (Fig. 3a), meaning that under the emerging stress caused by the pollutant on young FLs, they had already become more efficient in CO_2 assimilation in an attempt to cope with the O_3-stress (Fig. 2a). Moreover, when A_{FLsurf} was calculated to determine the impact of the pollutant on plant photosynthesis provided by the total FLs surface, A_{FLsurf} decreased in High-N with increasing O_3 exposure, due to the O_3-induced reduction on FL surface (Fig. 3b). Therefore, O_3-impact on A_{FLsurf} would be partly responsible for the subsequent crop yield loss. This result agrees with previous results analyzing the O_3 effect on the isotopic signature of Mediterranean wheat cultivars, including Artur Nick, showing that the O_3-stress reduced the selective use of ^{12}C for C fixation coherently with the observed A/Ci increase (Chang-Espino et al., 2021).

After a longer exposure period to the pollutant (DaE 51) Chl content of the FLs was reduced significantly by the highest O_3 levels (by 32 % across N treatments) and N was not able to limit the extended accumulated O_3-impact on FL foliar pigment deriving in accelerated senescence (Fig. 2c). Accelerated FL senescence, one of the most mentioned impacts on wheat due to O_3 exposure (Brewster et al., 2024; Broberg et al., 2017; Burkart et al., 2013; Z. Feng et al., 2016; Gelang et al., 2000; Ojanperä et al., 1998; Osborne et al., 2019), was also increased by O_3 in the present assay. This was not only found in the FLs but also in leaves from different age cohorts, and more intensely in the oldest ones (Chang-Espino et al., 2023), reducing the period of the entire plant physiological activity for carbon fixation and shortening the time for nutrient remobilization to the grain (Gelang et al., 2000; Ojamperä et al., 1998; Osborne et al., 2019). In the present study, the O_3-response functions for Chl loss identified this parameter as one of the most O_3-sensitive, showing the important impact of the pollutant on leaf senescence.

Growth and yield responses

Ozone and N treatment effects at the physiological level were reflected in growth and yield parameters. The increase in fertilizer application led to larger plants with a generally bigger biomass for all the different plant parts, and higher grain and protein yield. However, the pollutant induced a growth reduction in High-N that limited the N stimuli on growth (Fig. 4, 5), while O_3 effects were not found in Low-N. This result agrees with previous studies, showing no effect of O_3 on A and growth rate of spring wheat under low N availability, but significant effects under greater N supply (Cardoso-Vilhena & Barnes, 2001) and with results for some natural and semi-natural vegetation functional groups in a meta-analysis of O_3 interactions with N deposition levels (Feng et al., 2019). However, this contrasts with other studies showing O_3 effects on wheat irrespective of fertilization rates, which report an amelioration of O_3-induced reductions in different parameters by fertilization (Brewster et al., 2024; Pandey et al., 2018). Differences in the form and timing of fertilization in the various wheat studies, and the prevalence of ozone exposure for the vegetative or reproductive growth stages, may account for the observed differences in the responses. A greater agreement is found regarding the effect of O_3 on remobilization, showing that O_3 can reduce the translocation of N and assimilates during grain filling (Brewster et al., 2024; Broberg et al., 2017; Gelang et al., 2000). In this study, some parts of the plant such as the HS suffered the greatest growth loss (by 28%) which could be a potential indication of O_3 effect on remobilization, given the key significance of this organ during grain filling. Sanchez-Bragado et al. (2014) have indicated the relevance of reserves located in the ears for the protein content of the grain, which gains importance when wheat grows under other stresses such as water deficit or N deficiency.

The similarity in the intensity of the O_3 effects on growth and yield parameters is noteworthy. It could be expected that growth would suffer more intense O_3-effects given that 70% of the O_3-dose was absorbed during pre-anthesis, where plants display their maximum growth rate, and that the reduction of this absorption in the post-anthesis period would have facilitated the grain-filling processes and softened the final O_3-impact on yield. However, the similarity of both growth and yield effects could indicate the importance of pre-anthesis exposure. As mentioned before, anthesis is considered to initiate the most O_3-sensitive period of wheat (Pleijel et al., 1998) and the O_3 exposure during pre-anthesis is usually restricted to the flag leaf stage (CLRTAP, 2017; Pleijel et al., 2007). Nevertheless, in the Mediterranean basin pre-anthesis O_3-exposure could be an important determinant of the final effects on yield, since high O_3 levels during the tillering stage happen normally under non-limiting growing conditions favoring high g_s. This might be of particular importance for varieties that have adapted to abiotic stresses (water deficit, high temperature) during grain filling. Adapted cultivars give greater importance to the accumulation of reserves in pre-anthesis that are remobilized to the grain after anthesis to limit the impact of more stressful conditions during the late phenological stages (Sanchez-Bragado et al., 2014). Moreover, the results show that the effect of the pollutant on yield intensified slightly when considering Potential GY, which included the potential production of immature spikes at harvest. Thus, the impact of the pollutant could be intensified in wetter years, under more benign growing conditions, when the wheat lifespan is lengthened and a greater development of younger spikes can be expected.

Nitrogen Use indices

Both the NUE and AEN indices indicated a decline in the fertilizer stimuli under elevated O_3 levels, emphasizing again the impact of the pollutant on N fertilization. This decline was particularly pronounced in the highest O_3 treatment, where the enhancements in grain N use efficiency (NUE) and protein yield (AEN) due to additional N were strongly diminished or completely disappeared. While NUE reflects the overall contribution of the fertilizer to the grain, AEN specifically focuses on the N remobilized to the grain, and both indicators were strongly reduced by O_3 in this experiment. Previous findings suggested that O_3 exerts a greater influence on starch yield than on N uptake, via a reduction on carbon fixation rates from the other plant parts and the duration of grain filling related to accelerated leaf senescence (Broberg et al., 2015; Gelang et al., 2000; Pleijel et al., 2018; Wang & Frei, 2011). However, O_3 effects on N remobilization have also been described (Brewster et al., 2024; Broberg et al., 2017) which can result in lowered protein yield and NUE, as observed in the present assay.

Indeed, an increasing number of studies are reporting that O_3 reduced the fertilizer efficiency in wheat, rice and maize (Broberg et al., 2017; Peng et al., 2019; Pleijel et al., 2018) as well as other annual plant species (Héctor Calvete-Sogo et al., 2014). It is important to note, that given the costs of fertilizers for crop production, the impact of O_3 reducing the efficiency of N fertilization can significantly add to economic impacts directly related to yield losses. The point at which the fertilizer stimuli on GY was counteracted by O_3 pollution was in the range of 15 000 nL L^{-1} h for AOT40 and 11 mmol m^{-2} PLA for POD6. Although relatively high for Spain, these O_3-exposure values are already exceeded in cereal production areas of the Iberian Peninsula during high O_3 years (MITERD, 2022), showing that current O_3 levels could be considered a limiting factor for improving N fertilization efficiency in Mediterranean areas. However, current evaluations of the risk of O_3 pollution on agricultural production are only focused on yield and yield quality parameters, thus possibly underestimating the global effect of O_3 on agroecosystems.

Since results suggest that O_3 levels may be limiting the efficiency of N fertilization, the pollutant could be inducing an increase in the use of fertilizers in areas under chronic O_3 pollution to try to compensate for the lack of fertilization effectiveness, causing other environmental problems related to N losses from agriculture. For instance, O_3 exposure has been related to increases in nitrous oxide emissions from soils (Kou et al., 2018; Sánchez-Martín et al., 2017), a strong greenhouse gas. Therefore, tropospheric O_3 could be acting against the objectives of international forums, other than air pollution policies, aiming at reducing agricultural losses of N to the environment via leaching of N compounds into soil-water systems, or via the emission to the atmosphere of gaseous N compounds such as N_2O or NH_3. These efforts include the Objective 7 of COP15 for 2030 (United Nations, 2022), the European Green Deal in the Farm to Fork Strategy (European Commission, 2020a) and the Biodiversity Strategy (European Commission, 2020b).

Response functions, CL and Implications for O_3-Risk analysis

Exposure- and dose-response functions are currently used in risk assessment methodologies of O_3 effects in crops at regional and continental scales in the frame of the Air Convention, from which O_3 CLs to protect crop production are defined (CLRTAP, 2017). The exceedance of flux-based O_3 CLs levels is also considered an optional parameter for long-term monitoring of air pollution effects on ecosystems and agrosystems in the framework of the EU Directive on national emissions ceilings of atmospheric pollutants (EU 2016/2284). These indicators can help detect preferential areas of action for environmental policies, evaluate the efficiency of emissions abatement strategies, or analyze crop production under future climatic and air quality scenarios (CLRTAP, 2017; EEA, 2019c). Therefore, it is important to evaluate whether agricultural fertilization can affect or modulate O_3-responses and, consequently, the response functions and the CLs derived from them. This is especially important for wheat, which is considered the representative cereal for agricultural risk analyses (CLRTAP, 2017).

Response functions, both based on AOT40 and POD6, presented high and quite similar R^2. Generally, the POD6 index presents a better adjustment with the effects of O_3 than exposure-based indices (Pleijel et al., 2022), since it reflects the pollutant absorbed by the plant through the stomatal pores, which is considered the most important mechanism of O_3 damage (Emberson et al., 2018; Fiscus et al., 2005). In the present study, the similar behavior of both indices would be related to watering the plants during the whole O_3-exposure period. When there is no stomatal restriction to the O_3 flux towards plant tissues due to an absence of water stress, indices based on O_3-concentration in air or O_3-absorbed dose fit similarly when quantifying the O_3-effects (González-Fernández et al., 2014). Nevertheless, when considering the most sensitive responses to O_3 under High-N, POD6 is the index that best explains the effect of the pollutant.

Ozone CLs calculated for sensitive growth and yield parameters combining High-N and Low-N treatments, in the range of 3600 to 6275 nL L^{-1} h for AOT40 and 2-5 mmol m^{-2} PLA for POD6 (Table 4S), showed higher values than those established for wheat yield parameters in previous studies (Grünhage et al., 2012; Pleijel et al., 2007; 2022). However, it should be considered that in the present experiment, AOT40 O_3 was calculated from the tillering stage, which includes part of the vegetative growth phase of wheat. Past O_3 fumigation experiments, from which existing CLs were derived (CLRTAP, 2017; Grünhage et al., 2012), only consider O_3 exposures during the reproductive period based on the assumption that this is the most sensitive growth stage of wheat when growing under central and Northern European climatic conditions (Pleijel et al., 1998). Whether the O_3 exposure protocol used in this study resulted in greater effects, as suggested by higher POD values accumulated during the pre-anthesis period, or, contrarily to the initial hypothesis, that early O_3 exposure resulted in adaptations to O_3 stress on wheat growing under Mediterranean conditions, warrants further research.

The O_3 x N interactive effect found in different growth and yield parameters resulted in important differences in the slope of O_3 exposure- and dose-response relationships between N treatments and, thus, on the CLs derived from them. For all the sensitive parameters analyzed, CLs obtained from High-N functions were much lower than when Low-N functions were used (Table 4S). In the case of GY, CL based on AOT40 is 4 times higher in Low-N than High-N (12 525 *vs* 2976 nL L^{-1}); and if POD6 is considered CLs are 3-times higher in Low-N than High-N (9 *vs* 3 mmol m^{-2} PLA). Moreover, in some cases like the Protein Y CLs in Low-N could not be derived since neither the AOT40 nor POD6 relationships were statistically significant. Therefore, results point to a new source of uncertainty in the derivation and application of O_3 CLs for O_3 risk assessment, as crop management practices such as N fertilization change greatly across regions. Further O_3 x N interaction studies conducted under a range of growing conditions would be needed to address this issue.

Results of the present work stress the importance of N-fertilization management in the O_3-response for wheat, showing that O_3-response functions and the derived CLs can be affected by the N-dose used. The study opens the debate on the need to consider the N fertilization level in the O_3-risk assessment methodology, given its potential capacity to affect environmental policy decisions, and highlights the need for further experimental work to analyze the interactions between air quality and crop management.

5. Conclusions

Increasing N fertilization caused a general increase in the growth and yield parameters assayed, but the O_3 factor acted in the opposite direction reducing the growth and yield, limiting the fertilization efficiency and reducing NUE and AEN at the highest O_3 treatment. This interactive pattern between the O_3 and N factors was observed for most of the growth and yield parameters assayed, constituting an important effect that is currently not considered in the assessment of O_3 pollution impacts on agriculture.

The analysis of O_3 dose dynamics during the fumigation period in this experiment revealed that, on average, 71% of the POD6 was accumulated in the pre-anthesis period, despite similar O_3 exposures between pre and post-anthesis, as evaluated with the AOT40 index. This is in contrast with previous studies on the O_3 impact on wheat, giving greater importance to post-anthesis O_3 exposure. Whether this is a particular feature of O_3 exposure patterns under

Mediterranean conditions, resulting in differences in the response of wheat to O_3 pollution, needs further research.

Ozone response functions in High-N presented much steeper slopes than in Low-N. Therefore, lower CLs were derived from High-N than Low-N. The debate on the need to consider the N factor in the O_3 risk analysis methodology for crops is left open, given the N-fertilizer ability to modify the response functions to the pollutant and the CLs derived from them, thus potentially affecting environmental policy decisions.

Funding and Acknowledgements

This study has been funded by Ministerio de Ciencia e Innovación, Agencia Estatal de Investigación (MCIN/AEI/10.1303/501100011033) for funding (SUSCAP, PCI2019-103521/AEI) with support from EU SUSCROP-ERA-NET-FACCEJPI project-Horizon 2020, and the Comunidad de Madrid (Spain) AGRISOST-CM (S2018/BAA-4330) projects, and by the DGCEA - CIEMAT working program on atmospheric pollution and persistent organic pollutants (ACTUA-MITERD, Spanish Ministry of Ecological Transition). J. L. Araus and M. Chang-Espino acknowledge the support of Holistic Wheat Project (PID2022-138307OB-C21), from Ministerio de Ciencia e Innovación, (Spain). The OTC experimental facility located at La Higueruela Research Farm is supported by an agreement between MNCN-CSIC and CIEMAT. The farm personnel and especially the valuable work of Jose María Gómez Camacho on plant care and OTC facility maintenance are greatly acknowledged. R. Alonso, S. Elvira and H. Calvete are acknowledged for their appreciated help in the fieldwork.

Author Contributions

The authors contributed to the manuscript in the following ways: Conceptualization, M.C-E, J.L.A. and V.B-B.; Methodology, M.C-E., V.B-B., and I. G-F.; Formal Analysis, M.C-E., S.P-B., A.B.A. and V.B-B.; Investigation, M.C-E., V.B-B., I.G-F., A.B.A and J.L.A.; Resources, M.C-E. V.B-B. and J.L.A.; Data Curation, S.P-B, M.C-E. and V.B-B.; Writing – Original Draft Preparation, M.C-E., and V.B-B. ; Writing – Review & Editing, M.C-E., V.B-B., I. G-F and J.L.A.; Visualization, M.C-E.; Supervision, V.B-B. and J.L.A; Project Administration, V.B-B. and I. G-F; Funding Acquisition, V.B-B, and J.L.A.

Data Availability

The data presented in this study are available upon request from the corresponding author.

Conflict of Interest

The authors declare no conflict of interest.

Ethics Statements

The authors declare no ethical conflict.

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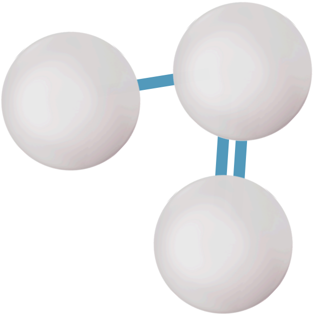
Chapter 3



The manuscript used for Chapter 3 has been prepared for submission to Environmental Pollution, which has an impact factor of 8.9 and CiteScore of 14.9, and is in the first quartile, Q1, in its research fields.

Resumen del Capítulo 3

El estudio profundizó en los mecanismos de respuesta del trigo blando a la exposición combinada de ozono (O_3) y fertilización nitrogenada (N). El ensayo se realizó en una instalación de cámaras de techo descubierto (OTC). Se utilizó una variedad mediterránea sensible al O_3 de uso extensivo, fumigándola desde pre-antésis para imitar los patrones de contaminantes en la zona. Se consideraron cuatro niveles de exposición al O_3 (aire filtrado con carbón (FA), aire no filtrado (NFA), NFA+20 ppb, NFA+40 ppb) y dos dosis de suplementación de N (de 100 kg N ha^{-1} y 200 kg N ha^{-1}). Se evaluaron los efectos en la absorción y removilización del C y N a los granos empleando composiciones isotópicas ($\delta^{13}C$, $\delta^{15}N$) en hojas bandera y granos, además de parámetros de intercambio gaseoso, contenido de pigmentos y anatomía foliar para comprender mejor la interacción O_3 x N.

Durante una etapa fenológica temprana, el efecto del O_3 fue menor con la suplementación más alta de nitrógeno, especialmente en los niveles más bajos de O_3, debido a un aumento de la actividad fotosintética. Esto estuvo relacionado con un mayor número de células mesófilas, conllevando a una reducción en la concentración de CO_2 intercelular, y a un aumento en el contenido de clorofila a medida que la exposición al contaminante aumentaba, indicando una resistencia a la pérdida de pigmentos proporcionada por el fertilizante adicional. Esta estrategia pareció ayudar en los niveles más bajos del contaminante, ya que la actividad fotosintética fue determinada más por la conductancia estomática que por la disminución en la carboxilación, pero esto no perduró bajo los niveles más altos del contaminante.

Además, las mediciones de $\delta^{13}C$ en el grano mostraron que el efecto compensatorio del nitrógeno no perduró más tarde en la vida de la planta, ya que el ozono causó un aumento significativo en $\delta^{13}C$ independientemente del nivel de fertilización con nitrógeno. Nuestro estudio también confirmó el rol de $\delta^{13}C$ en los granos como indicador del efecto crónico de la exposición al ozono al comparar las medidas en dos años diferentes, demostrando su fortaleza.

Assessing the nitrogen modulation of response of wheat to ozone through carbon and nitrogen isotopic signatures

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Abstract (300 words)

The study delved into the mechanisms of soft wheat response to the combination of ozone (O_3) and nitrogen (N) fertilization. The assay was conducted in an Open Top Chamber (OTC) facility. An O_3-sensitive Mediterranean cultivar of extensive use was exposed to O_3 levels from the tillering stage, mimicking the pollutant patterns in the area. Four O_3 exposure levels (charcoal-filtered air (FA), non-filtered air (NFA), NFA+20 ppb, NFA+40 ppb) and two N supplementation doses (Low-N of 100 kg N ha^{-1} and High-N of 200 kg N ha^{-1}) were considered. The effects on C and N uptake and remobilization to grains were assessed through isotopic compositions ($\delta^{13}C$, $\delta^{15}N$) in flag leaves and grains, as well as gas exchange parameters, pigment content, and leaf anatomy to better understand the O_3 x N interaction.

A higher nitrogen supply ameliorated the effect of the lower levels of ozone, at an early phenological stage, through increased photosynthetic activity. This was associated with an increased number of mesophyll cells, which led to a reduction in intercellular CO_2 concentration, as well as increased chlorophyll content with exposure to the pollutant, indicating a resistance to pigment loss provided by the additional fertilizer. This strategy appeared to be successful at lower pollutant levels, as photosynthetic activity was determined more by stomatal conductance than by decreased carboxylation activity, but this did not last under higher levels of the pollutant.

Moreover, $\delta^{13}C$ measurements in grain showed that the compensative effect of nitrogen did not last later in the plant life, as ozone caused a significant increase in $\delta^{13}C$ regardless of the nitrogen fertilization level. Our study also confirmed the role of $\delta^{13}C$ in grains as an indicator of the effect chronic ozone exposure affecting stomatal conductance through the comparison of measurements across different years, showing its strength.

Keywords

Isotopes; Photosynthesis; Wheat; Chlorophyll; Mesophyll

1. Introduction

Wheat is a staple crop grown across 217 M ha globally and accounts for 37% of all annual cereal consumption (Erenstein *et al.*, 2022), making it extremely important for the global economy and food security. Due to its ozone sensitivity, being only comparable to soybean as the most sensitive crop (Feng *et al.*, 2008; Pleijel *et al.*, 2018), it is a reference crop for O_3-risk assessments and to detect agricultural areas that are at risk due to high ozone pollution (González-Fernández *et al.*, 2013). Currently, over 20% of the crops in the EU region face a risk due to the pollutant (Mills *et al.*, 2007; Wilkinson *et al.*, 2012), and just wheat losses due to ozone surpass 8% in the Mediterranean, being higher than the northern parts of Europe which expect O_3-induced losses of 4% (Schucht *et al.*, 2021). Ample research on the impact of ozone on wheat in policy-making contexts has been done (Mills *et al.*, 2018; Pleijel *et al.*, 2019; EEA, 2022), however, if current trends in ozone precursor emissions persist and ozone concentrations continue to increase, wheat yields could decline by 0.26-0.95% per ppb increase in ozone, for daily concentrations exceeding 25pbb (Guarin *et al.*, 2019).

Modern wheat varieties tend to be more sensitive to ozone than landraces (Pleijel *et al.*, 2006; Fatima *et al.*, 2019). These modern varieties have been selected to enhance yield, often through higher stomatal conductance (g_s) to improve CO_2 exchange and increase photosynthesis (A) (Roche, 2015). However, this also facilitates the absorption of ozone through the stomatal pores (Ma *et al.*, 2022). When ozone enters the plant tissues, it initiates a burst of reactive oxygen species (ROS), which disrupts photosynthetic function in sensitive wheat varieties (Teixeira *et al.*, 2011). This disruption is caused by several factors, including a decrease in g_s due to O_3-induced stomatal closure (Hoshika *et al.*, 2015), alteration of Rubisco carboxylation (Emberson *et al.*, 2018), and a potential decreased mesophilic activity (Martin *et al.*, 2000). Additionally, ozone can impact the light reactions of photosynthesis by reducing the Fv/Fm ratio and altering PSII energy conversion and the rate of non-cyclic electron flux through PSII (Calatayud *et al.*, 2002, 2010; Pellegrini *et al.*, 2011).

Following O_3-exposure, these effects on plant CO_2 fixation accelerate senescence, promote chlorophyll degradation, affect nutrient uptake, and alter nutrient translocation, leading to grain yield loss and reduced grain quality (Pleijel *et al.*, 2019; Feng *et al.*, 2022; Brewster *et al.*, 2024). This behavior was also seen specifically in modern Mediterranean varieties, which responded to ozone with decreased g_s, diminished carbon assimilation, accelerated senescence, and significant yield loss (Monga *et al.*, 2015; Chang-Espino *et al.*, 2021; Marzuoli *et al.*, 2024).

When exploring ozone effects, other tools besides physiological parameters can be used to understand the plant response mechanisms. The impact that physiological changes can have on photosynthesis can be reflected through the ultrastructure of the foliar mesophylls, where a high mesophyll CO_2 diffusion conductance (g_m) is linked to higher photosynthetic rates (A) (Flexas *et al.*, 2016; Joffe *et al.*, 2022). Factors such as thicker cell walls can decrease g_m (Evans *et al.*, 2009; Terashima *et al.*, 2011; Tosens *et al.*, 2012), while increasing the surface area of chloroplasts exposed to intracellular airspace per unit leaf area can decrease mesophyll resistance, thereby increasing g_m (Evans *et al.*, 2009; Tosens *et al.*, 2012). Meanwhile, if the number of mesophyll cells occupying the intracellular space increases, a larger chloroplast surface area would be exposed to the intracellular air space, enhancing g_m and therefore A (Ren *et al.*, 2019).

Previous studies have shown that after exposure to high ozone levels, mesophyll cell walls thickened due to the deposition of callose between the plasma membrane and the cell wall. This prevents the diffusion of ozone into the protoplast by creating a mechanical barrier that may even contain compounds that act as antioxidants (Ljubešić and Britvec, 2006). This thickening however would decrease g_{m}, as explained before, due to an increased cell wall resistance, which accounts for up to 50% of the mesophyll diffusion resistance (Terashima *et al.*, 2011). Additionally, a denser leaf structure has also been seen under higher ozone levels, aiding in ROS damage mitigation by restricting O_3 diffusion, but therefore also decreasing g_m (Emberson *et al.*, 2018; Hoshika *et al.*, 2020).

In wheat, the isotopic signature of C ($\delta^{13}C$) and N ($\delta^{15}N$) has been widely used for measuring the stress caused by abiotic factors such as water input and nitrogen regimes (Cabrera-Bosquet *et al.*, 2009; Araus *et al.*, 2013; Sanchez-Bragado *et al.*, 2017), and salinity (Yousfi *et al.*, 2013; Chamekh *et al.*, 2016) and to a lesser extent due to the ozone stress (Saurer, *et al.*, 1991; Chang-Espino *et al.*, 2021; Ma *et al.*, 2022).

Discrimination against the heavier ^{13}C isotope compared to ^{12}C occurs in the leaf during CO_2 diffusion through the stomata, and by carboxylation (Farquhar *et al.*, 1989). As mentioned before, due to the stomatal closure and structural changes caused in the plant by exposure to the pollutant, which reduce CO_2 diffusion from the air, and CO_2 supply to the carboxylation sites for rubisco, discrimination against $^{13}CO_2$ decreases, therefore increasing the proportion of $^{13}C/^{12}C$ and therefore $\delta^{13}C$ within the plant (Saurer *et al.*, 1991; Jäggi *et al.*, 2005; Ma *et al.*, 2022).

A previous study (Chang-Espino *et al.*, 2021) revealed a strong negative correlation between $\delta^{13}C$ in grain ($\delta^{13}C_{grain}$) with grain yield (GY) and grain N yield (GNY), following an expected trend, as increasing O_3 concentrations decrease yield and discrimination against the heavier isotope, increasing $\delta^{13}C$. The study considered 8 Mediterranean varieties released after the Green Revolution, showing $\delta^{13}C_{grain}$ to be the best indicator of O_3 physiological stress accumulated throughout the wheat growing period. Incorporating the $^{12}C/^{13}C$ ratio over time also helps describe the balance between the stomatal conductance of the plant and its net photosynthetic assimilation (A), as $\delta^{13}C$ depletion is influenced by the intracellular versus atmospheric CO_2 concentration ratio (C_i/C_a), which is in turn affected by A and g_s (Tcherkez *et al.*, 2011; Chamekh *et al.*, 2016; Serret *et al.*, 2018).

The $^{15}N/^{14}N$ isotope ratio ($\delta^{15}N$) is frequently used to study N absorption, redistribution and assimilation (Yousfi *et al.*, 2013; Brewster *et al.*, 2024). During fertilizer uptake from the soil, N is assimilated and fractionated by different metabolites, which also discriminate against the heavier isotope ^{15}N (Cui *et al.*, 2020). As ^{15}N is discriminated against as is redistributed to the different plant parts, the N absorbed and redistributed to the aerial plant parts is ^{15}N-enriched, (Cui *et al.*, 2020) while the N assimilated into enzymatic reactions such as GS-GOGAT and NO_3 reduction result in products with a lower $\delta^{15}N$ than the source, as ^{15}N is discriminated against during the reactions (Evans, 2001). $\delta^{15}N$ is also discriminated against during volatilization, where due to stomatal closure, less ^{14}N volatilizes reducing the proportion of ^{15}N to ^{14}N left behind and therefore decreasing $\delta^{15}N$ (Sanchez-Bragado *et al.*, 2017; Cui *et al.*, 2020).

Though complex, the mechanisms of N isotope fractionation can also help explain alterations in N distribution within the plant due to stresses such as high O_3 levels. In water-stressed conditions, the stomata close, causing the decreased loss of ammonia and nitrous oxide mentioned before, reducing ^{15}N enrichment and decreasing $\delta^{15}N$ levels (Araus *et al.*, 2013). Lower $\delta^{15}N$ values in rainfed conditions, when compared to irrigated samples, have also been seen in shoots and grains, indicating the stress effects on redistribution (Sanchez-Bragado *et al.*, 2017). Similarly, in an assay performed under high salinity, which also induces stomatal closure, decreasing $\delta^{15}N$ values were observed in the shoot, again suggesting an influence of these stress conditions on nitrogen uptake and assimilation

(Chamekh *et al.*, 2016). Similar results were observed by Yousfi et al., (2013) in $\delta^{15}N$ values in biomass, where a strong negative correlation was noted between $\delta^{13}C$ and $\delta^{15}N$, reinforcing the effect that the stress-induced lower stomatal conductance has on nitrogen dynamics in the plant.

The detrimental effects of stress on decreasing stomatal conductance and nitrogen remobilization efficiency have also been seen with increasing O_3, particularly during the most O_3-suceptible phase which occurs post-anthesis (Brewster *et al.*, 2024). This would cause an overall reduction in $\delta^{15}N$ values due to a lower stomatal conductance and reduced volatilization (Chang-Espino *et al.*, 2021). The pollutant also affects the preferred N source by the plant, where for example Möcker *et al.*, (1996) noted that more ammonium nitrogen was absorbed under higher ozone. It should be noted that the complex nature of N remobilization can cause values to fluctuate with increasing ozone concentrations or over time as different mechanisms account for increases or decreases in $\delta^{15}N$ (Chang-Espino *et al.*, 2021; Ma *et al.*, 2022). It should also be noted that when the plant is exposed to the pollutant, antioxidants are produced to combat ROS formation (Möcker *et al.*, 1996; Pandey *et al.*, 2018), while due to tissue oxidation, more N is needed for tissue repair (Shang *et al.*, 2019), which can alter the $\delta^{15}N$ isotopic signature.

Despite ample research on the impact of ozone on wheat (Mills *et al.*, 2018; Pleijel, Broberg and Uddling, 2019), the investigation into the interactions between ozone and other abiotic factors is a more recent area of study. For example, Broberg et al., (2023) examined the interaction between ozone and drought, noting that the additional water ameliorated the O_3-induced effects on wheat. However, despite their importance, studies delving into the ozone x N interaction are scarcer and have had conflicting results (Broberg *et al.*, 2017; Pandey *et al.*, 2018). Depending on the species, fertilizer dose and pollutant exposure, or even depending on the response parameter considered, N supplementation can limit or extend the pollutant effects (Calvete-Sogo *et al.*, 2014; Broberg *et al.*, 2017; Brewster *et al.*, 2024).

Different factors can cause this disparity in the responses among species, like the plant capacity for fertilizer absorption, which can range from 30-50% depending on the species (Broberg *et al.*, 2017; Hawkesford and Riche, 2020); or the proportion of the N allocated to the vegetative parts of the plant *vs* yield as usually higher N is derived more towards vegetative parts than towards increasing yield (Peng *et al.*, 2020). As more N goes towards biomass, this could make the plant more susceptible under higher levels of ozone since there is a larger leaf surface area, therefore increasing the O_3-absorbing surface area. Furthermore, ozone reduces photosynthetic N use efficiency (PNUE) and leaf N allocation to photosynthetic components, as more N can be directed toward cell walls and defense compounds (Shang *et al.*, 2019). Recently, Broberg *et al.* (2017) and Brewster *et al.* (2024) indicated that the pollutant altered N remobilization within the wheat plant and reduced overall fertilizer efficiency, highlighting the importance of deepening experimental studies combining ozone and nitrogen factors for agricultural sustainability.

Regarding Mediterranean bread wheat, in a study about the modulation of the O_3-response by N fertilization considering a modern cultivar of ample use in the Iberian Peninsula, Artur Nick, it was observed that the fertilizer can limit yield and quality losses for medium levels of the pollutant, but not for higher levels. Results noted that under medium levels of the pollutant, the extra-N was allocated to functions other than leaf pigmentation, likely for repair and stress management to help the plant cope with the ozone stress rather than to enhance photosynthesis. However, exposure to high pollutant levels from the tillering stage caused a dramatic negative response in the growth and yield parameters, eliminating the fertilization stimuli on GY and GNY (Chang-Espino *et al.*, submitted for publication)

Given these results, the present study aimed to delve more into the ozone x N interaction by analyzing the isotopic signals of C and N in leaf and grain, while observing physiological, structural and gas exchange effects, to better understand the wheat response mechanisms to both factors.
The assay aimed to investigate the mechanisms through which elevated ozone exposure limited the N-fertilization efficiency of a Mediterranean wheat variety through a) the study of the CO_2 absorption based on gas exchange parameters and $\delta^{13}C$; b) the redistribution of carbon and nitrogen analyzed through $\delta^{13}C$ and $\delta^{15}N$ isotopic signals in both grain and flag leaf, and c) the potential early adaptation of the plant, to high ozone levels, through the study of the leaf anatomy, particularly the photosynthetic mesophyll tissue.

The initial hypotheses based on previous work, were:

a) the correlation between gas exchange parameters and $\delta^{13}C$ in grain, a robust indicator of chronic ozone stress, is expected to perdure but may be altered by additional nitrogen, as increased nitrogen resources should enhance gas exchange by bolstering investment in the photosynthetic machinery;

b) meanwhile, differences in $\delta^{15}N$ due to ozone should be larger under High-N because ozone alters N uptake and remobilization, this should be reflected by the difference between the grain and leaf $\delta^{15}N$;

c) early ozone exposure to ozone could trigger structural adaptations in wheat to better cope with high levels of ozone during its life cycle.

2. Materials and Methods

2.1 Experimental Design and OTC Field Site

The experimental assay was performed in an Open Top Chamber facility located in *La Higueruela/MNCN-CSIC* Agricultural Research Station, in Central Spain (Santa Olalla, Toledo, 40°3'N, 4°26'W). The 12 OTCs are distributed in three randomized blocks to provide 4 different levels of O_3-fumigation levels replicated 3 times (3 Lines): FA (charcoal-Filtered Air), NFA (Non-Filtered Air), which reproduces the ambient levels found at the field station; NFA+ (Non-Filtered Air + 20 nL L^{-1} of added O_3) and NFA++ (Non-Filtered Air + 40 nL L^{-1} of added O_3). The NCLAN type OTCs (Heagle *et al.*, 1989) had a 3 m high × 3 m diameter dimension and an open conic top (*frustum*) to prevent wind inflow. O_3 for the NFA+ and NFA++ fumigations were generated with an O_3 generator system (A2Z Ozone Systems Inc., Louisville, KY, USA) fed with pure O_2 for 8 h day^{-1} (6:00 to 14:00 GTM), seven days a week. The levels of ozone (ML® 9810B, Teledyne Monitor Labs, Englewood, CO, USA) and nitrogen oxide (NO_2 and NO; ML®9841, Teledyne Monitor Labs, Englewood, CO, USA) were monitored within each OTC above the canopy employing a timesharing system that sampled the gases every 10 minutes per plot. Air pollution monitors and flowmeters of the fumigation system were calibrated at the beginning of the experiment following company recommendations.

Air relative humidity (RH), air temperature (HOBO® Pro v2, Onset, Bourne, MA, USA), and photosynthetic active radiation (PAR; OSO-SUN HOBO®, Onset, Bourne, MA, USA) were measured within six of the OTCs above the canopy. More information on the OTC facility can be found in the literature (Calvete-Sogo et al., 2016).

2.2 Plant growing conditions and N treatments

Seedlings of the modern bread wheat (*Triticum aestivum* L.) cv. Artur Nick were sown in a specialized nursery and then transplanted into 3 liter pots, containing a 60/20/20 turve/vermiculite/perlite substrate. Two seedlings per pot were transplanted. On April 24, at tillering stage (stage 23, Meier, 2018), plants were distributed into the OTCs, 12 pots per OTC, to be exposed to the different O_3 treatments. Irrigation was supplemented manually following the climatic conditions and needs of the plant. The Artur Nick variety was selected due to its broad use in the Iberian Peninsula (MAPA, 2023) and its previous classification as a Mediterranean O_3-sensitive variety (Chang-Espino et al., 2021). The whole O_3-exposure period comprised 77 days from the start of fumigation on April 24 (0 Days after the start of the Exposure period, DaE 0), until harvest on July 10 (DaE 77). Pot nets were set at the start of grain development to avoid ant and bird damage on the grains.

To represent the fertilization range used in the commercial rainfed wheat fields in the area, two top dressing N fertilization doses were considered based on dissolved ammonium nitrate (26%): 100 kg N ha^{-1} (Low-N) and 200 kg N ha^{-1} (High-N), which were applied in two doses 18 days apart during the tillering stage, on April 30 (DaE 6) and May 18 (DaE 24). Of the 8 pots in each OTC, 4 received the Low-N dose and the other 4 received the High-N dose (12 pots per O_3 and N treatments).

2.3 Gas exchange, polyphenol and chlorophyll measurements

When plants reached anthesis (May 25, DaE 31), gas exchange parameters were measured: on May 31 and June 1 (DaE 37 and 38). The portable system Li-Cor 6400 (Li-Cor, Lincoln, NE, USA), calibrated by the company before measurements began, was used. Net photosynthesis (A), stomatal conductance (g_s), and intercellular concentration (Ci) were considered. Measurements were taken on healthy FLs of the most abundant secondary tillers, between 07:00-12:00 h GMT. The standardized conditions to reach high physiological activity were: PAR 1000 µmol m^2 s^{-1}, RH 55%, and temperature 20–22 °C. Intrinsic Water Use Efficiency (WUE) was calculated as the rate between A and g_s.

On June 5th (DaE 42), Anthocyanins (Anth), Flavonoids (Flav), and Chlorophyll Content (Chl) were performed on FLs with a portable leaf sensor (Dualex, Force-A, Orsay, France); at the same time the Nitrogen Balance Index (NBI), which relates C and N allocation (Cerovic *et al.*, 2012), was calculated as the ratio between Chl/Flav.

2.4 Elemental C and N and isotopic C and N signals Analysis

Fully expanded FLs of secondary tillers were taken for total C and N content and $^{13}C/^{12}C$ and $^{15}N/^{14}N$ signatures on June 5 (DaE 42). Samples were taken per pot, pooled per OTC and grounded (n=3 per O_3 and N treatment); and then analyzed using an isotope ratio mass spectrometer (Delta C IRMS, ThermoFinnigan, Germany) paired with an elemental analyzer (Flash 1112 EA, ThermoFinnigan, Germany) in the CCiT (Centres Científics i Tecnològics) at the University of Barcelona (Spain). One-mg samples of the pooled FLs, and reference materials, were weighed, placed in tin capsules, sealed, and loaded onto an automatic sampler for EA-IRMS analysis. Total carbon and nitrogen content were expressed as a percentage (%) of dry matter. Isotopic ratios were expressed in the δ notation, where carbon was calculated as

$\delta^{13}C$ (‰) = $(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1$ (Farquhar *et al.*, 1989)

In this equation, "sample" refers to the plant material and "standard" to international secondary standards where the $^{13}C/^{12}C$ ratios are known (IAEA CH7 polyethylene foil, IAEA CH6 sucrose, and USGS 40 L-glutamic acid), which were calibrated against Vienna Pee Dee Belemnite calcium carbonate (VPDB). These analyses had an analytical precision (SD) of 0.10‰. The δ notation was also used for the $^{15}N/^{14}N$ ratio ($\delta^{15}N$), using the international secondary standards IAEA N1 and IAEA N_2 ammonium sulphate and IAEA NO_3 potassium nitrate, which were calibrated against N_2 in the air, and had an analytical precision of 0.18‰.

Mature grains were harvested on July 10 (DaE 77) and analyzed at pot level (n=12 per O_3 and N treatment) by combustion in the CIEMAT lab of the Chemistry Division using a LECO TruSpec CHN elemental analyzer (Leco Instruments S.L., Michigan, USA).

The $^{15}N/^{14}N$ isotopic ratio was determined using an elemental analyzer Carlo Erba EA 1108 coupled in continuous flow mode to a VG Isochrom isotope ratio mass spectrometer (IRMS). The analyzes were carried out in the "Laboratorio de Isótopos Estables" del Servicio Interdepartmental de Investigación (SIdI) (Stable Isotope Laboratory of the Interdepartmental Research Service), Autonomous University of Madrid (UAM).

2.5 Leaf anatomy

On June 5th (DaE 42), FL cuts from secondary tillers were taken in the field considering the two most extreme O_3 treatments for the assay, FA and NFA++, for light microscopy imaging of semithin sections. About 1 mm^2 leaf sample cuts were made from the center of the lamina with a scalpel over a waxy block, while the leaf was covered with a fixative solution. The cuts were then submerged into pre-prepared glass vials containing the fixative solution, composed of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer. The fixative was then changed until the sample was completely transparent and the sample volume to fixative volume ratio was around 1:10. The fragments were then left in the fixative solution and stored at 4°C until they were taken to the Electron Cryomicroscopy Unit, Scientific and Technological Centers, University of Barcelona (CCiTUB) 24 hours later. There, they were then washed gently with Cacodylate buffer 0.1M and were processed through: 1) Cryoprotection with glycerol in cacodylate buffer 0.1M at 4ºC; 2) Cryofixation by immersion in liquid propane; 3) Freeze substitution in an EM AFS2 (Leica Microsystems, Vienna, Austria) with 2% osmium tetroxide (EMS, Hatfield, USA) 0.1% uranyl acetate (EMS, Hatfield, USA) in acetone at -90 °C for 72 h and warming up to 4 °C at a 5 °C/h slope. Samples were then kept at 4ºC for 3 h, transferred at room temperature and darkness for 2 h and gently washed with acetone. Samples were then 4) Resin embedded in Epon-812 (EMS, Hatfield, USA); 5) 60 nm-thick sections were obtained using a UC6 ultramicrotome (Leica Microsystems, Vienna, Austria), a 45 ° diamond knife (Diatome, Biel, Switzerland) and a clearance angle of 6 °. EPON sections were then mounted on formvar coated 200-mesh copper grids and stained with 2% uranyl acetate and lead citrate. The Acquired sections were then observed in a Tecnai Spirit microscope (EM) (FEI, Eindhoven, The Netherlands) equipped with a tungsten cathode. Images were acquired at 120 kV with a 1376 x 1024 pixel CCD Megaview III camera.

Total mesophyll area per unit leaf area, total mesophyll volume per unit leaf area, leaf thickness and epidermis thickness and individual mesophyll cell section area were assessed after Araus *et al.*, (1986). Thus, the total area of the leaf section, the abaxial and adaxial epidermis length and area, as well as the mesophyll cell perimeter and area, were measured with the Image software (J Fiji V1.0, National Institutes of Health, USA). These values were then divided over the average epidermis length to get an average per unit of the leaf area. In addition, number and area of the mesophyll cell sections were also evaluated.

2.6 *Ozone exposure indexes*

Ozone exposure was calculated based on two different frequently used indexes to describe O3-caused crop damage, the Accumulated Ozone over the Threshold of 40 nL L^{-1} index (AOT40), and the 7-hour mean O$_3$-index (7h-O$_3$). AOT40 was calculated by adding the O$_3$-hourly concentration values over 40 nL L^{-1} during daylight hours (PAR>100 □mol m^{-2} s^{-1}) for the O$_3$-exposure period (April 24, DaE 0- July 10, DaE 77), expressed as nL L^{-1} h (Fuhrer et al., 1997). This index has shown a robust linear relationship to quantify wheat yield losses induced by the pollutant (Mills *et al.*, 2007; Pleijel *et al.*, 2019) and is currently considered in the EU Air Quality Directive (2008/50/EU) and in the UNECE Air Convention for O$_3$-risk analysis on crops (CLRTAP, 2017). The 7h-O$_3$ index was calculated from 09:00-16:00 GMT expressed as nL L^{-1}; this index is also considered to quantify O$_3$ effects on crops (Lesser *et al.*, 1990).

2.6. Statistical Analysis

Mean differences among O$_3$ and N factors were studied through a Two-Way ANOVA analysis for samples where only one sample per chamber was taken, and split-plot analyses were done when more than one sample per chamber was acquired, considering O$_3$ and N as fixed factors and Line as a random factor. One-Way-ANOVAs were also performed separately for each N dose to analyze the ozone effect. Data was previously explored for outliers based on the studentized residuals and tested for homoscedasticity and normality with the Levene and Shapiro-Wilk tests. Tukey's honest significance test for multi-step comparisons was considered to study the differences among treatment levels. When p-values < 0.05 differences were referred as significant; when p-value < 0.1 differences were referred to as trends. Significant relationships between 2 variables were studied through Pearson correlation. Data were statistically analyzed using the IBM® SPSS® Statistics (V.27) software.

3 Results and Discussion

3.1 Ozone exposure and climatic conditions

The O$_3$ values throughout the 77-day fumigation period are presented in Table 1, where the 7-h mean of the diurnal ozone ranged from 23.44 to 65.30 nL L^{-1} and the AOT40 values ranged from 37 to 16 720 nL L^{-1} h for FA and NFA++ respectively. The maximum values of the NFA++ treatment are already common for the region during the spring months where it experiences maximum growth (Querol *et al.*, 2016).

Table 1. Ozone exposure throughout de experimental period from April 24 - July 10: 7-hour mean calculated from diurnal hourly means 09:00-16:00 GMT) (nL L^{-1}); AOT40 index, calculated as the accumulated ozone exposure values above 40 nL L^{-1}. FA=filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of added O_3, NFA++=non-filtered air + 40 nL L^{-1} of added O_3.

Indices	**7-hour mean** (nL L^{-1})	**AOT40** (nL L^{-1} h)
FA	23.44	37
NFA	44.97	5374
NFA+	53.90	10 057
NFA++	65.30	16 720

Weather conditions led to an increase in temperature, PAR and VPD from the tillering stage in April, at the beginning of O_3 exposure, until harvesting in July (Figure 1). Temperatures peaked around midday, while humidity reached its lowest points at the same time. This pattern intensified at the end of the experimental season causing all environmental variables to experience a wide range between April and July, with the highest monthly values ranging from 22-42 ºC, 18-49 %, 1.4-6.8 kPa for temperature, RH and VPD respectively.

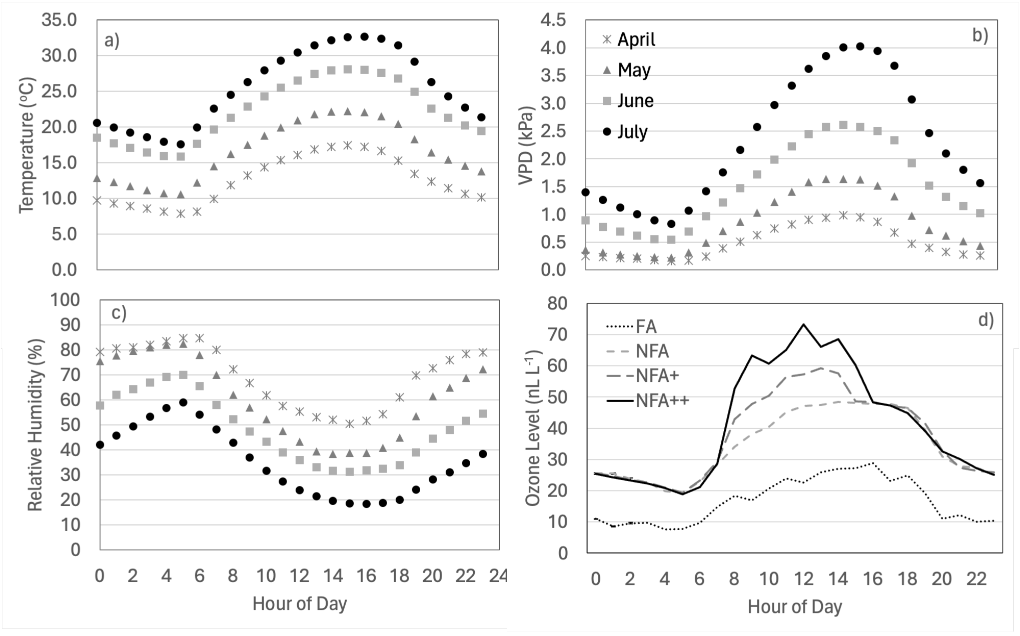

Figure 1. Mean daily profile of a) Temperature (ºC) b) Relative Humidity (%) and c) VPD (kPa) for the moths of the assay; and d) Mean daily profile of O_3 level (nL L^{-1}) for the the whole exposure period**.**

3.2 Leaf pigments

Measurements performed on FLs with the optical sensor Dualex© showed significant differences caused by N-dose for all the parameters (Table 2). Additional N increased the Chl index by 2.9% and the NBI by 5.1%; on the contrary, it decreased the Flavonols by 2.4% and Anthocyanins by 3.0%. The increase in Chl index is expected, as N is stored within the protein-chlorophyll thylakoids complex, making it a good indicator of N levels in the plant. It is after senescence that these organic

N compounds found in the chlorophyll break down to then be remobilized to the ear and grain (Brewster *et al.*, 2024).

Table 2. Leaf Chlorophyll (Chl), Flavonols (Flav) and Anthocyanins (Anth) content, the last two measured in relative absorbance units (RAU) and the Nitrogen Balance Index (NBI = Chl/Flav) in response to O$_3$ and N treatments. Measurements were performed on June 5th (DaE 42) using a handheld leaf sensor (Dualex) on the primary leaf of the secondary tiller, FA=filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of additional O$_3$, NFA++=non-filtered air + 40 nL L^{-1} of additional O$_3$; Low-N = Additional 100 kg ha^{-1} and High-N = 200 kg ha^{-1} (mean values ± SE).

		Chl (µg/cm^2)	**Flav (RAU)**	**Anth (RAU)**	**NBI**
Nitrogen					
100 kg ha-1		50.50^b ± 0.43	1.27^a ± 0.01	0.133^a ± 0.001	40.09^b ± 0.48
200 kg ha-1		51.88^a ± 0.38	1.24^b ± 0.01	0.130^b ± 0.001	42.05^a ± 0.44
p-value		**0.019**	**0.036**	**0.025**	**0.002**
Ozone					
FA		50.12 ± 0.51	1.26 ± 0.02	0.134 ± 0.001	40.17 ± 0.60
NFA		50.51 ± 0.59	1.25 ± 0.01	0.131 ± 0.001	40.58 ± 0.55
NFA+		52.16 ± 0.72	1.25 ± 0.01	0.129 ± 0.002	42.03 ± 0.79
NFA++		51.72 ± 0.44	1.26 ± 0.02	0.130 ± 0.001	41.27 ± 0.63
p-value		0.331	0.744	0.423	0.529
Nitrogen x Ozone					
p-value (N * O$_3$)		0.314	0.377	0.253	0.231
100 N kg ha^{-1}	FA	49.43 ± 0.63	1.28 ± 0.02	0.138^a ± 0.002	38.84 ± 0.89
	NFA	50.37 ± 0.80	1.27 ± 0.01	0.131^b ± 0.002	39.76 ± 0.56
	NFA+	51.90 ± 1.13	1.25 ± 0.02	0.131^b ± 0.003	41.92 ± 1.34
	NFA++	50.10 ± 0.69	1.27 ± 0.02	0.131^b ± 0.001	39.59 ± 0.63
p-value (N100)		0.206	0.755	**0.051**	0.110
200 N kg ha^{-1}	FA	50.81^b ± 0.78	1.23 ± 0.02	0.130 ± 0.002	41.50 ± 0.73
	NFA	50.70^b ± 0.90	1.22 ± 0.02	0.132 ± 0.002	41.68 ± 1.02
	NFA+	52.45^{ab} ± 0.87	1.25 ± 0.02	0.127 ± 0.002	42.14 ± 0.79
	NFA++	53.11^a ± 0.41	1.26 ± 0.02	0.130 ± 0.002	42.70 ± 0.97
p-value (N200)		**0.053**	0.656	0.310	0.756

Meanwhile, as NBI is formulated as the ratio of Chl to Flavonols content, and as it is related to N/C allocation (Cerovic *et al.*, 2012), increasing values would indicate that more N leads to more Chl than Flavonols. This follows previous works, as Flavonols have been previously seen to decrease at higher nitrogen levels because additional nitrogen inhibits phenyl alanine lyase (PAL) activity, which is essential for Flavonol production (Ibrahim *et al.*, 2011). This would explain why when wheat grown under the High-N was exposed to the highest ozone level NFA++, Flavonols increased, as N uptake assimilation is decreased by the pollutant (Broberg *et al.*, 2017).

The decrease in anthocyanins has also been previously seen, as values are often higher under nutrient deficiency (Yamuangmorn *et al.*, 2018; Zhang *et al.*, 2024). This follows with the observed values that were 2.3% higher under the Low-N treatment. Also specifically under Low-N, a significant anthocyanin reduction was measured induced by the O$_3$ values above NFA. This could be due to the degradation of the FL tissue as soon as the plant is exposed to ozone. Alternatively, this would not be seen under N-High because the additional N would be helping counteract the stress through antioxidants and/or repair.

Delving more into Chl, O_3 only significantly affected leaf pigment indexes under the High-N treatment, where chlorophyll content increased by 4.3% from FA to NFA++. This indicates that the additional N fertilizer could be allowing the plant to resist pigment loss when faced with increasing O_3 levels, coupled with a photosynthesis stimulation before the plant undergoes irreparable damage, which has been seen before (Salam and Soja, 1995). Moreover, as Chl correlated positively and strongly with foliar N (Figure 2), while it did not with foliar C, it could be inferred that the additional N would not be aiding carbonaceous structures, but possibly going towards antioxidation and repair (Calatayud *et al.*, 2002; Brewster *et al.*, 2024). This would be particularly important for the photosynthetic machinery, as chlorophyll is susceptible to ROS, and the plant could be prioritizing protection towards it by optimizing its synthesis or delaying chlorophyll breakdown, increasing antioxidant production to protect it (Janhom and Whangchai, 2023), and generally optimizing photosynthetic efficiency (Ren *et al.*, 2019; Lundgren and Fleming, 2020).

It should be noted that ozone usually induces a decrease in chlorophyll content (Pleijel *et al.*, 2006; Brewster *et al.*, 2024), but mainly after prolonged ozone exposure. However, in the present assay, the secondary stem FL measured had only emerged a week before measurements were taken, as it was phenologically delayed compared to the main stem, and an important chlorophyll loss was observed after prolonged exposure (Chang-Espino *et al.*, 2023).

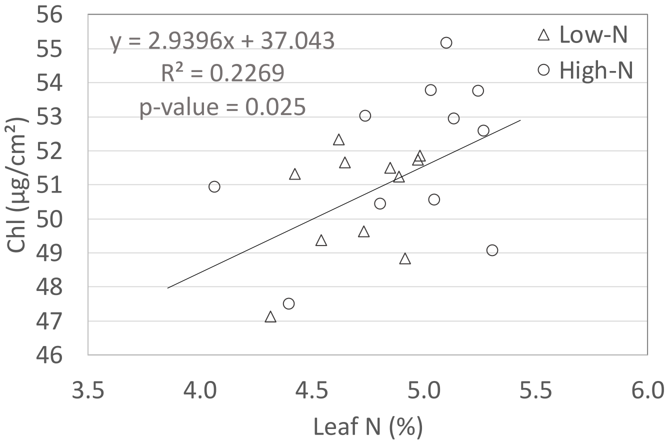

Figure 2. Correlation between Leaf N % and Chlorophyll Content Chl (□g cm^{-2}). The solid dark line shows the linear regression for all of the points.

3.3 Gas exchange

Nitrogen fertilization did not have a significant effect on any of the gas exchange parameters (Table 3). However, when ozone was analyzed across all N levels, all gas exchanged parameters, except for A, experienced significant effects. Stomatal conductance decreased significantly even under the lowest exposure level NFA (19% compared with FA across N levels) to then remain rather constant. The same percentage decrease in g_s was seen in a previous study with Artur Nick (Chang-Espino *et al.*, 2021) when ozone increased from FA to NFA. This might indicate that, as NFA represents the environmental values of the farm, current ozone pollution levels could already be affecting wheat.

Table 3. Gas exchange parameters response to the different O3 and N treatments: photosynthesis (A), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), ambient CO_2 concentration (C_a), intercellular to ambient CO_2 concentrations (C_i/C_a), and water use efficiency (WUE). FA=filtered air, NFA++=non-filtered air + 40 nL L^{-1} of additional O_3; Low-N = Additional 100 kg ha^{-1} and High-N = 200 kg ha^{-1} (mean values ± SE). Letters indicated significant differences among treatments ($p<0.05$).

		A (mmol CO_2 $m^{-2}s^{-1}$)	**g_s** (mmol H_2O m^{-2} s^{-1})	**C_i** (mmol mol^{-1})	**C_a** (mmol mol^{-1})	**C_i/C_a**	**WUE (A/g_s)** (units)
Nitrogen							
100 kg ha^{-1}		24.99 ± 0.44	0.55 ± 0.2	297 ± 4.01	389 ± 1.68	0.76 ± 0.01	46.58 ± 1.62
200 kg ha^{-1}		25.46 ± 0.69	0.54 ± 0.3	294 ± 3.63	388 ± 1.50	0.76 ± 0.01	48.49 ± 1.72
p-value		0.220	0.771	0.703	0.673	0.695	0.446
Ozone							
FA		26.39 ± 0.94	0.63a ± 0.02	304 ± 4.80	390 ± 2.06	0.78 ± 0.01	42.42^{b} ± 1.83
NFA		24.13 ± 0.73	0.51ab ± 0.03	296 ± 6.57	390 ± 3.20	0.76 ± 0.01	47.85^{ab} ± 2.27
NFA+		24.29 ± 0.78	0.51a ± 0.03	291 ± 4.79	386 ± 1.95	0.75 ± 0.01	49.06^{ab} ± 2.25
NFA++		25.91 ± 0.39	0.52ab ± 0.03	291 ± 5.26	387 ± 2.07	0.75 ± 0.01	51.15^{a} ± 2.33
p-value		0.302	**0.016**	**0.006**	**0.046**	**0.009**	**0.005**
Nitrogen x Ozone							
p-value (N * O_3)		**0.002**	0.214	0.828	0.996	0.700	0.862
100 kg ha^{-1}	FA	24.17 ± 1.08	0.60 ± 0.03	308 ± 8.12	391 ± 3.16	0.79 ± 0.02	41.01^{b} ± 3.32
	NFA	24.65 ± 0.77	0.52 ± 0.04	296 ± 9.04	390 ± 4.59	0.76 ± 0.01	47.96^{ab} ± 2.96
	NFA+	26.00 ± 1.16	0.58 ± 0.04	297 ± 8.88	387 ± 3.63	0.77 ± 0.02	45.52^{ab} ± 3.19
	NFA++	25.35 ± 0.56	0.49 ± 0.02	288 ± 5.31	388 ± 2.75	0.74 ± 0.01	51.64^{a} ± 2.09
p-value (N100)		0.539	0.127	0.355	0.896	0.175	0.103
200 kg ha^{-1}	FA	28.61^{a} ± 0.61	0.66^{a} ± 0.03	300 ± 5.38	389 ± 2.93	0.77 ± 0.01	43.83 ± 1.76
	NFA	23.28^{b} ± 1.55	0.50^{ab} ± 0.07	296 ± 11.44	389 ± 5.04	0.76 ± 0.02	47.66 ± 4.36
	NFA+	24.29^{ab} ± 0.80	0.46^{b} ± 0.04	287 ± 5.38	386 ± 2.43	0.74 ± 0.01	51.43 ± 2.88
	NFA++	26.61^{ab} ± 0.33	0.55^{ab} ± 0.06	296 ± 11.93	387 ± 3.60	0.76 ± 0.02	50.54 ± 5.01
p-value (N200)		**0.001**	**0.033**	0.575	0.874	0.486	0.363

Meanwhile, WUE increased across N levels, reflecting the stability of A when confronted with the decreasing g_s. Simultaneously, C_i/C_a decreased when exposed to ozone (across N treatments), suggesting some improvement in carboxylation efficiency. The C_i/C_a decrease and WUE rate increase indicate that exposure to ozone led to a more efficient CO_2 assimilation and water use to cope with stress across N levels.

Though these patterns give us some insight into the effect of ozone on gas exchange at that moment of the plant life, where some mechanisms were aiding in maintaining A despite increasing levels of the pollutant, more can be explained when delving into each N treatment.

The WUE values were largely driven by N treatment, as shown by Figure 3. Here, the slope for the N-High regression is steeper than for N-Low, indicating that the association between A/g_s would be largely driven by N supply, as given the R^2, 68% of the variability of A under High-N would be explained by g_s. Furthermore, the strong correlation between g_s and A ($p<0.001$) shows that g_s is playing a pivotal role in driving photosynthesis early in the plant life, even more so than carboxylation as, even though A and C_i/C_a also correlated significantly and positively under the High-N conditions, this correlation was not as strong as with g_s ($p=0.042$). This could indicate that while ozone was affecting the stomata, thereby limiting photosynthesis due to a reduced CO_2 diffusion, ozone may not have affected carboxylation as much by this stage, which has been seen before (Grandjean Grimm and Fuhrer, 1992).

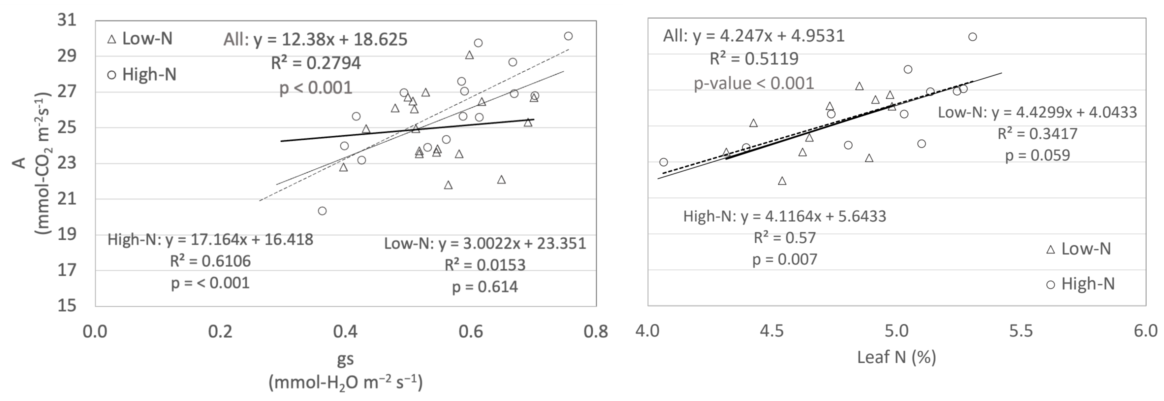

Figure 3. Correlation between A with g_s and Leaf N %. The solid line shows the linear regression for all of the points; the dark line shows the linear regression for Low-N, and the dotted line for High-N.

Meanwhile, as mentioned before, the additional N could be aiding in maintaining the chlorophyll structure, which can be seen by the positive and strong correlation of N_{Leaf} with A (Figure 3), which was only significant under N-High. These trends and distribution paralleled the findings of an experiment performed with different nitrogen and water treatments in durum wheat (Cabrera-Bosquet, 2009), where it was noted that the larger N input led to a larger biomass and a higher water input into the leaves, therefore causing a higher N_{Leaf} and therefore A. It should be noted that as ozone reduces the relative water content in leaves (Zheng *et al.*, 2011), and coupled with the strong effect it had on Chl, g_s and therefore A, this could explain the stronger correlations seen under High-N.

All of this supports the theory of improved photosynthetic efficiency due to the additional N in preparation to face O_3 stress. This was also reflected by the pattern in A seen under N-High, which decreased until NFA+, but then increased under the highest ozone level.

However, ozone exposure ultimately led to a larger production loss in High-N plants (Chang-Espino *et al.*, submitted for publication), indicating that the observed increase in A must be an early response occurring in recently expanded flag leaves, which were still subjected to a short period of ozone exposure. This increase in A would probably continue to then decline sharply under prolonged ozone exposure, something that was not detected with the instantaneous gas exchange measurements performed.

3.4 Nutrient Content and Isotopic Signatures

Carbon (Cabrera-Bosquet *et al.*, 2009; Tcherkez *et al.*, 2011) and nitrogen (Cui *et al.*, 2020; Effah *et al.*, 2022) isotopic signatures have been frequently used to analyze plant stress, but are not considered as much in ozone-induced stress studies (Saurer *et al.*, 1991; Jäggi *et al.*, 2005; Ma *et al.*, 2022). One main advantage is their ability to integrate the effects of the pollutant throughout the life cycle of the plant up to the point of analysis (Araus *et al.*, 2013; Chang-Espino *et al.*, 2021).

Nitrogen fertilization did not affect the N and C content in the FLs; nor was the isotopic signature of C significant. The most responsive foliar parameter was $\delta^{15}N_{leaf}$, which decreased by 1.23‰ due to the additional 100 kg ha^{-1} (Table 4). The observed differences in $\delta^{15}N_{leaf}$ could be due to the chemical fertilizer used, ammonium nitrate, which tends to have a very low $\delta^{15}N_{l5}$ (Choi *et al.*, 2006; Rezzouk *et al.*, 2023) and the fact the amount of fertilizer was doubled in High-N. Regarding pollutant effects, no significant responses were seen for any of the leaf parameters due to O_3 exposure, both where all values were compiled across N treatments and per N treatment separately (Table 4). Generally, isotopic signatures measured in leaf are not as significant in portraying ozone stress as those measure in grain (Saurer *et al.*, 1991; Jäggi *et al.*, 2005). This was also the case for this assay, where isotopic results in leaves contrast with the responsiveness of these parameters in grain, with C and N content, and their isotopic signatures, which showed significant responses to both N and O_3.

Following the leaf response pattern, additional N fertilization decreased $\delta^{15}N_{grain}$ by 0.66 ‰, which would be also related with the low $\delta^{15}N$ of chemical fertilizer. However, the observed $\delta^{15}N$ response in grains was milder compared to that observed in leaves, possibly due to discrimination effects during translocation, which causes the N to be ^{14}N-depleted by when it reaches the grain (Cui, 2020). Increasing exposure to the pollutant also caused a decrease in $\delta^{15}N_{grain}$ which could also be driven by an overall lower remobilization induced by the pollutant (Brewster *et al.*, 2024), but also because of the previously indicated O_3-induced effects on stomatal aperture. Stomatal conductance reduction could lead to lower volatilization of the lighter ^{14}N in the leaves, and therefore more ^{14}N would be left behind in plant, leading to a lower amount of ^{15}N in the remobilized N under the higher O_3 exposures (Farquhar *et al.*, 1980). Despite the non-significant O_3 x N interaction, the analysis revealed significant differences in $\delta^{15}N_{grain}$ among O3 treatments under the higher N treatment when data was analyzed individually. Differences between both N doses ranged from 0.44 to 0.58‰ for FA to NFA+, but then increased to 0.95‰ by NFA++ due to the large drop of $\delta^{15}N$ by the highest O_3 level and high fertilizer dose, indicating that the high N supplementation increased the susceptibility of the plant to the O_3 exposure. This large drop in $\delta^{15}N$ has been seen in previous experimental studies under other stress conditions due to a lower stomatal conductance induced by water stress or increased salinity (Yousfi *et al.*, 2013; Sanchez-Bragado *et al.*, 2017), where the decreased g_s would negatively affect the transpiration stream and therefore N assimilation (Sanchez-Bragado *et al.*, 2017). This trend was seen with modern Mediterranean cultivars of a previous assay, including Artur Nick (Chang-Espino *et al.*, 2021).

As expected, N_{grain} content increased significantly with the additional N, but was not significantly affected by the pollutant across N treatments. However, when each N treatment was analyzed separately for the effect of O_3, N_{grain} increased significantly under N-High. This result agrees with previous works (Feng *et al.*, 2008; Pleijel and Uddling, 2012), where the O_3-induced increase in N_{grain} was related with a greater decrease in starch accumulation than in N in the grain, leading to smaller grains that have a more concentrated N. This concentration effect in reproductive structures has been found in other species responding to O_3 like annual pastures seeds (Sanz *et al.*, 2005). However, despite the N concentration effect, the actual amount of N does decrease, as is shown by the GNY (Table 4) and in previous works (Wilkinson *et al.*, 2012; Broberg *et al.*, 2017; Ma *et al.*, 2022). It should be noted that the low GNY found under FA could be the effect of an early fungal infection that was treated, but which harmed the primary FLs (Chang-Espino et al., 2023), reinforcing the importance of the FL for grain filling later in the plant life (Sanchez-Bragado *et al.*, 2014).

N_{grain} and $\delta^{15}N_{grain}$ behave inversely regarding their response to N, showing that their responses are associated with each other, as while more N would lead to a higher $N_{grain,}$ it would also allow for more discrimination against ^{15}N. Additionally, the differences in their responses to ozone, where the effect of ozone was stronger on the isotope, also suggest that δ^{15}N$_{grain}$ would be a stronger indicator of pollutant stress.

Meanwhile, the response of δ^{15}N$_{grain}$ to O$_3$, which was only significant under High-N, would indicate a decrease in remobilization due to the lower effect of O$_3$ on δ^{15}N$_{leaf}$ under the same N treatment. More specifically, as the plant had been exposed to O$_3$ from tillering, and as the pre-anthesis time is the most important stage for N uptake (Sanchez-Bragado *et al.*, 2014), an effect on δ^{15}N$_{leaf}$ would have been related more to uptake at this stage of measurement. However as an effect was only seen on δ^{15}N$_{grain}$, this would indicate that ozone had a stronger effect on remobilization.

Significant differences were only found for the N x O$_3$ interaction for C$_{grain}$, which showed different patterns for the values measured under each N treatment, as values remained stable in Low-N, but decreased under N-High, though neither was significantly affected by O$_3$. Meanwhile, δ^{13}C$_{grain}$ presented great responsiveness to the N and O$_3$ factors individually without interaction (Table 4). This could be because δ^{13}C$_{grain}$ values increased similarly under each of the N treatments.

The significantly increasing values of δ^{13}C$_{grain}$ (0.16‰) due to additional N could be due to an increase in transpirative (i.e. aerial) biomass, which can induce some degree of water stress, therefore decreasing g$_s$ (Araus *et al.*, 2003; Cabrera-Bosquet *et al.*, 2007). Meanwhile, the significant increase (less negative value) in δ^{13}C$_{grain}$ (of 1.71% from FA to NFA++) in response to enhanced exposition to O$_3$ (Table 4) has been reported before (Saurer *et al.*, 1991; Ma *et al.*, 2022). Chang-Espino *et al.*, (2021) linked this response to its status as a good indicator of chronic O$_3$ stress associated with a decrease in g$_s$; as was found in this assay (Figure 4). The O$_3$-induced increase of δ^{13}C$_{grain}$ persisted when each N treatment was analyzed individually, both having a similar overall increase (1.66% under Low-N and 1.68 under High-N), and therefore showing that additional fertilization does not affect this index.

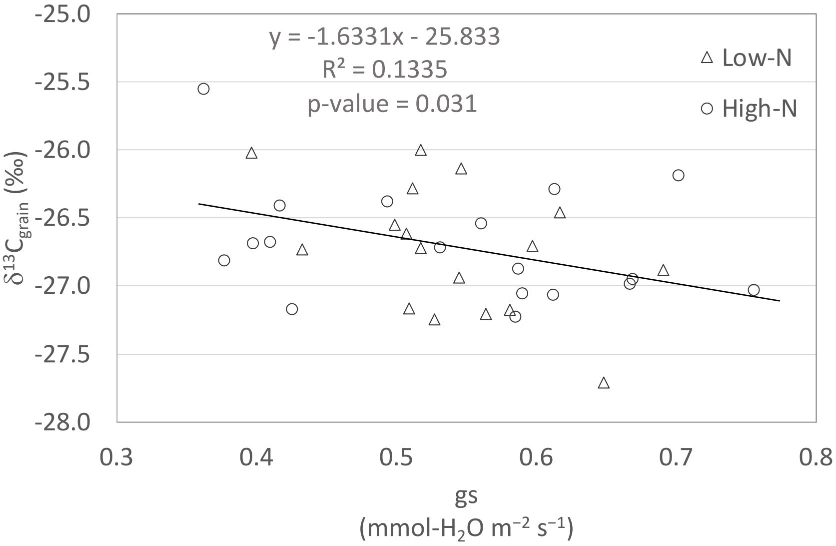

Figure 4. Correlation between g$_s$ and δ^{13}C$_{grain}$. The solid black line shows the linear regression for all of the points together.

The similar pattern of the N-Low and N-High O3-response of the present assay, would indicate that high additional N did not aid in reducing the accumulated stress affecting the grain, which is concordant with the strong O_3 effect observed on GNY and GY under N-High ozone, which almost eliminated the fertilizer stimuli (Chang-Espino *et al.*, submitted for publication). These results, coupled with the aforementioned effect that additional N counterbalances ozone at the earlier phenological stages but not later, strengthens the idea that O_3 stress was strongly reflected by $\delta^{13}C_{grain}$ (p=0.001), as has been mentioned in previous studies (Saurer *et al.*, 1991; Pausch *et al.*, 1996; Chang-Espino *et al.*, 2021), reinforcing the statement that isotopes can be a great tool to measure the chronic effects of a stressor like O_3 on the plant.

3. 5 $\delta^{13}C_{grain}$ response to O_3 of combined Mediterranean modern varieties

To further explore the strength of $\delta^{13}C_{grain}$ as an indicator of O_3-stress, the values measured for the present assay were combined with the values of a previous study conducted with different Mediterranean modern cultivars, following a similar experimental design, but only considering the O_3 factor (Chang-Espino *et al.*, 2021). The $\delta^{13}C_{grain}$ response to increased levels of the pollutant showed a similar range and pattern for all the varieties considered (Figure 5a). A One-way ANOVA analysis with pooled values of the 2015 and 2018 Artur Nick variety indicated that the $\delta^{13}C_{grain}$ values were strongly and significantly affected by the pollutant (p<0.001) and showed a significant regression (y = 3E-05x – 26.971, R^2 = 0.4920, p<0.001). The ANOVA analysis comprising all the modern varieties also showed a high significance for the $\delta^{13}C_{grain}$ response to O_3 (p<0.001) as well as a significant regression (y = 5E-05x – 26.855, R^2 = 0.5967, p<0.001). Compared with the other varieties, Artur Nick and Nogal presented the softest O_3-response, where the Artur Nick slope ranged from $2x10^{-5}$ to $4x10^{-5}$, while Berdun and Califa Sur had a slope of $5x10^{-5}$.

In the previous assay of Artur Nick, wheat plants were supplemented with 120 kg N ha^{-1}; thus being closer to the Low-N dose of the present assay; in fact when both regressions were compared there were no differences in the slopes between them (p-value =0.246). Moreover, when analyzed with a two-way ANOVA, significant differences were only seen due to the effect of ozone on both data groups (p < 0.001) but not by year (p = 0.181) nor by the interaction (0.088), again reinforcing the strength of the indicator.

Nonetheless, when the values of the present assay were compared to the pooled values of the previous analysis, differences were seen (p-value = 0.033), which could be due to the effect of the growth conditions between the years. The present assay was performed on a wetter year compared with the previous assay, and plants experienced a 10% higher RH, facilitating stomatal opening and therefore causing more general negative $\delta^{13}C_{grain}$ values, although following the same response pattern (Farquhar *et al.*, 1989; Saurer *et al.*, 1991).

Additionally, the 2015 measurements had a better goodness of fit and a steeper slope than the 2018 ones (Figure 5b), which could indicate that AOT40 would not be explaining all of the factors affecting $\delta^{13}C_{grain}$ in 2018. As this measurement only considers the accumulated ozone levels above 40 nL L^{-1} to which the plant is exposed, and coupled with the fact that 2018 was a wetter year, which would involve a more readily absorption of O_3, other factors influencing stomatal conductance should be considered when making these comparisons.

Table 4. Carbon and nitrogen content in leaf and grain (mean values in % ± SE), leaf and grain isotopic signature of C and N (mean values in ‰ ± SE) and grain N yield (GNY) (mean values± SE) responses to the different O_3 and N treatments. FA=filtered air, NFA=non-filtered air, NFA+=non-filtered air + 20 nL L^{-1} of additional O_3, NFA++=non-filtered air + 40 nL L^{-1} of additional O_3; Low-N = Additional 100 kg ha-1 and High-N = 200 kg ha-1. Different letters indicate significant differences among treatments (p-value <0.05).

		N_{leaf}	**C_{leaf}**	**$\delta^{15}N_{leaf}$**	**$\delta^{13}C_{leaf}$**	**N_{grain}**	**C_{grain}**	**$\delta^{15}N_{grain}$**	**$\delta^{13}C_{grain}$**	**GNY**
Nitrogen										
Low-N		4.72 ± 0.07	48.26 ± 0.23	2.36^{a} ± 0.12	-27.71 ± 0.12	2.35^{b} ± 0.02	40.70 ± 0.02	3.84^{a} ± 0.04	-26.74^{b} ± 0.06	0.33^{b} ± 0.01
High-N		4.92 ± 0.12	48.18 ± 0.21	1.13^{b} ± 0.14	-27.77 ± 0.10	2.55^{a} ± 0.02	40.72 ± 0.02	3.18^{b} ± 0.07	-26.58^{a} ± 0.06	0.36^{a} ± 0.01
p-value		0.387	0.829	**0.001**	0.959	**<0.001**	0.550	**<0.001**	**0.009**	**<0.001**
Ozone										
FA		4.90 ± 0.16	48.17 ± 0.32	1.83 ± 0.37	-27.80 ± 0.08	2.41 ± 0.05	40.70 ± 0.04	3.66 ± 0.09	-26.95^{b} ± 0.08	0.34 ± 0.01
NFA		4.62 ± 0.16	48.63 ± 0.20	2.00 ± 0.16	-27.62 ± 0.17	2.45 ± 0.03	40.76 ± 0.04	3.64 ± 0.09	-26.49^{a} ± 0.08	0.36 ± 0.01
NFA+		4.78 ± 0.10	48.13 ± 0.35	1.56 ± 0.23	-27.94 ± 0.16	2.37 ± 0.03	40.73 ± 0.03	3.57 ± 0.10	-26.70^{ab} ± 0.06	0.35 ± 0.01
NFA++		4.96 ± 0.12	48.10 ± 0.32	1.59 ± 0.44	-27.56 ± 0.17	2.52 ± 0.03	40.65 ± 0.02	3.26 ± 0.12	-26.49^{a} ± 0.08	0.33 ± 0.01
p-value		0.171	0.564	0.567	0.231	0.184	0.802	0.120	**0.003**	0.455
Nitrogen x Ozone										
p-value (N * O_3)		0.107	0.203	0.227	0.948	0.369	**0.052**	0.130	0.426	**0.003**
Low-N	FA	4.61 ± 0.19	48.15 ± 0.55	2.58 ± 0.16	-27.77 ± 0.14	2.27 ± 0.06	40.66 ± 0.05	3.93 ± 0.07	-27.08^{b} ± 0.12	0.35 ± 0.02
	NFA	4.74 ± 0.16	48.69 ± 0.28	2.23 ± 0.08	-27.47 ± 0.25	2.38 ± 0.03	40.78 ± 0.05	3.88 ± 0.08	-26.56^{a} ± 0.09	0.33 ± 0.01
	NFA+	4.82 ± 0.11	48.70 ± 0.45	2.03 ± 0.16	-27.94 ± 0.21	2.30 ± 0.04	40.68 ± 0.04	3.85 ± 0.09	-26.70^{a} ± 0.08	0.31 ± 0.02
	NFA++	4.69 ± 0.04	47.51 ± 0.32	2.48 ± 0.39	-27.64 ± 0.36	2.43 ± 0.05	40.66 ± 0.03	3.73 ± 0.08	-26.63^{a} ± 0.11	0.32 ± 0.02
p-value (N100)		0.785	0.219	0.480	0.624	0.059	0.215	0.365	**0.003**	0.493
High-N	FA	5.20 ± 0.08	48.19 ± 0.44	1.09 ± 0.33	-27.83 ± 0.13	2.55^{ab} ± 0.05	40.73 ± 0.06	3.49^{a} ± 0.13	-26.81^{b} ± 0.10	0.33^{b} ± 0.02
	NFA	4.43 ± 0.37	48.45 ± 0.00	1.65 ± 0.14	-27.85 ± 0.05	2.56^{ab} ± 0.04	40.75 ± 0.06	3.30^{a} ± 0.12	-26.39^{a} ± 0.13	0.41^{a} ± 0.01
	NFA+	4.74 ± 0.20	47.57 ± 0.32	1.24 ± 0.22	-27.94 ± 0.29	2.45^{b} ± 0.03	40.77 ± 0.03	3.29^{ab} ± 0.14	-26.71^{ab} ± 0.09	0.39^{ab} ± 0.02
	NFA++	5.14 ± 0.07	48.70 ± 0.22	0.71 ± 0.14	-27.49 ± 0.13	2.62^{a} ± 0.04	40.63 ± 0.03	2.78^{b} ± 0.12	-26.36^{a} ± 0.11	0.33^{b} ± 0.02
p-value (N200)		0.068	0.223	0.163	0.400	**0.049**	0.166	**0.007**	**0.009**	**0.003**

It is also interesting to note that the response of Artur Nick was milder in the present assay compared with the previous one, even though this experiment involved a longer O_3 exposure period as it started earlier, during tillering, as opposed to the previous assay where O_3 exposure started in anthesis. This leads to hypothesizing whether early exposure may allow for some type of plant adaptation to alleviate O_3 stress.

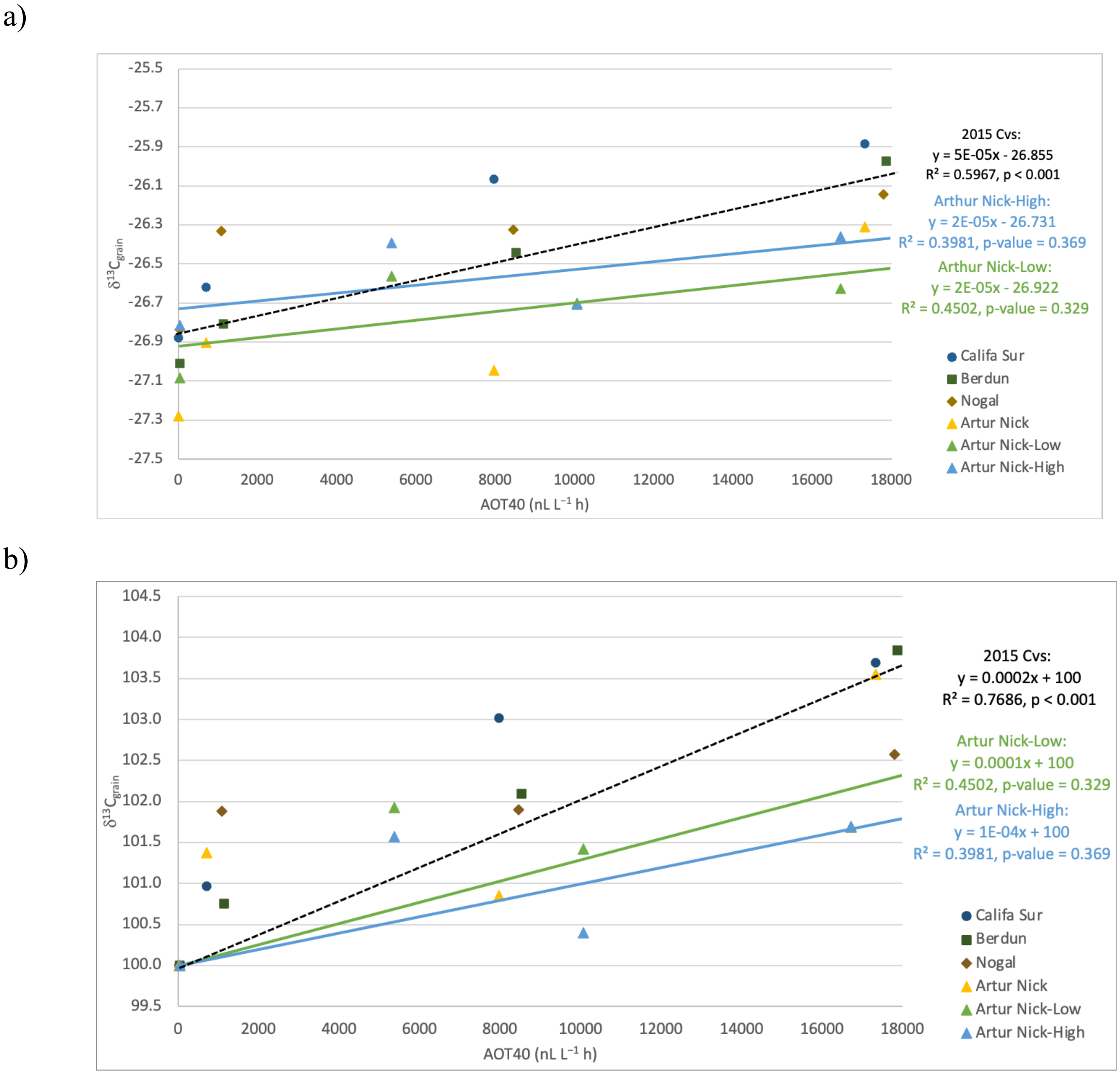

Figure 5. a) Response of $\delta^{13}C_{grain}$ to O_3 and b) Relativized response of $\delta^{13}C_{grain}$ to O_3 following the Air Convention Methodology, considering Mediterranean modern varieties Artur Nick, Berdun, Califa Sur and Nogal (combining data from previous assay, Chang et al., 2021). AOT40=Accumulated O_3 diurnal hourly values over 40 nL L^{-1}.

3.6 Leaf anatomy

In examining potential early adaptations in plant morphology to ozone exposure, and to better understand early ozone exposure, the leaf anatomy of the FLs was analyzed through light microscopy. Different parameters per unit leaf area were measured: wheat mesophyll volume (μm^3), mesophyll area (μm^2), number of mesophyll cells (μm^{-1}) and leaf thickness (μm). No significant differences were found in the leaf cross-sections for any of the traits analyzed in response to the N fertilization (Table 5).

Table 5. Wheat mesophyll volume per unit of leaf area (µm^3 µm^{-2}), mesophyll area per unit of leaf area (µm^2 µm^{-2}), number of mesophyll cells per unit leaf surface area (cells µm^{-1}), leaf thickness (µm), were extracted from microscopic images. FA=filtered air, NFA++=non-filtered air + 40 nL L^{-1} of additional O_3; Low-N = Additional 100 kg ha-1 and High-N = 200 kg ha-1 (mean values ± SE).

Nitrogen		**Mesophyll volume per unit surface leaf area (µm^3 µm^{-2})**	**Mesophyll area per unit leaf area (µm^2 µm^{-1})**	**Number of mesophyll cells per unit surface leaf area (cells µm^{-1})**	**Leaf thickness (µm)**
Low-N		44.60 ± 4.59	14.12 ± 0.71	0.22 ± 0.01	148.31 ± 12.35
High-N		44.33 ± 2.80	14.12 ± 1.17	0.20 ± 0.02	157.54 ± 9.54
p-val		*0.964*	*0.998*	*0.349*	*0.594*
Ozone					
FA		42.80 ± 2.89	13.23 ± 0.70	0.18^{b} ± 1.01	160.28 ± 9.34
NFA++		46.12 ± 4.41	15.01 ± 1.03	0.23^{a} ± 1.01	145.57 ± 11.97
p-value		*0.583*	*0.212*	***0.028***	*0.402*
Nitrogen x Ozone					
p-value (O_3 x N)		*0.644*	*0.361*	*0.777*	*0.866*
Low-N	FA	44.33 ± 5.90	13.86 ± 1.15	0.19 ± 0.01	154.22 ± 10.10
Low-N	NFA++	44.86 ± 8.39	14.38 ± 1.05	0.24 ± 0.02	142.40 ± 25.02
p-value (Low-N)		0.961	0.760	0.097	0.684
High-N	FA	41.27 ± 2.13	12.60 ± 0.84	0.17 ± 0.02	166.34 ± 17.42
High-N	NFA++	47.39 ± 5.03	15.65 ± 1.94	0.22 ± 0.03	148.75 ± 8.96
p-value (High-N)		0.325	0.222	0.151	0.416

However, regarding O_3-exposure, interesting findings were observed. Exposure to high pollutant levels significantly increased the number of mesophyll cells per section by 22%. This response would increase the surface area covered by chloroplasts that is exposed to the intracellular airspace. Several works on cell structure indicated that these changes, though small, enhance the CO_2 uptake and, therefore, increase the photosynthetic efficiency of the plant (Lundgren et al., 2019; Ren, 2019). In the present work, although the response of the mesophyll surface between O_3 treatments was not significant, it also shows an increasing pattern due to a higher O_3 exposure.

Concurrently, there is a trend of C_i decrease that occurs with the increasing number of mesophyll cells (p= 0.077) (Figure 6a). This relationship persisted only under the High-N (p=0.079) when both N doses were studied separately. This relationship strengthened when considering C_i/C_a (p= 0.059) and, similarly to C_i, persisted under High-N (p=0.041) (Figure 6b). It should be noted that both C_i and C_i/C_a values peaked under FA and Low-N, meaning that under this combined situation, more CO_2 was being absorbed, (due to the absence of the pollutant) and maintained within the intracellular space (as there were less mesophyll cells created by the Low-N to absorb it). The pollutant also increased WUE (p=0.027), possibly stimulating photosynthesis before irreversible damage was caused to the plant, as observed previous studies (Salam and Soja, 1995). This would indicate that the higher O_3 exposure caused the plants to become more photosynthetically efficient at the early stage but at the expense of a lowered g_s.

The difference in number of cells caused by the pollutant across N treatments was similar under both N treatments (increasing by 26% under Low-N and 29% under High-N); while differences were larger, though non-significant, between FA and NFA++ under High-N for mesophyll volume (+15%) and area (+24%). Results suggest again that while a higher exposure to the pollutant may cause the plant to improve its photosynthetic efficiency when supplemented with extra N, this supplemented N may still not be able to compensate for the effects of O_3, which agrees with the aforementioned increase in Chl, particularly under High-N.

Most of the results of this assay lie within range of another experiment examining the flag leaf anatomy of wheat in Spain (Araus *et al.*, 1986). The calculated mesophyll area in that experiment was very similar at 14.14 ($\mu m^2\ \mu m^{-1}$) while the number of mesophyll cells was 163.53 mm^{-1}. When transformed from μm^{-1}, the number of mesophyll cells for this experiment ranged from 170-190 cells mm^{-1}, only considering the non-fumigated values. However, the calculated volume was lower. When transformed into the same units, the values in this experiment ranged around 0.44 $cm^3\ dm^{-2}$, while Araus *et al.*, (1986) had an average of 0.84 $cm^3\ dm^{-2}$.

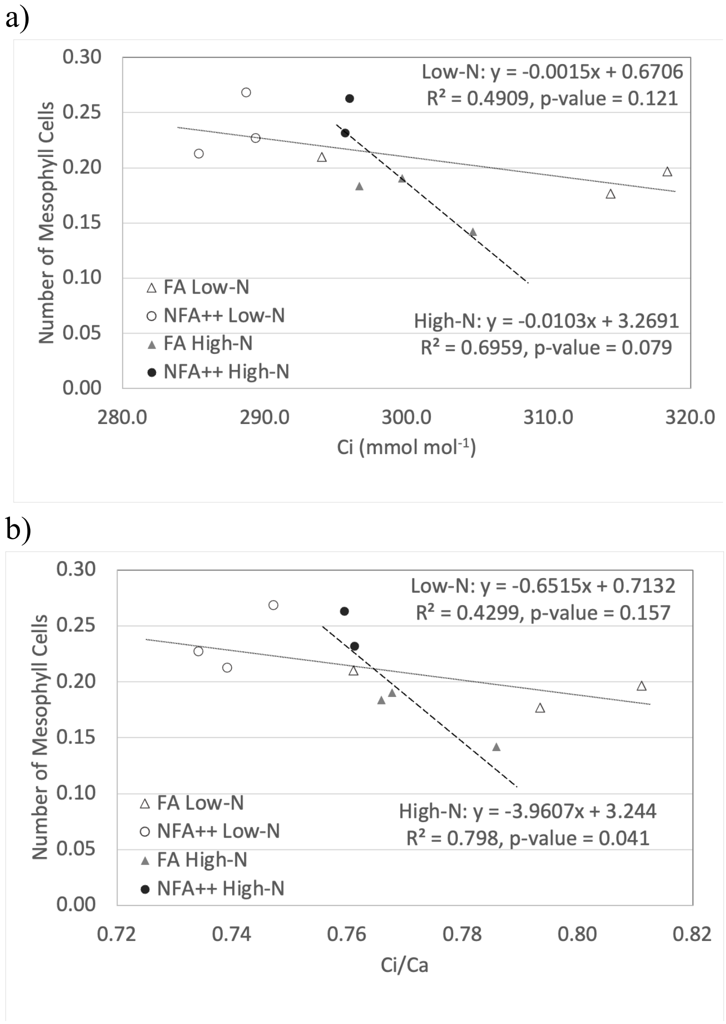

Figure 6. Correlation between number of mesophyll cells with a) Ci and b) Ci/Ca; the dashed line shows the linear regression for High-N, and the dotted line for Low-N.

The addition of N fertilizer in wheat crops alters the response of the plant to O_3 fumigation, particularly improving photosynthetic efficiency at the lower ozone levels and heightening its susceptibility to higher pollutant levels. The ameliorating effect could come from a higher availability of resources for defense and repair, while the detrimental effect could stem from the increased biomass facilitated by the additional fertilizer, higher stomatal conductance, and therefore increased O_3 uptake, which would lead to increased oxidation of the plant tissue. More specifically, under the High-N treatment, when exposed before anthesis, ozone had a predominant effect on the stomatal conductance of the plant rather than on carboxylation efficiency, which led to a decreased CO_2 diffusion, affecting A.

However, this was coupled with an attempt from the plant to counteract the effect of ozone effect by increasing its photosynthetic efficiency, due to an augmentation of the surface area of the mesophylls, where chloroplasts are located, which in turn reduced the Ci/Ca. Nonetheless, the counteraction was not successful by the highest ozone level, because there was not a corresponding increase in C_{leaf} and there was a decrease in the discrimination against $\delta^{13}C_{grain,}$ indicating that carbon structures were not being formed as readily through the process of photosynthesis. Consequently, despite the investment in additional fertilizer, which incurs higher costs for producers, ozone-induced damage compromises wheat photosynthetic efficiency, potentially resulting in economic losses and environmental issues like N_2O or NH_3 emissions, or leaching.

Moreover, while early ozone exposure induced a defensive reaction for the plants grown under the higher N treatment, these mechanisms did not perdure towards the end of the plant life, as $\delta^{13}C_{grain}$ showed that the plant had undergone stress throughout its life despite the additional N, reinforcing its use as an index of O_3 stress as its potential persisted even with other cultivars and in a different growth year.

4. Conclusions

The study investigated the multifaceted impacts of ozone exposure and nitrogen fertilization on wheat physiology and adaptation mechanisms. Following the initial hypothesis, it was noted that $\delta^{13}C$ in grain is a robust indicator of chronic ozone stress, being stronger at showing the detrimental effect of ozone on the plant than instantaneous gas exchange measurements. It was also a good indicator for comparing measurements performed in different years, where differences could be explained due to the experimental conditions. However, nitrogen did not alter the response of $\delta^{13}C_{grain}$ to ozone, indicating that the additional fertilizer did not ultimately aid in counteracting the effects of the pollutant. Meanwhile, differences in $\delta^{15}N$ in leaf and grain did show a decrease in N remobilization due to exposure to the pollutant.

Finally, the most interesting aspect seen was the early response of the plant to the pollutant. Additional N increased chlorophyll content, suggesting a potential for plants to resist pigment loss under high N availability and O_3 fumigation. This was coupled with an increase in the number of mesophyll cells and consequential reduced $C_{i,}$ suggesting an attempt to increase photosynthetic activity. This may have been successful under the lower levels of the pollutant as shown by the increased dependence on stomatal activity that A had under High-N, indicating that ozone was not hindering rubisco carboxylation as much. However as mentioned before, this effect did not perdure later in the plant life as shown by the response of $\delta^{13}C_{grain}$ to ozone.

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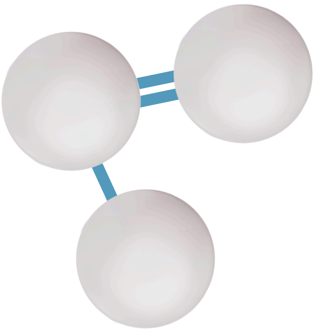
Chapter 4



Resumen del Capítulo 4

El aumento del ozono troposférico es una preocupación importante para la producción de cultivos en el marco del Cambio Global, especialmente en la cuenca mediterránea donde las condiciones climáticas favorecen su formación fotoquímica. Mientras tanto, el aumento de enfermedades comunes en los cultivos, como la roya amarilla —uno de los patógenos más importantes que afectan la producción de trigo en el mundo—, ha sido recientemente detectada en la zona. Sin embargo, el impacto del ozono en la ocurrencia e impacto de enfermedades fúngicas no se conoce bien.

Se llevó a cabo un ensayo en condiciones similares al campo (instalación de cámaras de techo descubierto) situadas en un área de cultivo de cereales mediterráneos de secano para estudiar el impacto del aumento de los niveles de O_3 y la fertilización con N en brotes fúngicos espontáneos en trigo. Se consideró cuatro niveles de fumigación con O_3 que reproducen niveles del contaminante desde preindustriales a futuros, con 20 y 40 nL L-1 adicionales sobre los niveles ambientales. Dos suplementos de fertilización nitrogenada (100 y 200 kg ha-1) se agregaron a cada fumigación con O_3; se midieron el daño foliar, el contenido de pigmentos y los parámetros de intercambio gaseoso. Los niveles de preindustriales de O3 favorecieron fuertemente la infección por roya amarilla, mientras que los niveles de O_3 actuales, que ya ocurren en la finca, beneficiaron al cultivo, mitigando la presencia de la roya en un 22%. Sin embargo, los futuros niveles esperados de O_3 neutralizaron el efecto beneficioso sobre el control de la infección al inducir una senescencia temprana en el trigo, disminuyendo el índice de clorofila de las hojas más antiguas hasta en un 43% bajo la mayor exposición a O_3. El nitrógeno promovió la infección por roya hasta en un 49.5%, sin interactuar con el factor O_3. Alcanzar los futuros estándares de calidad del aire podría requerir considerar nuevos programas de mejora varietal, para poder adaptar a los cultivos a una mayor tolerancia a los patógenos sin necesitar de la ayuda que proporcionan los actuales niveles de O_3.

Science of the Total Environment 880 (2023) 163370

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Science of the Total Environment

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Current ambient ozone levels mitigate the effect of *Puccinia striiformis* on wheat: Is Mediterranean wheat ready for pre-industrial background ozone levels?

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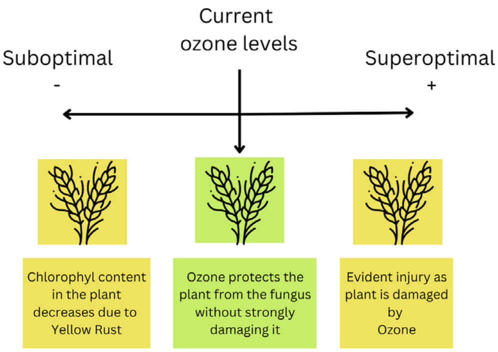
HIGHLIGHTS

- Pre-industrial O_3 levels favor yellow rust infection in modern Mediterranean wheat.
- Current O_3-polluted Mediterranean atmospheres mitigate wheat rust damage by 22 %.
- Projected O_3-levels induced early senescence decreasing the rust control benefit.
- Nitrogen fertilization increases rust incidence but did not interact with O_3.
- Achieving air quality standards might require improvements in pathogen tolerance.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Charlotte Poschenrieder

Keywords:
Pollution
Crop-pathogens
Fertilization
O_3-elicitor
Wheat
Yellow-rust

ABSTRACT

Increasing surface ozone is a main concern for crop production in the Global Change framework, especially in the Mediterranean basin where climate conditions favor its photochemical formation. Meanwhile, increasing common crop diseases, such as yellow rust, one of the most important pathogens affecting global wheat production has been detected in the area in recent decades. However, the impact of O_3 on the occurrence and impact of fungal diseases is scarcely understood.

A close-to-field-conditions assay (Open Top Chamber facility) situated in a Mediterranean cereal rainfed farming area was carried out to study the impact of increasing O_3 levels and N-fertilization on spontaneous fungal outbreaks in wheat. Four O_3-fumigation levels reproducing pre-industrial to future pollutant atmospheres with additional 20 and 40 nL L^{-1} over the ambient levels were considered (7 h-mean ranging from 28 to 86 nL L^{-1}). Two top N-fertilization supplementations (100 and 200 kg ha^{-1}) were nested within the O_3 treatments; foliar damage, pigment content and gas exchange parameters were measured. Pre-industrial natural background O_3 levels strongly favored the yellow rust infection, where the O_3-polluted levels currently observed at the farm highly benefited the crop, mitigating the presence of rust by 22 %. However, future expected high O_3-levels neutralized the beneficial infection-controlling effect by inducing early wheat senescence, decreasing the chlorophyll index of the older leaves by up to 43 % under the higher O_3 exposure. Nitrogen promoted the rust infection by up to 49.5 % without interacting with the O_3-factor. Achieving future air quality standards might require considering new varietal improvement programs, to be able to adapt crops to an increased pathogen tolerance without requiring the assistance provided by O_3-pollution.

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http://dx.doi.org/10.1016/j.scitotenv.2023.163370
Received 10 January 2023; Received in revised form 21 March 2023; Accepted 4 April 2023
Available online 6 April 2023

1. Introduction

Due to its high oxidative capacity, high ozone (O_3) ambient levels strongly affect agrosystems (Harmens et al., 2018; Wilkinson et al., 2012). In fact, O_3 is currently considered the most phytotoxic air pollutant (Sandermann et al., 1998) and a key factor in the effect of Global Change on crop productivity in the Northern hemisphere (McGrath et al., 2015; Tai et al., 2014). Its direct effects on wheat, one of the most O_3-sensitive staple crops (CLRTAP, 2017; Mills et al., 2018), are well-known, causing yield and quality losses (Pleijel et al., 1999; Broberg et al., 2017; Ma et al., 2022). However, other indirect O_3-effects on crops, such as its interaction with common crop pathogens, are less known, even when these can be highly detrimental to the plant, with economic and food security consequences even surpassing the direct toxicity of this pollutant (Bouvet et al., 2022).

As the strong oxidant capacity of O_3 eliminates or highly limits fungal growth and spores formation (Tzortzakis et al., 2008), the effect of O_3 on fungal infections has often been analyzed as a post-harvest treatment, including for example high-levelled trial ozonation to favor grain or fruit storage, and to avoid fungal growth and mycotoxin production, without affecting quality. This treatment has been successfully applied to various crops like cucumber, chickpea, garlic or vines (Botondi et al., 2015; De Santis et al., 2021; Khan and Khan, 1999; Pisuttu et al., 2023); as well as cereals like wheat (Bhattarai et al., 2015; Granella et al., 2018; Korzun et al., 2008; Sarooei et al., 2019), barley (Allen et al., 2003; Piacentini et al., 2017) and rice (Savi et al., 2020).

Nonetheless, the effects of increasing chronic tropospheric ambient O_3 levels on fungal infection development throughout the crop-growing cycle are much less known. In this case, O_3 levels can reach maximum hourly values of around 100–200 nL L^{-1} and extend over time. Therefore, the knowledge acquired for acute postharvest ozonation cannot be extrapolated to chronic crop exposure under field conditions. Experimental assays designed to analyze whether chronic high O_3 levels affect crop fungal diseases are scarce and results are very specie-specific and non-conclusive.

One of the most surveyed approaches to address this issue has been inoculating plants with fungi after exposing them to various O_3 levels and then to analyze whether the pollutant predisposes the plant against later fungal infections. Results generally indicated that fungal diseases progressed with increasing pollutant levels, but the response depended on the fungal species, the host growth stage at the time of inoculation and the O_3 level. Post-exposure wheat inoculations with the necrotrophic gender *Septoria* (*Parastagonospora)* or *Bipolaris* revealed a faster fungal lesion growth on previously O_3-exposed leaves, independently of the plant stage (Tiedemann et al., 1991; Tiedemann, 1992a). Responses were similar when other biotrophic fungi, such as *Puccinia* or *Erysiphe sps* were considered, although the latter was crop-stage dependent (Tiedemann, 1992b). However, in some cases such as for *Bipolaris or Erysiphe* there was a critical O_3 value under which spot blotch symptoms declined.

An alternative closer to a field approach was fungal inoculations during the plant's growing cycle under controlled O_3 levels. In the earliest studies following this concomitant approach, wheat infected with stem rust (*Puccinia graminis)* was less injured by the infection when peak O_3-exposures were applied (Heagle and Key, 1973). Employing more realistic O3 levels, Tiedemann and Firsching (2000) followed the development of induced yellow leaf rust (*Puccinia striiformis*) at tillering, finding that the pollutant strongly inhibited the fungal presence. Furthermore, the apparent weakening of the leaves by the fungus led infected plants to suffer four times more severe O_3 lesions, which also appeared 2–4 weeks earlier than the non-infected ones.

As most of these assays were developed in laboratory-controlled chambers and the fungal infection was artificially induced by single or repeated re-inoculations, usually requiring specific incubation climate periods within the chambers for the inoculum to grow (Tiedemann et al., 1990), all these procedures cause assays to differ from real conditions and difficult extrapolation to field-grown crops.

Fewer studies consider closer-to-field conditions and natural fungal infection outbreaks to study the interactions between O_3 and fungal diseases. Tiedemann et al. (1991) followed powdery mildew outbreaks caused by *Erysiphe graminis* on wheat during the development of an O_3-exposure assay for 31 days (from seedling until ear emergence) in open-top chambers (OTC) under six different O_3 levels. Under these seminatural conditions, the pollutant significantly increased the disease. For Mina et al. (2016) the results pointed in the same direction, and the growth of *Bipolaris* in fumigated wheat plants was significantly higher in plants exposed to elevated O_3 compared to control plants. Meanwhile, the opposite response, the reduction of fungal infections due to chronic O_3 levels, has been observed in wheat in northern Germany (Tiedemann and Firsching, 2000); and even in other species like beech with an endophytic fungus of the *Apiognomonia* gender (Olbrich et al., 2010). However, other studies (e.g. Pfleeger et al. (1999)), where wheat was grown in OTC at different densities, found no interaction between O_3 and wheat leaf rust (caused by *P. recondita f.* sp. *tritici*) regardless of the cultivar, density or plant response variable measured.

Nitrogen (N) fertilization can modulate both fungal infection and plant response to O_3. Previous studies have shown that supplemental N affects fungal crop pathogens. The direction of this effect can either reduce the infection severity or facilitate its development (Devadas et al., 2014; Liu et al., 2017). However, more frequently, studies have denoted a fertilizer-induced surge in the infection, which is why a limited N fertilization is recommended among the agronomic measurements for controlling some fungal diseases like yellow rust (YR) (Almacellas, 2010; Devadas et al., 2014).

Fertilizer also modulated the O_3-response, although the observed enhancement or limitation of N on O_3-induced effects depends on the species, the parameters considered and both the O_3 exposure and N fertilization levels (Sanz et al., 2005, 2015; Wyness et al., 2011). Wheat studies considering the combination of N and O_3 factors have focused on how O_3 can affect N fertilizer efficiency (Broberg et al., 2017; Peng et al., 2020), N metabolism (Sanz et al., 2014; Yendrek et al., 2013), or how N availability can mitigate (Sanz et al., 2015) or exacerbate (Wyness et al., 2011) the effects of $O_{3.}$ Nonetheless, very few studies have delved into the combined N and O_3 effect on the plant-fungus relationship, and in this case, they focus more on the effects of mycorrhizal fungi on N uptake (Cui et al., 2013) than on the effects on fungal pathogens. The scarce results on this subject indicate that N-effects on fungal wheat diseases can change under O_3-polluted atmospheres: at elevated pollutant levels, N-modulation on the *Septoria* spontaneous outbreak reversed and led to stronger fungal infections (Tiedemann, 1996). Meanwhile, in the same assay, O_3 also strongly enhanced the disease-promoting effect of N on two other inoculated biotrophic pathogens, *Erysiphe* and *Puccinia.*

The aforementioned studies reveal the complexity of the plant-fungi interaction under O_{3}-polluted atmospheres and N fertilization regimes. Ozone may stimulate or suppress plant pathogenic fungi, and N can help limit or favor it. Thus, understanding this interaction is still far from clear and complete, and may depend on plant and fungal species, plant phenological stage, O_3 exposure (concentration and duration), or N level. Moreover, expanding the knowledge of the complex O_3 x N x pathogens interaction under the Global Change framework is becoming increasingly concerning, especially as crop diseases have been increasing in recent decades, even without considering the potential importance of the air quality factor (Fones et al., 2020).

Yellow rust (YR) is a fungal disease caused by *Puccinia striiformis* that causes large yield losses all over the world (Hao et al., 2016). This wind-dispersed fungus can spread for hundreds of kilometers, and due to its large dispersal capacity, quick germination at low temperatures, and genetic variation, it is highly infectious (Hovmøller et al., 2002, 2016). This disease can decrease yield by 24 to 39 % in winter wheat cultivars in Central Asia (Sharma et al., 2016) and is currently considered as one of the most important diseases affecting European wheat (Willocquet et al., 2021). Up until the early 2000s, YR was not of much concern in the Central Iberian Peninsula, due to the prevalent Mediterranean continental climate which involves low humidity and extreme cold and hot temperatures, as well as the common agronomic management of wheat as a rainfed cereal, which limited fungal spread. However, since the new and more aggressive rust races are better adapted to warm conditions, their presence has increased in the area (Aparicio Gutierrez et al., 2014). One of the new races that have been particularly noted as having had a large effect on Spanish wheat crops (Warrior/Ambition race) arrived after spreading through the UK, Denmark, France and into northern Spain and is currently causing important yield losses (Hovmøller et al., 2016; Vergara-Diaz et al., 2015).

The present study was performed to improve the understanding of the O_3-wheat fungal disease interaction while maintaining close-to-field conditions and considering the modulation of the N-fertilization response. A Mediterranean wheat cultivar was grown in semi-natural conditions in OTCs, where periodic monitoring was conducted to detect spontaneous fungal pathogen outbreaks, especially *Puccinia striiformis*. The YR disease appears spontaneously every year in the area where the OTC facility is located, and where rainfed wheat is extensively grown. We hypothesized, that after a certain level, O_3 would limit YR because its biotrophic behavior would expose the hyphae to the excessive oxidant stress induced by the pollutant. Moreover, biotrophic pathogens do not possess the detoxification mechanism for responding to phytoalexin or polyphenol oxidase, which can be induced in plants in response to ozone exposure (Dowding, 1988). We also hypothesized that N fertilization would help wheat counteract the effects of O_3 by being able to divert more resources to combat the fungal pathogen.

2. Materials & methods

2.1. Plant material and growing conditions

An Open Top Chamber (OTC) experiment was performed under Mediterranean conditions in the CIEMAT facility located in Central Spain in “La Higueruela”-MNCN/CSIC Research Farm (Santa Olalla, Toledo, 450 m.a.s.l., 40°3′ N, 4°26′ W). OTC design was based on (Heagle et al., 1989) with a 3 m diameter and 3 m height, adding a conic *frustum* structure on the top to limit wind from entering the chamber.

Four O_3 treatments with 3 repetitions (3 OTC per O_3 treatment) were considered in a complete randomly block design: Filtered Air (FA), reproducing pre-industrial O_3 levels; Non-Filtered Air (NFA), reproducing the ambient O_3 levels at the farm; Non-Filtered air $+20$ nL L^{-1} of added O_3 (NFA+) and Non-Filtered Air $+40$ nL L^{-1} of added O_3 (NFA++), to reproduce future pollutant atmospheres with the additional 20 and 40 nL L^{-1} over the ambient levels, exposing the plants from 7:00 until 14:00 GMT, 7 days week^{-1}.

Ozone over ambient levels in the fumigated OTCs NFA+ and NFA++ were obtained by an O_3 generator (A2Z Ozone Systems Inc., Louisville, KY, USA) system fed with pure O_2, and diverted to the OTCs throw electronic valves to achieve the programmed O_3 levels. From the control cabin, air pollution levels were monitored continuously above the canopy with a timesharing system, having a sampling period of 10 min per plot. Ozone (ML. 9810B, Teledyne Monitor Labs, Englewood, CO, USA) and nitrogen oxides (NO_2 and NO; ML.9841, Teledyne Monitor Labs, Englewood, CO, USA) monitors were calibrated at the beginning of the experiment following the standard protocol of the company. Six of the OTCs were monitored for meteorological parameters, recording air relative humidity (RH) and temperature (Pro-v2 HOBO, Onset, Bourne, MA, USA) and photosynthetic active radiation (PAR) (OSO-SUN HOBO, Onset, Bourne, MA, USA). More information about the OTC site can be found in previous studies (Calvete-Sogo et al., 2016).

Two top N fertilization regimes, consisting of a total of 100 (Low N) and 200 (High N) kg N ha^{-1}, respectively, were applied in two partial doses 18 days apart. The spring Mediterranean wheat cultivar (CV) Artur Nick, which is among the most widely used modern CV in Spain and is even used as a reference cultivar for yield analysis of new cultivars (GENVCE, 2021a), was selected for the assay due to its O_3-sensitivity, proved on previous studies (Chang-Espino et al., 2021). Two seedlings per pot (20 cm diameter) were transplanted using 60/20/20 turve/vermiculite/perlite substrate. A total of 24 plants (2 plants pot^{-1}) per O_3 and N treatment were considered. Ozone exposure began on April 24th (Day of the Year-DOY 114, Days after the start of the Exposure-DaE 0) when wheat was at tillering (stage 23 according to Meier (2018) and lasted till the last sampling date on June 15th (DOY 165, DaE 52).

To describe O_3 exposure in the period between the beginning of O_3-fumigation and the YR quantification among the treatments, two O_3 indexes were considered: the 7 h-mean (10-percentile of the 7-h daily mean from 08:00–16:00, h GMT); and the AOT40 index (Accumulated Ozone exposure over a 40 ppb Threshold) calculated as the sum of O_3 hourly values over 40 nL L^{-1} during the daytime hours (PAR > 100 μmol m^{-2} s^{-1}) throughout the exposure period (expressed as nL L^{-1} h). The latter is considered in the United Nations Air Convention (UNECE) for the derivation of O_3 critical levels (threshold limits) for plant protection (CLRTAP, 2017). The EU Directive of Air Quality (2008/50/CE) also considers the AOT40 index to define the Objective and Long-Term values required to achieve plant protection.

2.2. Plant monitoring

Plants were monitored every 2–3 days to detect the beginning of a rust outbreak, which occurred on May 24th (DOY 144, DaE 31). On May 28th the infection was treated with a specific fungicide to stop its progress. Plant recovery was analyzed two weeks later on June 13–14 (DOY 164–165, DaE 51–52). The disease affected the main stems but did not reach the secondary stems, which were slightly phenologically delayed.

YR and O_3 damage intensity were assessed on June 1st (DOY 152, DaE 39) at the late milk grain development stage (stage 77 Extended Scale BBCH; Meier, 2018). Damage was visually quantified in the flag leaf (FL) and the other three main stem leaves of increasing age (12 plants per O_3 and N treatment) through percentage classes of damage considering both intensity and extent (by 5 % steps). Foliar pigments were evaluated through chlorophyll index with a single-photon avalanche diode (SPAD) on May 30–31 (DOY 150–151, DaE 37–38) (SPAD-502Plus; Chlorophyll Meter, Konica Minolta, Japan), and gas exchange parameters, photosynthetic activity (A) and stomatal conductance (g_s), were measured with a portable IRGA system (Licor 6400; Li-Cor, Lincoln, NE, USA) on June 1st, considering Flag Leaves (FL) from the main stem. Intrinsic water Use Efficiency (WUE) was calculated as the ratio between A and g_s.

Foliar pigment content was re-evaluated 12–13 days later, on June 13–14, to analyze plant recovery. This was performed on all four leaves (the FL and the three leaves under) to measure all the differently aged physiologically active leaves. Furthermore, this was done on one main and one secondary stem per pot.

2.3. Statistical analyses

All ANOVA analyses were performed with the IBM® SPSS® Statistics 20 (Chicago, IL, USA) package. The database was analyzed for outliers with the SPSS Descriptive Statistics function, and normality and homoscedasticity were checked using Shapiro–Wilk and Levene tests respectively. When the data did not meet the requirements, they were log or square root transformed. Then a Split Plot Analysis was performed employing O_3 as the complete randomized factor and N as the split-plot factor. Block was considered a random factor. Significant level differences ($p < 0.05$) were based on the Tukey Honestly Significant Difference test (HSD) when ANOVA allowed it. Ozone symptoms were analyzed using the non-parametric Kruskal-Wallis rank sum test in R (R Core Team, 2022). Then, post hoc analyses were performed using the dunnTest function (FSA package; Ogle et al., 2021).

3. Results and discussion

3.1. Experimental conditions

A spontaneous YR outbreak caused by *Puccinia striiformis* was visibly detected on May 24th (DaE 31), during the end of heading and fully emerged inflorescence (stage 59 Extended Scale BBCH; Meier, 2018). Since the beginning of O_3 exposure to that date, ambient conditions followed the common evolution for the area for the spring season (Fig. 1S). Daily mean temperature values increased from 6.4 to 21.1 °C, while air RH decreased from 85.7 % to a minimum of 38.4 %, both leading to an increasing daily mean VPD ranging from 0.14 to 1.3 kPa. However, specifically around 10 days before infection (between 12 and 14/05 – DOY 132–134), a cool short event occurred with low RH values (mean hourly values of 39.4 %) coupled with low temperatures (mean hourly values of 13.0 °C) causing VPD values of 0.87 kPa, which are favorable for rust outbreak (Aparicio Gutierrez et al., 2014).

A couple of decades ago, Artur Nick CV was qualified as highly yellow rust-resistant (GENVCE, 2003; Martínez-Moreno and Solís, 2019), but the later appearance of aggressive rust races altered this endurance and their current tolerance to this rust is not well-defined (GENVCE, 2021b). In fact, even before the spread of the aggressive races, Spanish wheat CVs varied in their YR susceptibility, and to this date no Warrior/Ambition strain-resistant variety is recommended in Spain (Aparicio Gutierrez et al., 2014; GENVCE, 2021a) although recently a new variety claims resistance (LimaGrain, 2020). Therefore, in the last decade, concern about this fungal disease has risen. A high YR incidence has been reported throughout the Iberian Peninsula, especially in humid years with cooler spring temperatures (Martínez-Moreno et al., 2022; Martínez-Moreno and Solís, 2019), causing durum wheat losses of 18 % in Central Spain, but even suffering losses up to 57 % with the most sensitive cultivars (Vergara-Diaz et al., 2015). Thus, any abiotic influence on the development of this disease is an important subject to consider.

3.2. Main effects of ozone and nitrogen on yellow rust

38 days after the beginning of exposure (DaE) (9 days from the beginning of the fungal foliar damage) O_3 diminished the disease's progress significantly ($p < 0.05$) in all leaves, despite their age, although the differences were greater in the older leaves, which were subject to a longer exposure to the pollutant (Fig. 1a, Table 1S). The farm's O_3 levels, reproduced by the NFA treatment, were enough to strongly limit the fungal infection without interacting with the N factor. By just considering the differences caused by increasing O_3 from pre-industrial to current ambient values (NFA versus FA), the percentage of YR damage decreased by 22 %, 54 %, 69 % and 71 % on the FL, Leaf 2, Leaf 3 and Leaf 4 respectively (mean values across N treatments). When NFA+ and NFA++ were evaluated, the infection rate strongly decreased too, more intensely in the older leaves (leaves 2–4). The infection decreased by a maximum value of 94 % under Leaf 4 of the NFA++ treatment, compared to the plants grown under FA. In the youngest leaf (i.e. FL), the disease presented a more progressive decline with O_3, and the maximum differences were found in NFA+ plants, with 50 % less infection than in FA. It is well known that the flag leaf holds an important role in grain filling (Sanchez-Bragado et al., 2017), therefore the mitigated effect of current O_3 levels on YR can be seen as highly beneficial for later production.

These observed positive effects of O_3 on controlling YR differ from previous assays where a powdery mildew outbreak caused by *Erysiphe graminis* in wheat increased significantly due to all O_3 levels (Tiedemann et al., 1991). Other studies based on induced post-exposure inoculations also found that the pollutant favored other fungal diseases like the blotch caused by the necrotrophic fungi *Septoria nodorum* (*Parastagonospora nodorum)* or leaf rust caused by the biotrophic *Puccinia recondita f.* sp.*tritici* (Tiedemann et al., 1991; Tiedemann, 1992b, 1992a). Disease severity was O_3-enhanced even on undamaged leaves, although O_3 lesions were the starting point of the *Septoria* infection. However, when O_3 levels reached the highest values, the pollutant reversed the impact of some of the pathogens and limited infection, such as the necrotrophic *Bipolaris sorokiniana* (Tiedemann, 1992a), or powdery mildew caused by the biotrophic *Erysiphe graminis f.* sp. *tritici* (Tiedemann, 1992b). Fungal disease symptoms were stronger at mean O_3 values of 160 μg m^{-3} (80 nL L^{-1}), but significantly decreased at 240 μg m^{-3} (120nL L^{-1}) although in the case of *Erysiphe* this response also depended on wheat stage and the infection was only limited by O_3 when the inoculations were induced at the younger stages.

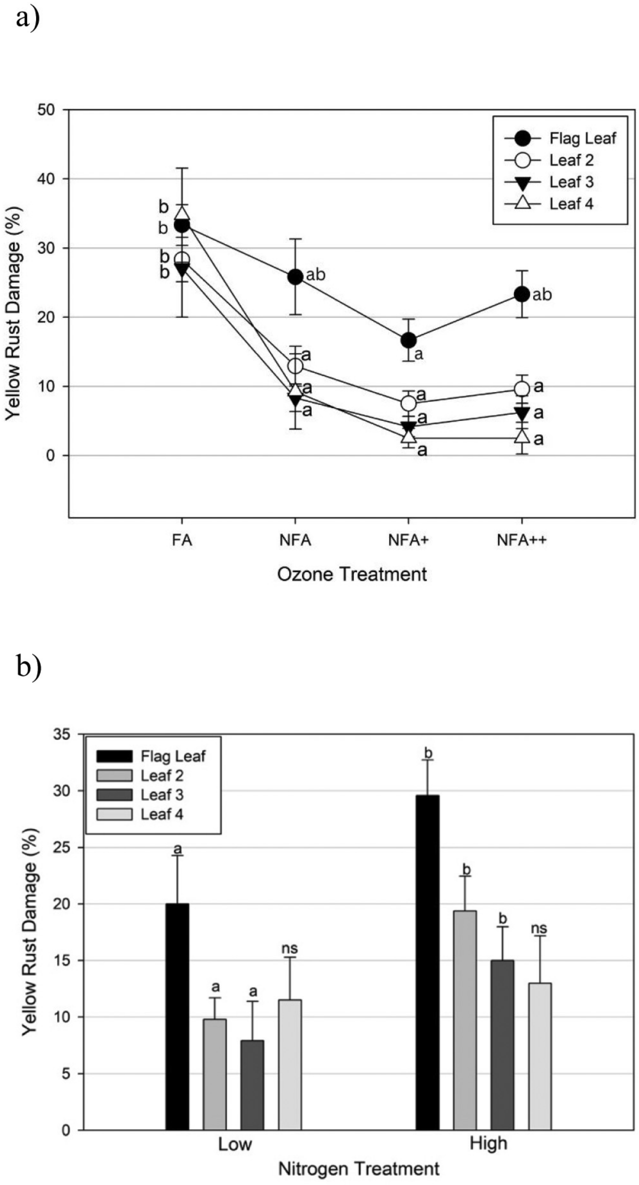

Fig. 1. YR leaf damage (means ± SE) on leaves of different ages for the O_3 (1a) and N treatments (1b) on June 1 (38 DaE). FA (filtered air), NFA (non-filtered air), NFA + (non-filtered air + 20 nL L^{-1} of O_3) and NFA + + (non-filtered air + 40 nL L^{-1} of O_3); Low $N = 100$ kg N ha^{-1}, High $N = 200$ kg N ha^{-1}. Significant differences among O_3 levels (across N treatment) and significant differences between N levels (across O_3 treatments) ($p < 0.05$) are shown with different letters: *p*-values for O_3 treatments: FL = 0.011; L2 = 0.019; L3 = 0.002; L4 = 0.008; p-values for N treatments: FL = 0.007; L2 = 0.000; L3 = 0.040; L4 = ns. There was no significant interaction between factors.

In the present assay, held under semi-natural conditions, the spread of the biotrophic *Puccinia striformis* outbreak was significantly reduced by medium levels of the pollutant currently found in the ambient (NFA), which limited disease spread in all leaves from different ages. Results follow Tiedemann and Firsching (2000), which examined the development of induced YR at tillering, finding that the pollutant strongly inhibited the fungus presence when mean O_3 values reached 60 nL L^{-1} compared with the filtered treatment which ranged around 20 nL L^{-1}. The O_3 levels in the Artur Nick assay for the 38-day period between the beginning of O_3-fumigation (April 24th) and the quantification of the YR impact (June 1st), as well as the 51-day period until recovery (June 14th) are shown in Table 1. The NFA treatment that sufficed to abruptly reduce the disease presented a 7 h-mean of 49 nL L^{-1} and corresponded with an AOT40 index of

Table 1
Ozone levels for the different O3 treatments during the exposure period from the start of the fumigation on April 24 till the analysis of YR outbreak impacts on June 1, and till sampling of the recovery on June 14. FA (filtered air), NFA (non-filtered air), NFA + (non-filtered air + 20 nL L^{-L}) and NFA + + (non-filtered air + 40 nL L^{-L}). 7-h mean (nLL^{-1}) = 10 percentile of 7-h daily mean from 08:00–16:00 h GMT; AOT40 = Accumulated O3 diurnal hourly values over 40 nL L^{-1}.

	FA	NFA	NFA+	NFA++
April 24 to June 1				
7-h mean (nL L^{-1})	28.8	49.1	68.0	87.8
AOT40 (nL L^{-1}h)	10	2341	6377	11,762
April 24 to June 14				
7-h mean (nL L^{-1})	28.3	47.4	64.5	85.5
AOT40 (nL L^{-1} h)	10	2559	7257	13,879

2341 nL L^{-1}h. Thus, the control of *Puccinia* by O_3 happened at levels that were very much lower than in the *Erysiphe* or *Bipolaris* inoculations, but which lies in range with what has been observed previously for *Puccinia* (Tiedemann and Firsching, 2000). As mentioned before, we hypothesized that high O_3 would limit YR because of the direct effect of the oxidative pollutant on the hyphae of the biotrophic pathogen (Dowding, 1988); however, the pollutant levels required to control de fungus were much lower than expected. Therefore, other (additional) mechanisms explaining the beneficial effects of low O_3-level exposures on the plant should be considered.

3.3. Defense mechanisms

There are different mechanisms by which O_3 can elicit plant reactions comparable to the ones generated by fungal pathogens, which may result in improved resistance to the pathogen as O_3 prompts the plant's defense mechanisms (see review by Zuccarini, 2009). Among these cross-induction mechanisms, are the production and accumulation of phytoalexins (Sandermann, 1996), cell wall strengthening (in most cases with lignin) (Guidi et al., 2005), pathogenesis-related protein stimulation through ethylene liberation (van Loon et al., 2006), the promotion of signal substances (like ethylene and salicylic acid) to activate plant defense or influence cell death (Tuomainen et al., 1997), or the stimulation of the antioxidative cellular system to block the oxidative burst caused by O_3 inducing reactive oxygen species (ROS) when in contact with plant tissue (Lamb and Dixon, 1997). All these cross-induction mechanisms can be related to the O_3-induced pathogen resistance. For wheat, several studies showed that ROS-related mechanisms can be highly important in this protection. The O_3-induced tolerance of wheat against powdery mildew (*Blumeria graminis*) has been related to ROS-blocking antioxidative system signaling, as well as triggering the salicylic acid (SA) and jasmonic acid (JA) pathways (Pazarlar et al., 2017).

Hao et al., 2016 also found that stressing plants with O_3 in the earlier phenological stages could grant some defense against pathogens, as the continually accumulating ROS caused by O_3 could make the plant more resistant to the disease, better suiting the adult plant to inhibit YR growth and development. Correspondingly, for Artur Nick, the strong differences between FA and NFA might be produced by the accumulated ROS caused by O_3 stress at the time of outbreak, considering that the outbreak occurred late in the season, giving plants above the FA treatment time to accumulate ROS defensiveness, even at the NFA treatment pollutant levels (7 h-mean 49 nL L^{-1}).

3.4. Is there a threshold for ozone benefits against infection?

Nevertheless, in the Artur Nick assay, although fungal infection in the FL leaf diminished progressively between FA and NFA+, it then rebounded under the highest O_3 exposure, the NFA++ treatment (7 h-mean of 87.8 nL $L^{-1}$and AOT40 index of 11,762 nL L^{-1} h.). Thus, O_3 values close to 100 nL L^{-1} began to reverse the infection-limiting beneficial effect of the pollutant, probably due to the excessive ROS caused by the highest O_3 levels, which may have overwhelmed the plant's defense systems (Fatima et al., 2018). This does not only happen with wheat, but other studies with horticultural crops have also indicated an O_3 threshold for limiting crop diseases. A study by Khan and Khan (1999) revealed that under O_3 concentrations in the 50–100 nL L^{-1} range, powdery mildew in cucumber remains uninfluenced, whereas higher levels suppressed the disease. For the Artur Nick CV, values of around 100 nL L^{-1} seem excessive for the plant and favor infection rebound. Therefore, the most suitable O_3 range for fungal crop disease control during crop growth cannot be generalized, and needs to be specifically studied.

The foliar O_3 damage on CV Artur Nick after the exposure period augmented progressively with the increasing pollutant levels, thus healthy leaf surfaces decreased with the progressive O_3 levels (Fig. 2a; Table 1S). The effect was more intense in the older leaves, which were exposed to the pollutant for longer. The youngest FL did not present O_3 damage, not even under NFA++, due to its short exposure time; meanwhile the oldest Leaf 4 presented a sharp decline even after the NFA treatment. Some previous assays have indicated that it is not only O_3 that affects fungal infection, but fungal pathogens can also sensitize wheat to the pollutant, and in those assays O_3-foliar lesions appeared earlier and were more severe in infected plants compared to non-infected plants (Tiedemann et al., 1990; Tiedemann and Firsching, 2000). However, in the present assay the pathogen and O_3 symptoms had opposite trends (Fig. 2a; Fig. 2S), where the plant presented more rust symptoms under FA and more O_3 symptoms under NFA++. Thus, leaves with more damage due to the fungal disease did not show more O_3 symptoms, and vice versa.

3.5. Consequential foliar pigments

When foliar pigments were evaluated, the results followed the evolution of both the fungal infection damage and the intensity of the O_3 symptoms. The O_3 effect was significant without interacting with the N factor (Fig. 3a, Table 2S). The FA treatment presented the lowest chlorophyll values in all the leaves despite their age due to the intensity of the YR infection. Under the preindustrial background O_3 levels (FA), the chlorophyll loss was in the range of 7–28 % when compared with NFA, which reproduced the current pollutant levels (mean value for the different leaves). When younger leaves were measured (FL and Leaf 2), no differences among NFA, NFA+ and NFA++ treatments were found; however, older leaves (Leaf 3 and Leaf 4), following their longer and higher exposure to the pollutant and greater O_3 damage, presented a significant chlorosis gradation with increasing O_3 levels. The greater pollutant-induced chlorophyll loss occurred between NFA and NFA++ in Leaf 4 (43 %) and coincided with the lower rust damage. The senescing effects of O_3 have been assumed to be a major factor in predisposing wheat to necrotrophic leaf pathogens (Tiedemann et al., 1991). However, in the present assay, senescing seems to be a consequence of the pathogen infection under FA, or a consequence of O_3 damage under NFA+ and NFA++, where senescent leaves caused by the O_3 impact did not boost the progress of the infection. NFA produced plants with the highest foliar pigment content, where its medium O_3 levels did not cause significant losses while strongly limiting the fungal infection.

N fertilization caused a significant impact both on foliar damage and chlorophyll content due to the infection, ($p < 0.05$) but not on O_3 foliar injury. Regarding the influence of N on the disease spread, the added dose of 200 kg of N significantly enhanced YR damage similarly in all the leaves and despite their age, except for the oldest Leaf 4 (Fig. 1b, Table 1S). Rust symptoms increased under High N by 32.4 %, 49.5 % and 47.3 % on the FL, Leaf 2 and Leaf 3 respectively compared with Low N (mean values across O_3 treatments). Meanwhile, foliar pigment content was significantly lower ($p < 0.05$) in all the leaves under the High N treatment except for Leaf 4, and the pigment loss was in the range of 7–14 % (Fig. 3b, Table 2S). Although previous studies showed that high N levels reduced the severity of some fungal infections, like leaf blotch caused by *Septoria nodorum* (Simón et al., 2003), literature more frequently indicates the opposite effect, that the fertilizer helps infection, which was also observed in the

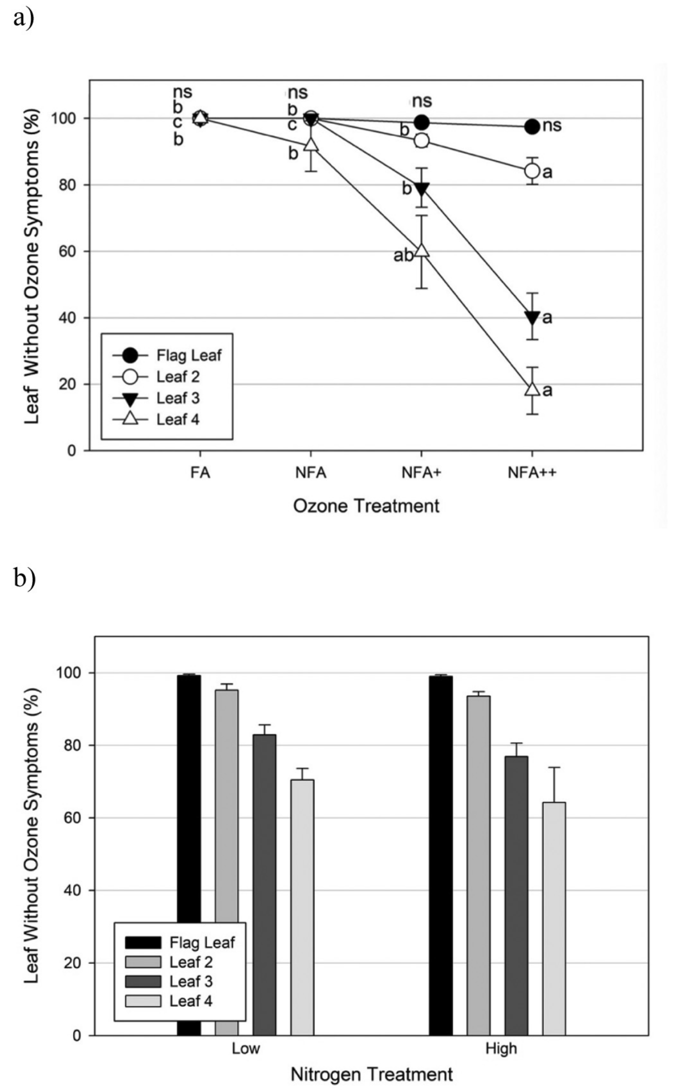

Fig. 2. Foliar O_3 damage on leaves of different ages expressed as a percentage of healthy leaf surface (means ± SE) for the O_3 (2a) and N treatments (2b) on June 1st (DaE 39). FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air +20 nL L^{-1}) and NFA++ (non-filtered air +40 nL L^{-1}); Low N = 100 kg N ha^{-1}, High N = 200 kg N ha^{-1}. Significant differences among treatments ($p < 0.05$) are shown with different letters; corresponding p-values are for O_3 treatments: FL = ns; L2 = 0.007; L3 = 0.011; L4 = 0.002; for N treatments: all p-values were ns. There was no significant interaction between factors.

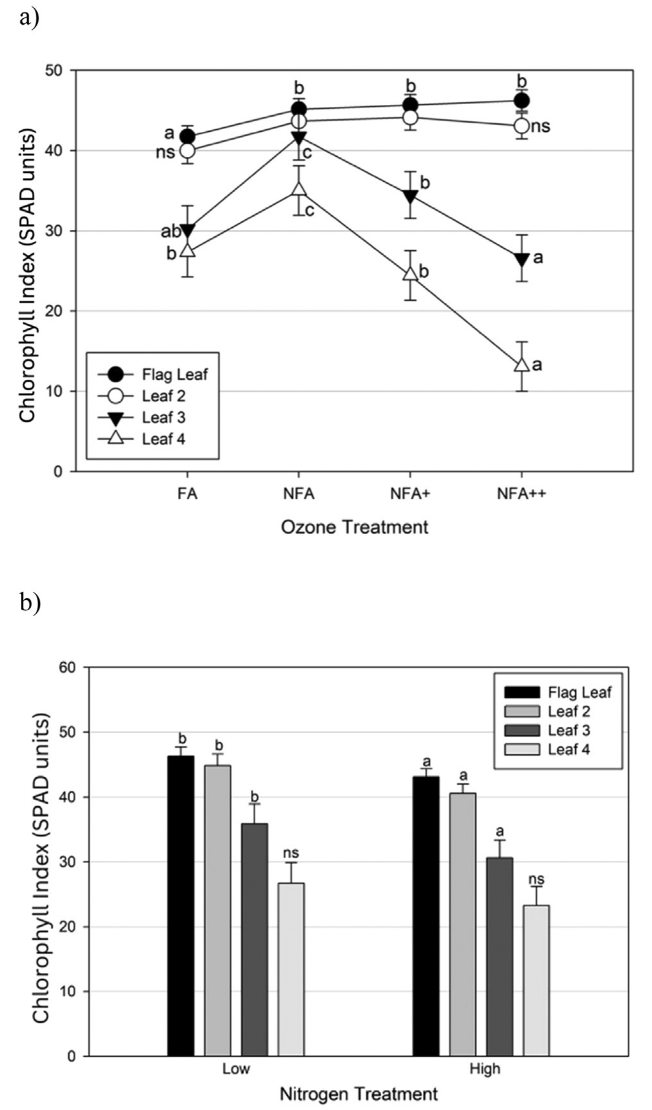

Fig. 3. Chlorophyll Index (SPAD units) (means ± SE) for the O_3 (3a) and N (3b) treatments on May 30–31. FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air +20 nL L^{-1}) and NFA++ (non-filtered air +40 nL L^{-1}); Low N = 100 kg N ha^{-1}, High N = 200 kg N ha^{-1}. Significant differences among treatments ($p < 0.05$) are shown with different letters; corresponding p-values are for O_3 treatments: FL = 0.015; L2 = ns; L3 = 0.019; L4 = 0.003; p-values for N: FL = 0.001; L2 = 0.000; L3 = 0.009; L4 = ns.

present assay. Tiedemann (1996) found that N facilitates powdery mildew (*Erysiphe graminis f.* sp. *tritici*) or leaf rust (*Puccinia recondita f.* sp. *tritici*) expansion, and Devadas et al. (2014) also found that N favors the spread of *Puccinia striformis*; thus N management seems an important factor on controlling fungal crop diseases.

Nitrogen fertilization can also modulate the O_3 response. This has been observed in different species where the nutrient can counterbalance O_3 effects (Calvete-Sogo et al., 2016) or exacerbate them (Wyness et al., 2011). Regarding wheat, the fertilizer has been seen to modulate yield losses at the lower O_3 levels, but these benefits are rapidly counteracted by the higher O_3 levels (Broberg et al., 2017; Chang-Espino et al., unpublished results; Peng et al., 2020). Some studies also indicate that the N effect on fungal diseases can change under O_3-polluted atmospheres where for example the pollutant strongly enhanced the disease-promoting effect of N on two other biotrophic pathogens; *Erysiphe* and *Puccinia* (Tiedemann, 1996). However, in the present assay no significant interaction was found for rust leaf damage and the O_3 effects on *Puccinia* were almost parallel under both N regimes (Fig. 2S).

3.6. Physiological parameters

Gas exchange measurements were performed on the FLs to quantify the potential effect of the pathogen and O_3 on yield, considering that the grain-filling capacity depends on the physiological activity of this youngest leaf (Sanchez-Bragado et al., 2014). At the time of the gas exchange measures, FL was not affected by the pollutant strongly enough to cause visual foliar damage (Fig. 2a), but the pollutant had caused effects at a physiological level. Ozone led to a g_s decrease pattern, which was more intense than for A, causing a slight, non-significant WUE enhancement with increasing pollutant levels (Fig. 4). This decrease in g_s with a lesser effect on A and WUE in young leaves has been seen in previous assays (Grandjean Grimm and Fuhrer, 1992), suggesting that in the younger FL, the O_3 affects the stomata directly, limiting photosynthesis mainly due to a CO_2 diffusion reduction, while in the older senescent leaves O_3 could be decreasing carboxylation.

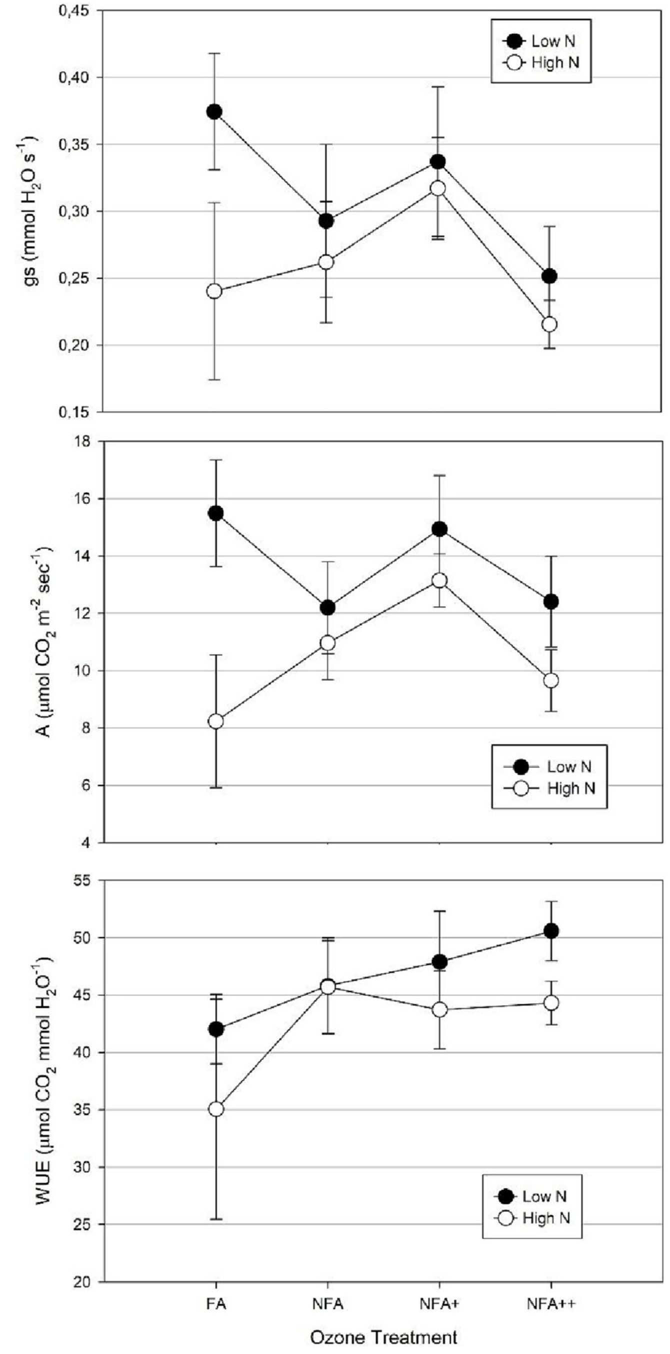

Fig. 4. Gas exchange parameters, stomatal conductance (g_s), photosynthetic activity (A) and water use efficiency (WUE) (means ± SE), for the different O_3 and N treatments on June 1. FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air +20 nL L^{-1}) and NFA+ + (non-filtered air +40 nL L^{-1}). Low N = added 100 kg N ha^{-1}, High N = added 200 kg N ha^{-1}. There were no significant differences by N treatment.

However, along with the general pattern caused by the pollutant, the infection also affected the physiological parameters assayed, especially in the FA plants, which presented the maximum rust damage levels. Following the enhanced rust damage caused under the low O_3 exposure and high fertilizer dose, FLs grown under the combined FA and N-high treatments presented the lowest A, g_s and WUE values. The observed reduction in N-high was 26.7 %, 17.6 % and 9.4 % for A, gs and WUE respectively than those observed in N-low, highlighting the importance of N allocation in the evolution of YR on wheat. The 100 Kg N ha^{-1} value is usual for the top fertilization applied in rainfed commercial wheat fields in Central Spain, which would somewhat restrict infection if pre-industrial O_3 background levels were again achieved. However the 200 Kg N ha^{-1} dose, which is recommended to increase wheat flour quality in Spanish rainfed wheat (López Bellido, 2009), would favor the progress of the infection under cleaner atmospheres.

Tiedemann and Firsching (2000) considered that the enhanced O_3-sensitivity of rust-infected wheat was related to stomatal aperture, which increased strongly on infected plants, causing additional oxidative stress. However, in the present study, O_3-induced stomatal closure and the maximum rust spread happened under the high g_s of the FA treatment, and therefore under minor oxidative stress by the pollutant. Meanwhile, following the minimum infection observed in the FL of NFA+ plants (Fig. 1a), g_s and A peaked, but then under NFA+ +, when the infection rebounded under the highest O_3 exposure, wheat physiological activity reached its lowest values. Thus, both rust and O_3 play important roles in affecting the photosynthetic activity of the plant, but without interacting; because at low O_3 levels the rust determines plant health and activity, while at high O_3 levels the pollutant becomes the key factor (Fig. 2S). Therefore, and given the importance of the FL health for grain filling (Sanchez-Bragado et al., 2017), O_3 levels should not surpass the NFA+ and NFA+ + levels, but current O_3 does seem to be beneficial concerning YR mitigation and therefore for preserving production.

3.7. Post-infection follow-up

A subsequent foliar pigment content evaluation was taken on June 13–14, 16 days after chemically controlling the disease (Fig. 5, Table 2S). Flag leaves were measured at 50 DaE to evaluate the recovery of the main stem affected by the rust, and to compare these with the slightly phenologically delayed secondary stems, which were not infected.

Foliar pigments evaluated at the time of the YR outbreak were high, but significantly lower under the High-N fertilization and FA treatment, which relates to the higher YR impact on FL. Two weeks later, foliar pigment content experienced a general decrease, which is consistent with the aging process; but the N-induced differences between FA and NFA had disappeared. This would entail some recovery from the infection, considering the slowing of additional infection-induced senescence. However, the pollutant caused a progressive chlorophyll loss with increasing O_3 levels, and differences between FA and NFA+ + were 57.9 %; 87.5 %, 79.8 % and 92.9 % in the FL, Leaf 2, Leaf 3 and Leaf 4 respectively. Pigment losses were more pronounced at 59 DaE in the oldest leaves, which were exposed to the pollutant for longer, an effect that had already been observed on the first sampling date in May, but for NFA. At this earlier date, FL and Leaf 2 presented positive differences between FA and NFA+ + of 10.8 % and 5.6 % respectively due to the pathogenic impact on these treatments, while losses of 11.9 % and 50.9 % were found at NFA+ and NFA + + respectively. Therefore, chemically eliminating the rust did not help wheat combat O_3 and two weeks later the impact of the pollutant was stronger despite the N level.

The secondary stems were never affected by the rust infection and were less exposed to the pollutant due to their phenological delay, so they had a generally higher chlorophyll content than the main stems (which lay within the range observed at the early main stems sampling). Following the O_3 effect seen in the main stem measured on the same dates, a significant O_3-induced pigment loss was observed in Leaves 2 to 4, with parallel drops of 62.5 %, 84.8 % and 78.0 % (in FA versus NFA+ +) respectively, again showing an exacerbation of the pollutant's effect in the older leaves, although slightly less so than in the main stem. Pigment content after recovery from the rust did not present any influence due to the N treatment, reinforcing the effect that the fertilizer has on the rust but not on the O_3 response. Decreasing foliar chlorophyll is a usual response to the pollutant, as it relates to O_3-induced accelerated senescence (Reichenauer, 1998). However, the intense effect of the pollutant on the Artur Nick CV should be noted, particularly because the phenological stage at which effects were observed was so close to anthesis, which is recognized as the most O_3-susceptible stage for wheat (Pleijel et al., 1998).

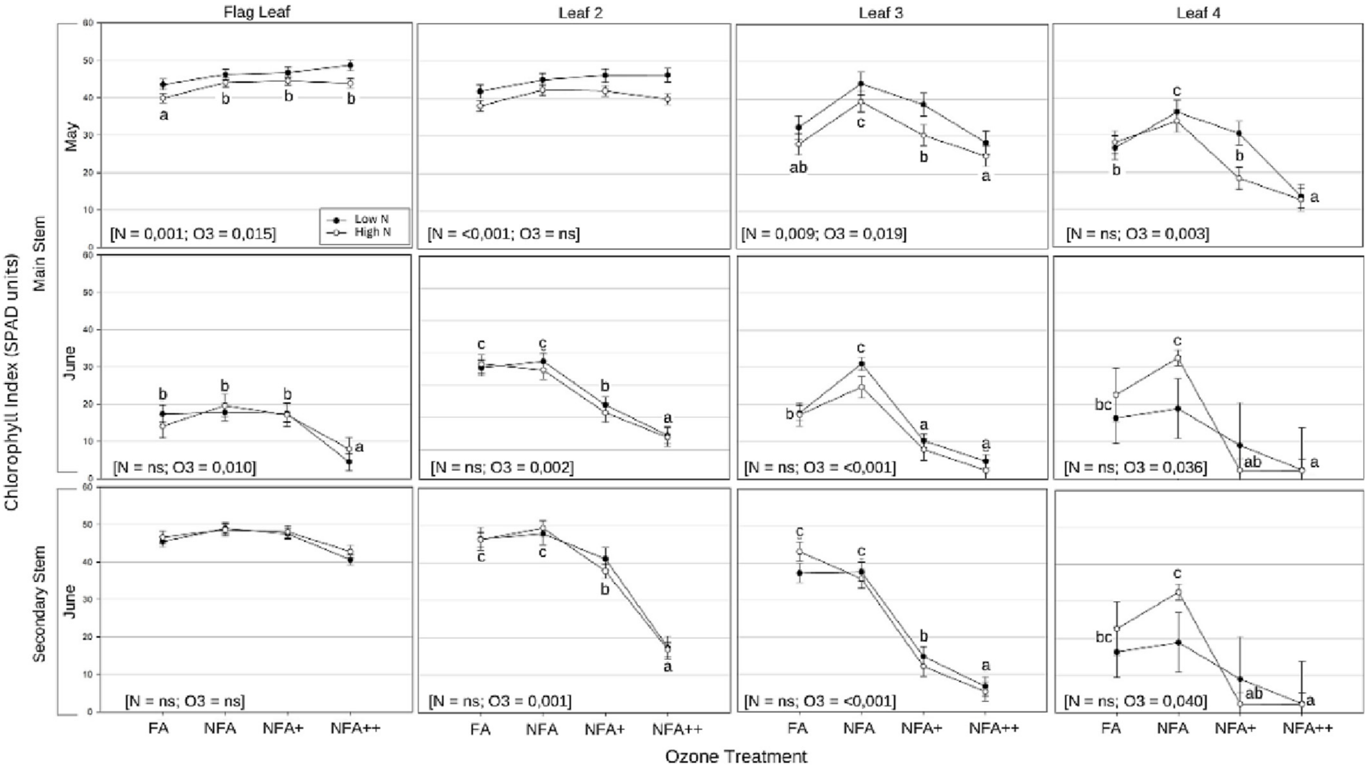

Fig. 5. Chlorophyll Index (SPAD units) (means ± SE) for the different leaves under O_3 and N treatments on May 30–31 (main stem) and June 12–13 (main and secondary stems). FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 nL L^{-1}) and NFA++ (non-filtered air + 40 nL L^{-1}); N-Low = added 100 kg N ha^{-1}, N-high = added 200 kg N ha^{-1}. Significant differences among O_3 treatments ($p < 0.05$) are shown with different letters. *p*-value for N and O_3 factors are indicated in the graphs. No significant interactions were found (see Table 2S).

3.8. The implications of approaching pre-industrial ozone levels

The results presented here raise concerns about the ideal return to the natural background (preindustrial) O_3 levels pursued in environmental policies. Considering overall experimentally-caused differences due to FA and NFA fumigations on wheat in OTC assays, Pleijel et al. (2018) indicated that returning to pre-industrial O_3 levels would improve wheat grain yield by an average of 8.4 %, which is a considerable percentage for the Food Security arena. However, the potential role of O_3 in limiting fungal pathogens should also be considered due to their current increase and evolving resistance.

The O_3-values of the FA treatment, which well reproduced the natural O_3 background levels at pre-industrial times, which were 25–50 % lower than current levels (Yeung et al., 2019), favored the rust infection significantly (7 h-mean of 28.8 nL L^{-1} and AOT40 index of 10 nL L^{-1} h). In the Central Iberian Peninsula, O_3 hourly 50–60 nLL^{-1} range values are commonly reached almost every day during the early wheat growing season (MITECO, 2022) where rainfed cereal is a resource of economic importance (MAPA, 2021). Thus, the results prompt a reflection on the potential role of O_3-contaminated environments in controlling wheat fungal diseases in Mediterranean wheat fields. Considering the similar high O_3-sensitivity of Spanish CV released after the Green Revolution (Chang-Espino et al., 2021), modern wheat CVs in the Central Iberian Peninsula might benefit from growing under O_3 levels within the 50–80 nL L^{-1} range due to the O_3-induced control on plant fungal diseases.

Experimental sciences have proved that clean air provides great benefits for crop growth, yield and yield quality (Li et al., 2021; CLRTAP, 2017), but the present assay reinforces previous data on the beneficial effects of medium O_3 levels and low N fertilization for limiting fungal diseases. Usually, the pollutant effect on crops has been studied as an isolated factor and scarcely considering its potential interaction with pathogens and fertilization. Screening studies testing crop O_3 tolerance have frequently shown the higher O_3-sensitiviy of the modern cultivars (Barnes et al., 1990; Pleijel et al., 2006; Chang-Espino et al., 2021), even though crop breeding in the Northern hemisphere would be carried out under an environment with increasing O_3 levels. It is possible that breeding also drifted inadvertently towards a selection of varieties that have been optimized for a balance between O_3 tolerance and pathogen resistance.

Under the United Nations Air Convention (UNECE) framework, the O_3 threshold for crop protection (Critical Level, CL) based on wheat is currently established at an AOT40 value of 3000 nL L^{-1} h calculated for a 3-month period during the daily hours of the crop growing season (CLRTAP, 2017); this is also the objective value established by EU Air Quality Directive for plant protection (EU/80/50). Thus, ideally O_3 ambient levels must be reduced progressively to achieve this O_3 ambient limit. However, in the present Artur Nick assay an AOT40 index of 2341 nL L^{-1} h accumulated over only 1 month was beneficial enough to strongly limit YR infection; meaning that O_3 levels above the current threshold values for crops protection would be required for wheat to grow under some O_3-induced limitation in fungal diseases (Fig. 6). The AOT40 response function of the present assay indicated values of around 6000 nL L^{-1} accumulated 1 month before the outbreak to achieve the best rust control. At these O_3 values, there is no N-modulation in the YR response to the pollutant, which does happen at low ozone values, where N aids in infection development.

The response function presented in Fig. 6 is based on the results of the present assay; however, to generalize the observed response, a consistent AOT40 function is needed based on different independent trials and under different growth conditions. More experimental trials considering O_3, N, and pathogens are required to understand this complex interaction and clarify whether future achievement of crop protection air quality standards might necessitate new varietal improvement programs that adapt crops to an increased pathogen tolerance without involving the aid of O_3 pollution.

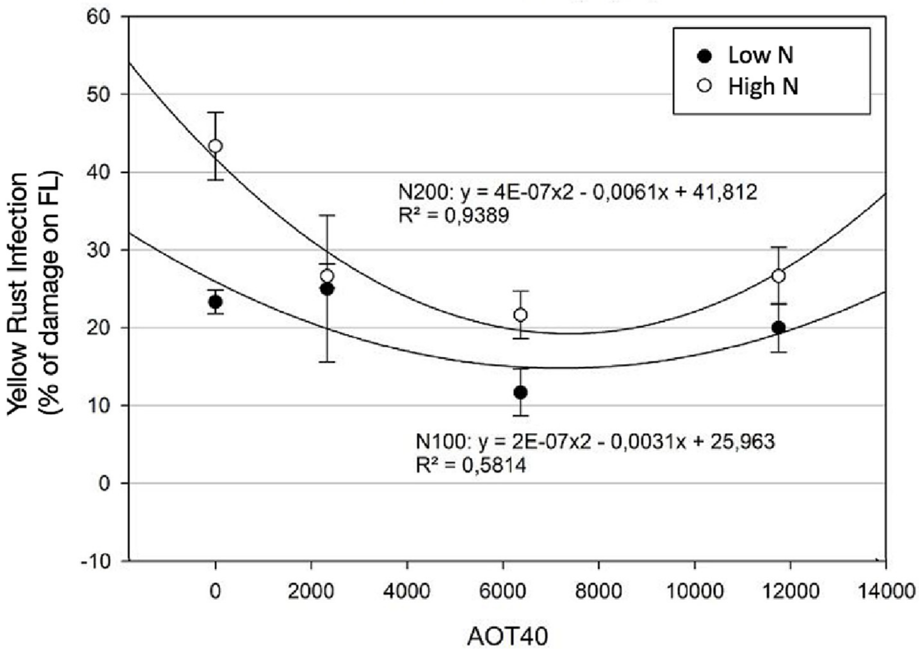

Fig. 6. Response function for YR outbreak based on 1 month-AOT40 index previous to the infection, expressed as a percentage of the affected section of the flag leaf (FL) (means ± SE) for the different nitrogen treatments: N-Low = added 100 kg N ha^{-1}; N-high = added 200 kg N ha$^{-1.}$

4. Conclusions

Preindustrial background O_3 levels (FA) strongly favored YR spread by 22 % when compared to NFA, and this percentage for Leaf 2 until Leaf 4 lay in the range 54–71 %. This led to rust-induced chlorophyll losses of 7–28 % in the different leaves, where the fungal disease was exacerbated in the older leaves and aggravated by the supplementation of N-fertilizer. Meanwhile, current central Iberian Peninsula O_3 ambient levels (NFA) strongly inhibited the rust, limiting the chlorophyll loss overserved in FA and achieving maximum physiological activity (g_s and A). The lowest fungal infection level was observed under the NFA+ treatment. However, in the FL, O_3 levels above ambient (NFA++) switched the O_3 effect in the opposite direction and rebounded the fungal disease. Results of the present assay showed an optimal O_3 range to limit the spread of YR on Mediterranean wheat in the range of 50–70 nL L^{-1} for the 7-h mean, which corresponded with a 1 month-AOT40 value in the range 2400–6400 nL L^{-1} h.

Ozone foliar damage augmented progressively with the increasing pollutant levels and the exposure duration, causing more intense effects in the older leaves, which were exposed to the pollutant for longer. The youngest FL did not present O_3 damage, not even under NFA ++, due to its short exposure time, although effects were observed at the physiological scale (A and gs reduction). Ozone damage caused progressive foliar pigment loss. Nitrogen did not affect O_3 foliar damage. Nonetheless, N and O_3 did not interact in their effects on YR or O_3 damage.

Based on the results of the present assay, the response function for YR incidence on the FL suggested an optimal value of around 6000 nL L^{-1} h accumulated 1 month before the outbreak to achieve the best pollutant-aided rust control, providing values above the current threshold for crop protection established under the EU and UNECE frameworks. At this O_3 exposure, no N-modulation was found for the YR response due to O_3. However, more experimental effort is needed to generalize these findings.

Despite the undeniable detrimental effect of O_3 on wheat, its beneficial effects in limiting fungal pathogens may be important when considering air quality standards for plant protection. Therefore, breeding programs may require adjustments for a better fungal pathogen tolerance that does not require the aid of O_3.

CRediT authorship contribution statement

The authors contributed to the manuscript in the following ways: Conceptualization, M.C-E, J.L.A. and V.B-B.; Methodology, M.C-E., V.B-B., I. G-F. and J.M.G-C; Formal Analysis, M.C-E., S.P-B. and V.B-B.; Investigation, M.C-E., V.B-B., I.G-F. and J.L.A.; Resources, M.C-E. V.B-B. and J.L.A.; Data Curation, S.P-B, M.C-E. and V.B-B.; Writing – Original Draft Preparation, M.C-E., V.B-B. and S.P-B. ; Writing – Review & Editing, M.C-E., V.B-B. and J.L.A.; Visualization, M.C-E.; Supervision, V.B-B. and J.L.A; Project Administration, V.B-B. and I. G-F; Funding Acquisition, V.B-B, and J.L.A.

Ethics statements

The authors declare no ethical conflict.

Funding and acknowledgments

This study has been funded by the ERANET/SUSCROP SUSCAP (PCI2019–103521/AEI) and the Comunidad de Madrid (Spain) AGRISOST-CM (S2018/BAA-4330) projects. M.C-E. and J.L.A. acknowledge the support from PID2019-106650RB-C21, from MICIN, Spain. J.L.A. thanks the support from the ICREA Academia program, Generalitat de Catalunya, (Spain). H. Calvete-Sogo and R. Alonso are acknowledged for their appreciated help in the fieldwork. Thanks are given to La Higueruela-MNCN/CSIC Research Farm staff for their valuable help during the development of the experimental assays.

Data availability

The data presented in this study are available upon request from the corresponding author.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary information consists of: Table 1S. O_3 and N effect on YR damage and O_3 damage; Table 2S. O_3 and N effect on foliar pigment content; Fig. 1S. Meteorological conditions during the exposure period and Fig. 2S. Yellow rust and O_3 damage relative to total leaf area (%) for the different O_3 and N treatments. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163370.

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Appendix A. Supplementary data

Table 1S. O_3 and N effect on yellow rust damage (% of affected leaf) and O_3 damage (% of healthy foliar surface). Means ± SE. FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 nL L^{-1} of O_3) and NFA++ (non-filtered air + 40 nL L^{-1} of O_3); N-Low=added 100 kg N ha^{-1}, N-high =added 200 kg N ha^{-1}. Letters indicate significant differences among levels within each factor ($p<0.05$).

		Yellow Rust damage (%)				**O3 damage (% senescence)**			
		Flag Leaf	**Leaf 2**	**Leaf 3**	**Leaf 4**	**Flag Leaf**	**Leaf 2**	**Leaf 3**	**Leaf 4**
Nitrogen									
N-Low		20.0^{a} ± 2.4	9.8^{a} ± 1.4	7.9^{a} ± 2.4	11.7 ± 2.9	0.8 ± 0.3	4.8 ± 1.3	17.1 ± 2.4	29.4 ± 4.8
N-High		29.6^{b} ± 2.4	19.4^{b} ± 1.4	15.0^{b} ± 2.4	12.8 ± 3.1	1.0 ± 0.3	6.5 ± 1.3	23.1 ± 2.4	35.4 ± 5.1
p-value N		***0.007***	***<0.001***	***0.04***	*0.795*	*0.625*	*0.355*	*0.084*	*0.4*
Ozone									
FA		33.3^{b} ± 3.3	28.3^{b} ± 2.0	27.1^{b} ± 3.3	34.4^{b} ± 4.1	0.0^{a} ± 0.4	0.0^{a} ± 1.8	0.0^{a} ± 3.4	0.0^{a} ± 6.8
NFA		25.8^{ab} ± 3.3	12.9^{a} ± 2.0	8.3^{a} ± 3.3	9.9^{a} ± 4.1	0.0^{a} ± 0.4	0.0^{a} ± 1.8	0.0^{a} ± 3.4	7.4^{a} ± 6.8
NFA+		16.7^{a} ± 3.3	7.5^{a} ± 2.0	4.2^{a} ± 3.3	2.5^{a} ± 4.5	1.3^{ab} ± 0.4	6.7^{a} ± 1.8	20.8^{b} ± 3.4	39.4^{ab} ± 7.5
NFA++		23.3^{ab} ± 3.3	9.6^{a} ± 2.0	6.3^{a} ± 3.3	2.2^{a} ± 4.1	2.5^{b} ± 0.4	15.8^{b} ± 1.8	59.6^{c} ± 3.4	82.6^{b} ± 6.8
p-value O_3		***0.011***	***0.019***	***0.002***	***0.008***	***0.300***	***0.007***	*0.011*	***0.002***
Nitrogen x Ozone									
N-Low	FA	23.3 ± 4.7	20.0^{b} ± 2.9	20.8 ± 4.7	37.5^{b} ± 5.5	0.0 ± 0.6	0.0^{a} ± 2.5a	0.0^{a} ± 4.8	0.0^{a} ± 9.2
	NFA	25.0 ± 4.7	9.2^{a} ± 2.9	5.0 ± 4.7	7.3^{a} ± 6.1	0.0 ± 0.6	0.0^{a} ± 2.5a	0.0^{a} ± 4.8	0.0^{a} ± 10.1
	NFA+	11.7 ± 4.7	5.8^{a} ± 2.9	4.2 ± 4.7	2.5^{a} ± 5.5	0.8 ± 0.6	5.8^{ab} ± 2.5	18.3^{b} ± 4.8	34.2^{b} ± 9.2
	NFA++	20.0 ± 4.7	4.2^{a} ± 2.9	1.7 ± 4.7	0.0^{a} ± 6.1	2.5 ± 0.6	13.3^{b} ± 2.5	50.0^{c} ± 4.8	85.2^{c} ± 10.1
p-value N-Low		*0.425*	***0.002***	*0.068*	***0.034***	*0.088*	***0.007***	***0.04***	***<0.001***
N-High	FA	43.3^{b} ± 4.7	36.7^{b} ± 2.9	33.3^{b} ± 4.7	31.2 ± 6.1	0.0^{a} ± 0.6	0.0^{a} ± 2.5	0.0^{a} ± 4.8	0.0^{a} ± 10.1
	NFA	26.7^{a} ± 4.7	16.7^{a} ± 2.9	11.7^{a} ± 4.7	12.5 ± 5.5	0.0^{a} ± 0.6	0.0^{a} ± 2.5	0.0^{a} ± 4.8	16.7^{a} ± 9.2
	NFA+	21.7^{a} ± 4.7	9.2^{a} ± 2.9	4.2^{a} ± 4.7	2.5 ± 7.2	1.7^{ab} ± 0.6	7.5^{b} ± 2.5	23.3^{a} ± 4.8	44.7^{ab} ± 11.8
	NFA++	26.7^{a} ± 4.7	15.0^{a} ± 2.9	10.8^{a} ± 4.7	5.0 ± 5.5	2.5^{b} ± 0.6	18.3^{c} ± 2.5	69.2^{b} ± 4.8	80.0^{b} ± 9.2
p-value N-High		***0.04***	***0.058***	***0.008***	*0.096*	*0.053*	***0.025***	***0.002***	***0.015***
*p-value N * O3 interaction*		*0.272*	*0.14*	*0.599*	*0.719*	*0.866*	*0.726*	*0.169*	*0.621*

Table 2S. O_3 and N effect on foliar pigment content (SPAD units). Means ± SE. FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 nL L^{-1}) and NFA++ (non-filtered air + 40 nL L^{-1}); N-Low=added 100 kg N ha^{-1}, N-High=added 200 kg N ha^{-1}. Letters indicate significant differences among means within each factor.

		Foliar Pigment – May (main stem)				**Foliar Pigments-June (main stem)**				**Foliar Pigments - June (secondary stem)**			
		FL	**Leaf 2**	**Leaf 3**	**Leaf 4**	**FL**	**Leaf 2**	**Leaf 3**	**Leaf 4**	**FL**	**Leaf 2**	**Leaf 3**	**Leaf 4**
Nitrogen													
N-Low		46.3^{b} ± 0.6	44.9^{b} ± 0.7	35.9^{b} ± 1.4	26.5 ± 1.3	14.8 ± 1.5	18.2 ± 1.2	15.7 ± 1.2	15.7 ± 2.2	46.4 ± 0.5	38.9 ± 0.7	24.2 ± 1.2	15.2 ± 2.4
N-High		43.1^{a} ± 0.6	41.0^{a} ± 0.7	30.6^{a} ± 1.4	24.0 ± 1.4	15.3 ± 1.4	16.4 ± 1.1	13.2 ± 1.2	13.3 ± 1.7	46.5 ± 0.5	38.1 ± 0.8	24.1 ± 1.2	14.9 ± 3.9
p-value		***0.001***	***<0.001***	***0.009***	*0.204*	*0.8*	*0.256*	*0.153*	*0.618*	*0.85*	*0.464*	*0.983*	*0.917*
Ozone													
FA		41.7^{a} ± 0.9	40.8 ± 1.0	30.2^{ab} ± 1.9	27.7^{b} ± 1.9	15.7^{b} ± 1.9	27.1^{c} ± 1.6	17.3^{b} ± 1.8	17.0^{b} ± 2.3	46.4 ± 0.8	46.4^{c} ± 1.0	40.2^{c} ± 1.7	14.1^{bc} ± 5.5
NFA		45.2^{b} ± 0.9	43.7 ± 1.0	41.7^{c} ± 1.9	35.9^{c} ± 1.9	20.6^{b} ± 2.3	26.1^{c} ± 1.6	27.9^{c} ± 1.7	24.2^{c} ± 1.8	48.8 ± 0.7	49.4^{c} ± 1.0	36.7^{c} ± 1.7	31.8^{c} ± 3.2
NFA+		45.7^{b} ± 0.9	44.1 ± 0.9	34.5^{b} ± 1.9	23.7^{b} ± 2.0	17.3^{b} ± 1.9	12.7^{b} ± 1.6	9.1^{a} ± 1.7	13.6^{b} ± 2.0	47.8 ± 0.7	40.9^{b} ± 1.0	13.5^{b} ± 1.7	8.8^{ab} ± 3.4
NFA++		46.2^{b} ± 0.9	43.1 ± 0.9	26.6^{a} ± 1.9	13.6^{a} ± 2.0	6.6^{a} ± 1.9	3.4^{a} ± 1.7	3.5^{a} ± 1.8	1.2^{a} ± 3.9	42.9 ± 0.8	17.4^{a} ± 1.1	6.1^{a} ± 1.7	3.1^{a} ± 5.9
p-value		***0.015***	*0.325*	***0.019***	***0.003***	***0.01***	***0.002***	***<0.001***	***0.036***	*0.262*	***0.001***	***<0.001***	***0.040***
Nitrogen x Ozone													
N-Low	FA	43.5 ± 1.2	41.9 ± 1.3	32.5^{a} ± 2.7	26.9^{b} ± 2.6	17.4^{ab} ± 2.6	27.6^{c} ± 2.4	16.7^{b} ± 2.4	14.7 ± 1.8	46.1 ± 1.1	46.4^{c} ± 1.4	37.3^{b} ± 2.4	13.7 ± 5.5
	NFA	46.2 ± 1.2	45.0 ± 1.3	44.1^{b} ± 2.7	35.9^{b} ± 2.8	19.7^{b} ± 3.8	27.4^{c} ± 2.2	31.0^{c} ± 2.4	27.4 ± 1.6	49.0 ± 1.0	47.8^{c} ± 1.4	37.7^{b} ± 2.4	30.5 ± 4.7
	NFA+	46.8 ± 1.2	46.2 ± 1.3	38.5^{ab} ± 2.7	28.7^{b} ± 2.6	17.5^{ab} ± 2.6	13.9^{b} ± 2.2	10.3^{ab} ± 2.4	12.8 ± 1.4	47.5 ± 1.0	41.1^{b} ± 1.4	14.9^{a} ± 2.4	10.6 ± 5.3
	NFA++	48.6 ± 1.2	46.3 ± 1.3	28.4^{a} ± 2.7	14.5^{a} ± 2.8	4.4^{a} ± 2.6	4.0^{a} ± 2.4	4.7^{a} ± 2.4	0.2 ± 3.5	42.9 ± 1.1	20.2^{a} ± 1.6	6.8^{a} ± 2.4	3.9 ± 4.3
p-value N-Low		***0.076***	*0.433*	*0.058*	***0.003***	***0.015***	***0.003***	***0.002***	*0.065*	*0.262*	***0.002***	***0.001***	*0.149*
N-High	FA	39.9 ± 1.2	39.7 ± 1.3	28.0^{a} ± 2.7	28.6^{bc} ± 2.8	14.1^{ab} ± 2.6	26.6^{c} ± 2.2	17.8^{b} ± 2.6	14.3 ± 3.3	46.7 ± 1.0	46.3^{bc} ± 1.4	43.1^{b} ± 2.4	14.6 ± 8.4
	NFA	44.1 ± 1.2	42.3 ± 1.3	39.4^{b} ± 2.7	36.0^{c} ± 2.6c	21.4^{b} ± 2.8	24.7^{c} ± 2.2	24.7^{b} ± 2.4	21.0 ± 3.2	48.6 ± 1.0	51.0^{c} ± 1.5	35.8^{b} ± 2.4	33.0 ± 4.7
	NFA+	44.6 ± 1.2	42.1 ± 1.3	30.4^{ab} ± 2.7	18.6^{ab} ± 3.1	17.1^{ab} ± 2.6	11.5^{b} ± 2.2	7.9^{a} ± 2.4	12.6 ± 3.6	48.1 ± 1.0	40.6^{b} ± 1.5b	12.2^{a} ± 2.4	7.0 ± 4.1
	NFA++	43.9 ± 1.2	39.9 ± 1.3	24.8^{a} ± 2.7	12.6^{a} ± 3.0	8.7^{a} ± 2.8	2.8^{a} ± 2.4	2.3^{a} ± 2.6	2.2 ± 7.1	42.8 ± 1.0	14.6^{a} ± 1.5	5.3^{a} ± 2.4	2.3 ± 10.6
p-value N-High		***0.072***	*0.582*	***0.034***	***0.007***	***0.044***	***0.008***	***<0.001***	*0.108*	*0.299*	***<0.001***	***0.003***	*0.415*
*p-value N * O3*		*0.673*	*0.406*	*0.846*	*0.169*	*0.559*	*0.977*	*0.521*	*0.067*	*0.951*	*0.052*	*0.258*	*0.925*

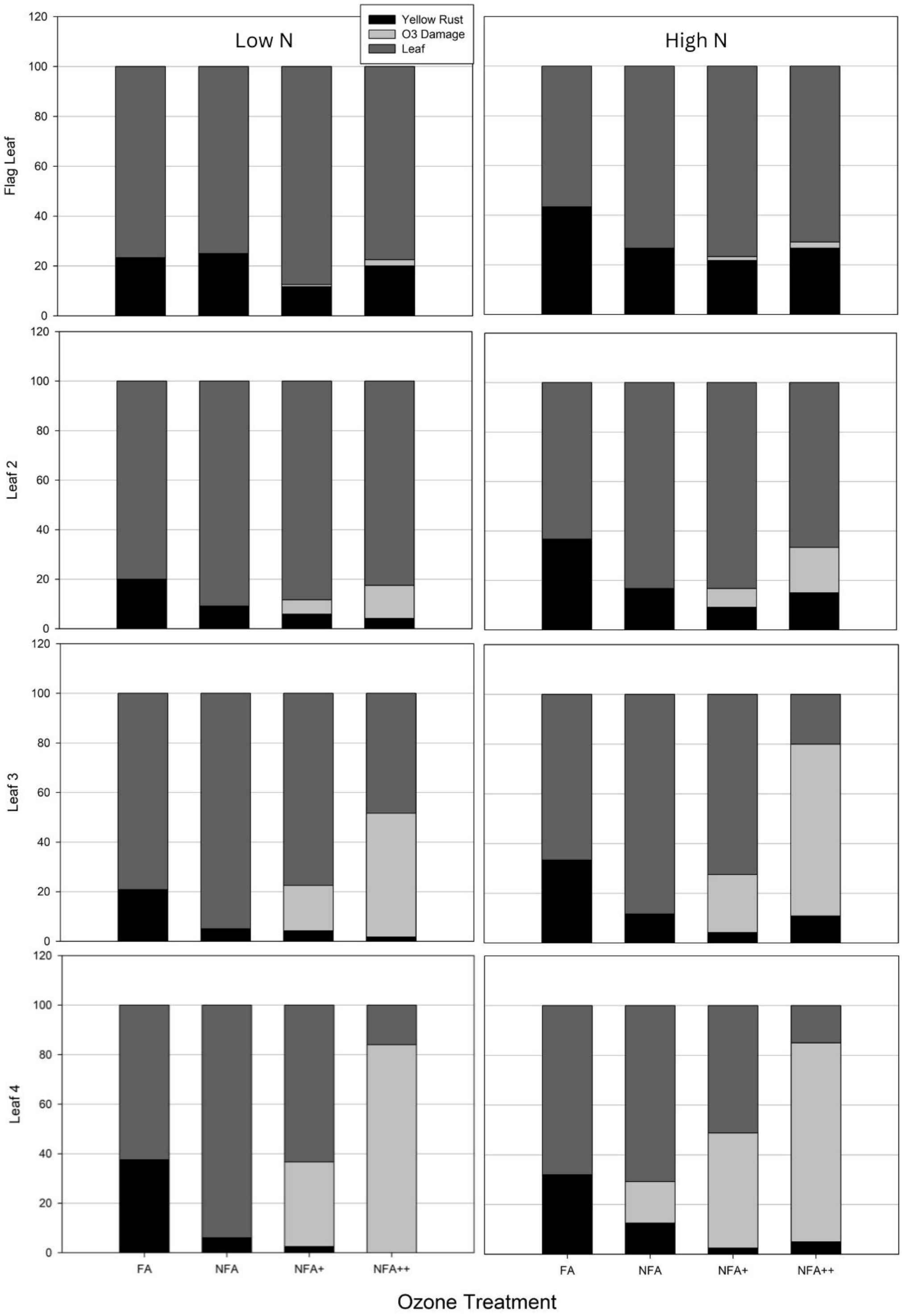
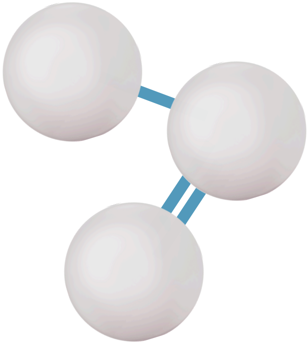


Figure 1S. Yellow rust and O_3 damage (%) relative to total leaf area (Means □ SE) for the different O_3 and N treatments on May 30th-June 1st. FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 nL L^{-1}) and NFA++ (non-filtered air + 40 nL L^{-1}); N-Low=added 100 kg N ha^{-1}, N-High=added 200 kg N ha^{-1}. Significant differences are shown in Table 2S.

GENERAL DISCUSSION



General Discussion

Due to the harmful effects of ozone (O_3), and its implications for food security in the Mediterranean region, this research aimed to investigate the interactive effects of this pollutant with various factors such as cultivar oldness, pathogen infections, and supplemental fertilization on wheat in a Mediterranean environment. Different research methodologies and techniques, which provide a particular angle by themselves, were combined to provide a more comprehensive view. The study focused particularly on the use of the natural abundances of isotopic signatures, which have been used before to assess plant response to factors such as water status or nitrogen fertilization, as valuable indicators of chronic O_3 stress. This knowledge can broaden our understanding of the effects of O_3 in the region, providing valuable information for crop management and policy-making in a changing environment.

1. *Pollution, climate, and experimental conditions of the assays*

Ozone pollution is an issue that affects the entire Northern Hemisphere (Ainsworth *et al.*, 2012; Schultz *et al.*, 2017), and within the European Union (EU), it is especially significant in the Mediterranean region (EEA, 2022) due to its climatic conditions, which favor the photochemical production of ozone (Proietti *et al.*, 2016), and the regional transport of precursors within the basin (Gangoiti, 2001).

FA chambers are often used to compare current and increased ozone levels with pre-industrial pollutant levels (Pleijel et al, 2011). The 7-hour mean FA level measurements acquired in both experiments ranged from 23.4 to 26.5 nL L-1 (AOT40 11-43 nL L^{-1} h), which lie within the range of surface ozone concentrations calculated by Young et al., (2013). These were 25 nL L-1 lower in pre-industrial times in the Mediterranean, matching the levels we got when compared to the NFA measurements.

The experiments of the present study simulated O_3 levels and profiles typical for wheat-growing areas in Central Spain, one of the main wheat production areas that accounts for 57.7% of all of the production in Spain (MAPA, 2021). Current O_3 levels, represented by NFA treatment, increased from April to June-July, and followed the seasonal spring-summer O_3 variability, ranging from 45-55 nL L^{-1} for the 7-h mean, and AOT40 values of 1140-5374 nL L^{-1} h. These daily profiles are common in Spain (Querol *et al.*, 2016) and often exceed the 40 nL L^{-1} EU plant protection threshold for plants, actually surpassing 60 nL L^{-1} and causing chronic exceedances over the AOT40-based objectives to protect vegetation (MITECO, 2022; EEA, 2023). These values also surpass the specific limit values (Critical Levels, CL) for wheat crop protection established under the Air Convention to avoid yield and quality losses of over 5% (CLRTAP, 2017).

The NFA+ and NFA++ treatments reproduced the highest O_3 values in the experiments, ranging from 53.9 to 90 nL L^{-1} for NFA+ and NFA++ for the 7-hour mean for both experiments, as well as 7968-10057 nL L^{-1} h and 16720-17872 nL L^{-1} h respectively following the AOT40 index. These values are already sporadically reached in the area (Alonso *et al.*, 2001; Elvira *et al.*, 2016; MITECO, 2022), and are characteristic of the Mediterranean basin (EEA, 2023). Moreover, modeling exercises have predicted O_3-level increases of 18% globally (Young et al., 2013), or up to 5 nL L^{-1} in Europe (Colette *et al.*, 2015) by 2100. Compared to NFA, these values lie within range for future increases from current average values.

Even though mitigation strategies have already been implemented in the EU and thus in the Northern Mediterranean countries, O_3 is still expected to remain at current levels or increase despite the reduction of precursor emissions due to the effect of climate change (Colette *et al.*, 2015). The Mediterranean area is a hotspot for climate change (Giorgi and Lionello, 2008) and temperatures are expected to increase by 4 ºC by 2100 (Paeth *et al.*, 2017), coupled with more regular heat waves (Meehl *et al.*, 2018) increased solar radiation, and drier summers (Dewan and Lakhani, 2022; Essa et al., 2023), which are already favoring O_3 photochemical formation. The detrimental effect of increasing temperatures on O_3 is known as the O_3 climate penalty (Dewan and Lakhani, 2022; Zanis et al., 2022) and is expected to counteract O_3 mitigation strategies. Based on measurements performed from 2005-2014, this anticipated rise in temperature is expected to worsen and increase O_3 levels by 0.2-2 nL L^{-1} $ºC^{-1}$ from 2015-2100 in regions influenced by O_3 precursors (Zanis *et al.*, 2022).

The climatic conditions experienced during the assays followed the typical patterns for the area. Temperatures and vapor pressure deficit increased throughout the growth period, while relative humidity decreased. Values ranged from 8 to 42 ºC for temperature and 18 to 89 % for relative humidity, which led to Vapor Pressure Deficit (VPD) values of 1.2 to 6.8 kPA. These factors reached their most extreme values by the end of the growth season in June/July.

Meanwhile, as the lower VPD values experienced around April favor stomatal conductance, this would therefore favor pre-anthesis absorption, as seen in Chapters 2 and 3, which considered wheat grown in semi-natural conditions that were fumigated from tillering onwards. As most studies focus on anthesis, and coupled with modeling exercises indicating that the Mediterranean basin experiences O_3-induced wheat yield losses of more than 8%, compared to the 4.7% average for Europe (Schucht et al., 2021), pre-anthesis absorption could be an important factor to explore for Mediterranean wheat. Analyzing the effects of O_3 in this, while considering Mediterranean pollution, climate and growing conditions, could help take appropriate steps to facilitate informed policy-making on Mediterranean varieties, given the limited information available on them (Mills et al., 2018b; Pleijel et al., 2019; EEA, 2022).

2. *Ozone sensitivity of Mediterranean wheat genotypes*

Wheat is the most ozone-sensitive staple crop (Avnery, 2013; Singh et al., 2018; Pleijel et al., 2019; Broberg et al., 2020) as ozone-induced stress can lead to reduced growth, and decreased nutrient remobilization, causing yield and quality losses (Mills *et al.*, 2007; Pleijel, 2011; Ma *et al.*, 2022). The implications of these O_3 effects are profound for food security (see section 9), even affecting the economic viability of wheat production (Brewster et al., 2024).

Different wheat varieties have an expansive variability in terms of ozone response. Just considering the age of the cultivars, significant O_3-response differences have been observed between landraces and CVs when Northern and Central European, as well as Asian varieties, are considered, with landraces exhibiting a greater tolerance than CVs (Barnes et al., 1990; Pleijel et al., 2006; Biswas et al., 2008).

This particular analysis had not been conducted for Mediterranean spring wheat cultivars, which is why this thesis focused on filling that knowledge gap. This was addressed through the experiments conducted in Chapter 1 and Chapters 2-4. The studies focused on Mediterranean varieties, using 8 cultivars and 4 landraces for Chapter 1, while Chapters 2-4 centered on the third most important cultivar in Spain, Artur Nick, which is often used as a "witness" or reference cultivar in most studies (GENVCE, 2023; MAPA, 2023)

Studies performed in other regions showed that modern cultivars have a higher sensitivity to O_3 due to their selection for a higher harvest index, which has consequentially led to a higher stomatal conductance and therefore a higher O_3 absorption. The same response was observed for the Mediterranean varieties. In the assay performed for Chapter 1, O_3 increased senescence and decreased wheat growth for post-Green Revolution varieties, causing GY losses of 15%, and 11% for GNY from NFA to NFA++, while no significant changes were seen for the pre-Green Revolution varieties. This also followed for the effect of ozone on Artur

Nick under the second experiment (Chapter 2), where O_3 induced a GY decrease of 21% and protein yield by 20% from NFA to NFA++, even when treated with 200 kg ha^{-1} of nitrogen.

The observed losses in the sensitive Mediterranean cultivars due to increasing ozone coincide with the values observed in other cultivars from different agroclimatic areas, which range from 7-22% (Mills et al., 2007; Feng et al., 2008; Pleijel and Uddling, 2012; Broberg et al., 2015; Mills et al., 2018a). At the European level, losses were also in the range of 20% when cultivars of Belgium, Finland, Sweden, and Italy were joined for the O3-response function (Pleijel *et al.*, 2004, 2007). Regarding protein yield losses, results from the first assay indicated a similar effect to previous works which lie in the range of 6.2-16% (Pleijel and Uddling, 2012; Broberg *et al.*, 2015; Pleijel *et al.*, 2018), but a higher effect was seen in the second assay, which the additional nitrogen fertilizer can explain, as this would cause a larger contrast under the lower and higher ozone levels.

It is important to note that the response of the modern Mediterranean cultivars to O_3 stress, in terms of GY, seems to be similar to that of sensitive European cultivars, despite their adaptation to the Mediterranean climate. This suggests that the combined effect of ozone and the Mediterranean climate may counteract the wheat cultivars' adaptation to their environment.

Though adapted to the dry conditions of the Mediterranean, these cultivars are still significantly affected by the fluctuating water conditions of the year, something that is very common in the Mediterranean (Rezzouk *et al.*, 2022), where the effect of drought on wheat yield could be exacerbated in some years by more intense or longer droughts. The effects of this climatic interannual variability were seen in our experiments, as while the second experiment underwent a longer O_3 exposure than the first, effects on GY and GNY/Protein Yield lay in a similar range. The second experiment was performed in a wetter year, which would lead to an increase in stomatal conductance (g_s) and therefore higher yields. This, however, would also lead to a higher O_3 uptake, which would be related to lower yields under the higher O_3 levels. Nonetheless, as discussed in Chapter 3, earlier exposure to the pollutant could be causing early defenses against the detrimental effects of ozone.

Similarities in the effect O_3 on Mediterranean and other European cultivars could also be attributed to an increase in surface ozone levels. When experiencing drought, plants close their stomata to decrease water loss, limiting ozone uptake (Broberg *et al.*, 2023). This would decrease the damage caused to wheat plants due to the oxidative properties of the O_3-generated ROS, but it would also mean a higher surface ozone level because it would not be up-taken by plants. This was seen in a model simulation for 1960-2019, where a decrease in ozone removal under drought increased ozone pollution in Europe, counteracting some of the improvements caused by emission control (Lin *et al.*, 2020). Moreover, concerning ROS, the combined impact of O_3 and drought has also been observed to elevate ROS to a level that surpasses the plant's detoxification capacity (Cotrozzi *et al.*, 2016; Hoshika *et al.*, 2020).

As both ozone and climate could be determining yield in Mediterranean cultivars, where more droughts would decrease ozone uptake but decrease yield due to climatic stress, while more rainfall would help increase yield but also increase ozone uptake and stress, further experiments on this interaction should be considered. This will be expanded upon in Section 10.

Regarding quality, for the first assay (Chapter 1) the effect of ozone on GNY in cultivars differed more than under the second assay (Protein Yield; Chapter 2), which could be attributed to the timing of O_3 exposure. For the first experiment, O_3 exposure began near anthesis, and as N absorption typically declines significantly after anthesis (Perez *et al.*, 1989), N uptake may not have been as affected as in the second assay (Chapter 2), which was fumigated from tillering.

Moreover, for CVs, N aimed at grain filling is mainly remobilized from vegetative tissues, such as the flag leaf (Cui, 2020), which is greatly harmed by O_3. This difference was smaller for the landraces (10% for GNY), as grain filling depends on carbohydrates stored in a plant part that is not as affected by ozone, the ears (Simpson et al., 1983; Sanchez-Bragado et al., 2017). This indicates that cultivars could also be more susceptible to the effects of O_3 on resource remobilization, which should be considered in breeding programs.

The beneficial effects of breeding lasted despite the higher O_3 levels. Post-green revolution cultivars, having been selected for a higher Harvest Index, had a grain yield 26% higher than the landraces, as well as a higher GNY (30%) and HI (34%), even under the highest O_3 level. However, the strong effect of O_3 on the CVs as opposed to the landraces still leads to considering the broader genetic base of the latter, which could help improve the O_3-tolerance of the CVs.

3. *Mechanisms affecting Mediterranean wheat*

O_3 significantly affects gas exchange and the photosynthetic machinery (Hoshika *et al.*, 2015), altering carbon fixation factors (Ainsworth et al., 2012; Emberson et al., 2018) and reducing photosynthetic rates that in the end are greatly responsible for the O_3-induced yield losses (Feng and Kobayashi, 2009; Emberson et al., 2018; Sampedro et al., 2020). These effects on gas exchange were also observed in the experimental work of the thesis. However, the responses differed based on wheat genotype or nitrogen fertilizer dose.

In Chapter 1, landraces displayed an overall lower g_s, which remained rather steady throughout the different fumigation levels. Meanwhile, the stomatal conductance of the modern and old varieties, which at the lowest O_3 level was 46% larger than for the landraces, decreased with the pollutant to reach similar values to the landraces by NFA++. This would indicate that, even though cultivars have been selected to provide a greater production, which is coupled with an improved photosynthetic activity through greater g_s, this breeding effect on stomatal conductance can be canceled by increasing O_3 levels.

In Chapters 3 and 4 a difference in the effect of O_3 on g_s and net photosynthetic assimilation (A) was also observed in recently expanded flag leaves, where the effect was stronger on g_s. The main impact of the pollutant on g_s compared to A has been seen before in younger leaf measurements, where the early plant reaction to O_3 is considered to be mainly caused by the effect of a lowered g_s on A as opposed to a decreased carboxylation (Grandjean Grimm and Fuhrer, 1992).

It has been observed, from the results of Chapters 2 and 3, that sensitive wheat varieties such as Arthur Nick, can have an early response to try to compensate for ozone stress through an increase in photosynthetic efficiency, facilitated by an additional N supply. According to the results of Chapter 2, the intracellular CO_2 concentration (C_i) decreased with ozone levels while A remained steady, indicating an efficient CO_2 assimilation despite the increasing O_3 stress at earlier flag leave development. Exploring this in more detail in Chapter 3, the effect of ozone on decreasing stomatal conductance was only significant under the highest nitrogen treatment, where the beneficial effects of fertilizer were offset by the ozone fumigation level NFA+. The additional nitrogen also caused an interesting fluctuation in photosynthesis (A) by the highest fumigation level, where after decreasing, it recovered, almost reaching the values it had under FA. This effect has been seen before, where photosynthetic efficiency is stimulated early in the plant life (Ren et al., 2019; Lundgren and Fleming, 2020) in preparation for further damage (Salam and Soja, 1995).

This observed early attempted improvement in photosynthetic efficiency was also seen due to the increased number of mesophyll cells under the higher N fertilization (Chapter 3). This increase enhances mesophyll CO_2 diffusion conductance (g_m) (Ren et al., 2019; Lundgren and Fleming, 2020) by expanding the chloroplast surface area exposed to the intracellular airspace (Evans *et al.*, 2009; Tosens *et al.*, 2012).

However, it has been noted that what defines the O_3-sensitivity of modern cultivars is not only g_s, but also its antioxidant capacity to cope with the ROS burst caused by the pollutant when in contact with the vegetative tissues (Feng et al., 2016), and the protection they exercise in the physiological activity of the mesophyll cells (Moldau, 1998). This mechanism was suggested in Chapter 2, based on the Chlorophyll to N contents, PNUE pattern as well as the proportion of foliar carbon to nitrogen (C/N rate), which indicated that the additional nitrogen was not being used proportionally to construct carbonaceous structures. Therefore suggesting that the additional N up-taken by the leaves could be derived for other purposes such as tissue repair or increasing the expression of antioxidant molecules and enzymes (Wang et al., 2014; Feng et al., 2016). This should be considered in breeding, as plant varieties with more antioxidant substrates would then be less sensitive to ozone. Moreover, cultivars that can better exploit additional nitrogen for defense and repair processes could provide an advantage. However, by prioritizing the protection of the chlorophyll cells from the damaging ROS and increasing antioxidant production, additional N seems to initially be going towards conferring resistance to ozone-induced stress as opposed to carbon fixation and growth (Calatayud *et al.*, 2002), which would be detrimental to production. Additionally, prolonged exposure to the pollutant would cause excessive ROS to overwhelm the defense system of the plant, ultimately nullifying these benefits and decreasing the nitrogen use efficiency and yield (Broberg et al., 2017; Fatima et al., 2018).

In the assays of this thesis, the mentioned early defense mechanisms triggered to protect the plant from ozone did not last later in the plant life or were not strong enough to fully avoid the damages. In Chapter 2, while leaf chlorophyll (Chl) content increased with ozone fumigation, particularly under the higher N treatment, early in the flag leaf expansion, a later Chl measurement showed a significant drastic reduction, where the additional N could not counteract the prolonged exposure to the pollutant or decelerate senesce. This was also seen in differently aged leaves (Chapter 4), where the older tissue, which was exposed to ozone for longer, showed more intense senescence and lower physiological activity. As grain filling and nutrient remobilization times are shortened (Pleijel et al., 2006; Pandey et al., 2018), particularly affecting starch content in the grain (Wang and Frei, 2011) and causing smaller grains, this led to a decrease in yield and growth parameters, as well as an increase in $\delta^{13}C_{grain}$, discussed in sections 2 and 4 respectively.

4. *The use of C and N isotopic signatures to analyze plant mechanisms against ozone pollution*

Understanding plant responses to ozone stress requires a comprehensive approach and utilizing different parameters that can provide valuable insights into plant response mechanisms. Frequently, portable equipment has been used in the field to measure gas exchange and to analyze the effect of ozone on parameters related to photosynthetic activity, stomatal conductance, or transpiration. However, these methods only offer instantaneous measurements during the plant life that are extrapolated to the entire life cycle of the plant. On the contrary, the study of the isotopic signatures of C and N in plant tissues, can integrate the entire accumulated plant response through simple field sampling, providing very satisfactory results for the study of chronic stress.

Isotopic discrimination is driven by the ratio of intercellular to atmospheric CO_2 concentration (C_i/C_a) (Farquhar and Richards, 1984; Farquhar, 1989), and therefore provides an insight into the balance between g_s and A that the plant experienced up until the sampling date (Farquhar, 1989; Tcherkez et al., 2011). When the stomata close due to increased ozone exposure, this decreases CO_2 available to the carboxylation site, decreasing C_i/C_a and therefore discrimination, increasing $\delta^{13}C$.

The nitrogen isotopic signature ($\delta^{15}N$) measured in different plant parts can be influenced by various factors. For example, $\delta^{15}N$ can vary depending on the source of N, as chemical fertilizers have a lower $\delta^{15}N$ than organic fertilizers (Serret *et al.*, 2008). In other processes, such as assimilation, metabolism, remobilization into other plant parts, and volatilization, fractionation is driven by the preferential use of the lighter ^{14}N isotope (Tcherkez, 2011; Yousfi et al., 2012; Cui et al., 2020). The fractionation of $\delta^{15}N$ as N moves towards the grain (Cui et al., 2020) explains why any alterations caused by ozone, such as reduced remobilization times (Brewster et al., 2024), a different N storage organ (considering the reliance of modern cultivars on the flag leaf for grain filling) (Sanchez-Bragado et al., 2017), a lower stomatal conductance (which can affect N remobilization through the phloem as stated by Sanchez-Bragado et al., (2014b), or allocating nitrogen towards antioxidant defense mechanisms or repair (Shang *et al.*, 2019) can alter the $\delta^{15}N$ isotopic signature.

Generally, the effect of ozone on these isotopic signatures on leaves is not as evident as in the grains (Saurer et al., 1991; Jäggi et al., 2005), as the grain is the ultimate resting stop for these resources. However, coupled with other factors such as those previously mentioned, they can provide insights into resource remobilization and use.

$\delta^{13}C$ in grain exhibited a consistent response across various modern Mediterranean wheat varieties (Chapter 1, (Chang-Espino *et al.*, 2021)), and when under different nitrogen fertilization treatments (Chapter 3), suggesting its viability as an indicator of cumulative O_3 stress. Moreover, it also served as a tool to gain insights into the effects of ozone on resource redistribution. In Chapter 1, it was noted that ozone-tolerant landraces showed no significant effect on the $\delta^{13}C$ of mature grains despite reduced yield parameters, displaying its reliance on carbohydrate storage in the stem and ear and its redistribution to the grain under stress conditions. This, however, was not the case for the modern varieties, for which both a decreased stomatal conductance and reliance on carbohydrates stored in the more susceptible flag leaf were coupled with a decreased discrimination, causing $\delta^{13}C$ in the grain to increase significantly.

Meanwhile, in Chapter 3, additional N did not cause a differential response to ozone for $\delta^{13}C_{grain}$, displaying its reliability as an indicator despite the N fertilizer treatment. Also, as mentioned before, despite the improved photosynthetic efficiency seen at an earlier phenological stage provided by the additional N, $\delta^{13}C_{grain}$ showed that O_3 counteracted the effect of the fertilizer by the end of the plant life. In the same chapter, a comparison of $\delta^{13}C_{grain}$ values measured in different years showed the same response pattern to O_3 for the modern varieties in general, and more specifically for Artur Nick, where differences were driven by the year-specific experimental conditions. This again shows the strength of this parameter as an indicator of cumulative O_3 stress.

Regarding $\delta^{15}N$, in Chapter 1, ozone-sensitive cultivars showed a pattern in $\delta^{15}N_{grain}$ that increased up to NFA+, which could be related to an increase in the use of N (preferentially ^{14}N) in the leaf for other metabolic processes, such as defense mechanisms, thereby leading to an ^{15}N enrichment of the N remobilized to the grain. It then decreased by the higher O_3 level which could be related to the detrimental effect of O_3 on g_s, which would decrease the volatilization of ^{14}N, therefore increasing the amount of the lighter isotope in the leaf and consequentially in the grain, decreasing $\delta^{15}N$ (Farquhar *et al.*, 1980).

This decreased $\delta^{15}N$ due to increasing O_3 was also seen in Chapter 3, both in leaf and grain. Nonetheless, the effect was only significant in the grain. As wheat in Chapter 1 was exposed to the different O_3 treatments after N uptake (anthesis) and due to the non-significant response of $\delta^{15}N$ in Chapter 3 (O_3-exposure from tillering), both experiments would indicate an O_3 effect on remobilization.

In the present thesis, the importance of isotopic signatures on O_3-effect studies is highlighted, due to their insights into the effect of ozone on nitrogen and carbon uptake and redistribution mechanisms, as well as their resulting impact on quality.

5. The effect of pre-anthesis O_3-exposure on Mediterranean wheat

Anthesis is often considered the most O_3-sensitive period for wheat based on studies performed on central and northern European cultivars and conditions (Pleijel *et al.*, 1998). In fact, the ozone response functions and the critical levels derived for O_3-risk analyses, following the methodology of the Air Convention, are mostly based on OTC assays where O_3 exposure began at anthesis (CLRTAP, 2017). However, the most O_3-sensitive stage for wheat growing in the Mediterranean basin can differ from that observed in other agroclimatic zones, because of the different behavior of the varieties, pollution levels, and climatic conditions.

To assay the effect of the pollutant on wheat productivity following the O_3 pollution profile and timing in the Central Iberian Peninsula, plants were exposed from the tillering stage, during the pre-anthesis period (Chapters 2-4). Ozone exposure began in April, when ozone levels in the region had already reached and exceeded EU threshold levels (EEA, 2022) due to high solar radiation, and warm and stable conditions that favor the photochemical production of O_3 (Proietti *et al.*, 2016). As mentioned before, these pollution and climatic conditions coincide with the non-water limited period in the area, allowing for a heightened stomatal activity and gas exchange that would increase O_3 absorption and its effects (Emberson et al., 2018; Mills, Frei, et al., 2018a; Hoshika et al., 2020). This was clearly reflected by the POD6 index of the second assay, where 70% of the O_3 absorption occurred in pre-anthesis.

Given that the high pre-anthesis O_3 absorption coincides with FL development, the high dose could affect the resource reserves used for grain filling later in the season, when g_s decreases due to the more stressful climatic conditions, a mechanism that is of special relevance in drought-adapted cultivars (Sanchez-Bragado et al., 2014a). In Chapter 2, the effects on both yield and growth factors were similar, even though they were expected to be higher on growth due to a higher O_3 absorption pre-anthesis. This would reinforce what was commented in section 3. The protective responses caused by an early O_3 exposure, like increased chlorophyll content, stomatal closure or increased mesophyll cell numbers, particularly under high fertilization conditions, are indicative of the early plant response to the pollutant, and suggest that the effects happening pre-anthesis could be affecting the plant post-anthesis.

Results point to the importance of considering early O_3 exposure in the response of Mediterranean wheat, but further experimental work is needed to determine the extent to which this early preparation against O_3 benefits the plant, and to consider O_3 uptake before anthesis in modeling exercises for the region and within the risk assessment methodologies used for policy-making.

6. N-fertilization modulates the O_3-response and affects fertilizer efficiency

Only 30-50% of the fertilizer applied to croplands is up-taken by the plant (Broberg *et al.*, 2017; Hawkesford and Riche, 2020). Therefore, reaching a high Nitrogen Use Efficiency (NUE), upon which a larger part of the N applied results in a higher GY, is imperative to avoid economic and environmental losses. Although there is still little experimental information, the interaction between ozone and nitrogen fertilization can profoundly impact wheat cultivation, with nitrogen availability playing a pivotal role in modulating plant responses to ozone stress (Calvete-Sogo et al., 2016; Pandey et al., 2018; Peng et al., 2020), while ozone has been seen to counteract the benefits of nitrogen fertilization, reducing its effectiveness (Broberg *et al.*, 2017).

The experimental assays of Chapters 2 and 3, showed that the effect of counteracting the effect of O_3 on the plant due to additional nitrogen happens under low O_3 exposure but not under higher levels of the pollutant.

Additional nitrogen increased plant growth and yield, and lowered Harvest Index by 5%, showing that the fertilizer was contributing largely towards vegetative growth as opposed to the reproductive parts, which is the aim of improving NUE and which has been observed before (Peng *et al.*, 2020). As mentioned, N supplementation can also help during early flag leaf development, aiding in some pigment-loss avoidance and in maintaining photosynthetic efficiency, but this early protection did not last later in the plant life. Consequently, high ozone levels counteracted the beneficial effects of nitrogen fertilization, resulting in decreased growth and crop yields and almost eliminating the fertilizer stimuli. While the additional N fertilizer caused GY and Potential GY to increase, these decreased in the range of 21-23% by NFA++, reaching the levels of the lower N fertilizer treatment under the same pollutant level. It also strongly affected plant parts that are imperative for grain filling in modern wheat varieties, such as the hulled spikelet weight, which decreased by 28% due to the pollutant (Sanchez-Bragado et al., 2014a), also suggesting a lower remobilization efficiency.

When calculating the NUE and Agricultural Efficiency of Nitrogen (AEN) indices, the O_3 effect blocking the fertilizer stimuli became even more evident. Both indicated the beneficial effect of additional nitrogen on GY and Protein Yield respectively, and both followed the same trend, remaining steady under NFA and NFA+ to then drop significantly by NFA++. This was also reflected when calculating the Critical Levels (CL) under each of the N treatments individually, as the CL values under High-N were two times lower than those seen under Low-N.

These results highlight the importance of integrating nitrogen fertilization management into ozone risk assessment strategies to accurately inform policy-making and risk analysis concerning agricultural practices and ozone pollution mitigation strategies. This not only includes the yield and quality-related losses caused by the impact of O_3 on N fertilization, but also the economic and environmental consequences that unused fertilizer can have. The latter specifically has been shown to increase nitrous oxide emissions from the soils (Sánchez-Martín *et al.*, 2017; Kou *et al.*, 2018), which goes against several EU and global pollutant mitigation strategies, such as Objective 7 of COP15 for 2030 (United Nations, 2022), the European Green Deal in the Farm to Fork Strategy (European Commission, 2020a) and the Biodiversity Strategy (European Commission, 2020b).

While nitrogen fertilization initially enhanced resistance to ozone stress, prolonged exposure can lead to diminished photosynthetic efficiency and carbon assimilation, ultimately impacting both yield and quality. Moreover, as nitrogen fertilization can exacerbate fungal infections, nitrogen management practices need to be considered to effectively mitigate risks to wheat production.

7. The O_3-fungal disease interaction and its modulation by N-fertilization

While nitrogen fertilization may promote certain aspects of wheat growth, it can paradoxically exacerbate fungal infections (Almacellas, 2010; Devadas *et al.*, 2014), as was seen in this assay, further complicating the issues surrounding food security and quality in wheat production systems. Meanwhile, ozone exposure can influence the spread of fungal diseases such as yellow rust (*Puccinia striiformis*) in wheat, with medium ozone levels potentially limiting yellow rust infection, while excessively high ozone levels may reverse this beneficial effect, underscoring the importance of understanding this interaction for agricultural policy-making and risk assessment.

Chapter 4 (Chang-Espino *et al.*, 2023), delved into the effects of O_3 and N fertilization on yellow rust. As this fungal disease can decrease grain yields far more intensely than O_3 (Vergara-Diaz *et al.*, 2015), and as the pollutant has been seen to mitigate the fungi (Tzortzakis et al., 2008), the interaction seems important to consider in crop management and policy-making. Particularly because the fungus affects the grain-filling essential flag leaf, and because more aggressive rust races, which have adapted to the warmer conditions of the Mediterranean, have emerged in the last decade (Aparicio et al., 2014).

In this assay, the lowest pre-industrial O_3 levels (reproduced by the FA treatment), caused Chl losses ranging from 7-28% for the younger to older leaves and favored fungal infection by 22-71%, compared to NFA which reproduced the ambient pollution in central Spain. However, once current (NFA) levels were achieved, the pollutant limited fungal infection. Ozone can trigger various resistance mechanisms in the plant (Zuccarini, 2009), such as reinforcing the cell walls (Guidi *et al.*, 2005), accumulating ROS (Lamb and Dixon, 1997), stimulating pathogenesis-related proteins (van Loon et al., 2006), and activating defense signaling pathways mediated by ethylene and salicylic acid (Tuomainen *et al.*, 1997), all of which can prepare the plant for fungal infections. This means that by the 7 h-mean 49 nL L^{-1} of the NFA level, upon which the outbreak occurred, enough ROS had accumulated to defend the plant against the fungus. However, this was the optimal level of anti-fungal defense. At NFA++, as values approximated 100 nL L^{-1}, the effect of O_3 became predominant on the plant, possibly as the excessive ROS overwhelmed the defense system of the plant (Fatima et al., 2018). As both the fungal infection and excessive ozone have a detrimental effect on plant senescence and production, to maintain current yields, O_3 levels within the 50–80 nL L^{-1} range could be required to help limit common fungal infections like yellow rust without causing excessive O_3-induced losses.

These results also suggest that meeting air quality standards set by environmental policies, such as reaching pre-industrial O_3 levels, may require improvements in pathogen tolerance in Mediterranean wheat cultivars to compensate for the absence of O_3 in mitigating the effects of rust. This opens a new and interesting debate regarding the applications of scientific knowledge in environmental policies. A deeper understanding of the interaction between ozone and pathogens is required to contribute more information to this debate.

8. *Ozone response quantification based on exposure-based (AOT40) and dose–based (POD6) indexes, considering the modulation of N fertilization*

Due to its sensitivity to O_3, wheat is considered a reference crop for O_3 risk assessments (Feng et al., 2008; González-Fernández et al., 2013; Pleijel et al., 2018). The methodologies for risk analysis developed under the framework of the UNECE Air Convention are continually under revision following new scientific knowledge (CLRTAP, 2017), since for the best development of these analyses, information and quantification of the response of varieties from different agroclimatic zones and the factors that modulate them are required.

In Chapters 2 and 3, exposure-based (AOT40) and dose–based (POD6) indexes were calculated to quantify the effects of O_3 on Mediterranean wheat under two N fertilization regimes. Functions based on both indices are currently considered in the Air Convention to assess the effect of O_3 on wheat yield and quality (CLRTAP, 2017; EEA, 2022) and to model the impact of O_3 on food security under different future Global Change scenarios (Emberson et al., 2018; Feng et al., 2022). However, up to now, these methodologies do not consider how N fertilizer may modulate the O_3 response on wheat, nor do they consider the potential importance of ozone exposure in pre-anthesis crop periods. This work highlights the importance of both.

Section 4 showed the importance of the pre-anthesis period in Central Spain, where high levels of O_3 coincide with the wettest period, mild temperatures and thus, with the lower atmospheric stress for plants (low VPD), providing conditions when gas exchange is not limited and O_3 absorption is facilitated. In fact, based on the POD6 index, 70% of the pollutant-absorbed dose occurred during this pre-anthesis period (Chapter 2). This percentage is too high to expect that this early O_3 dose could compromise production at the end of the plant life. This is of major importance considering that Mediterranean wheat varieties, which have adapted to water stress, store early reserves for later remobilization into the grain when later water deficit limits photosynthetic activity (Sanchez-Bragado et al., 2014a).

Chapter 2 showed that both AOT40 and POD6 functions under High-N resulted in more negative slopes, and consequently lower critical values derived from them, than those calculated for Low-N. In the case of GY critical levels were 4 and 3 times higher for AOT40 and POD6 respectively under High-N, showing the reliance of the O_3-response on N fertilization.

As calculated by the NUE and AEN indices, which remained rather stable up to NFA+ to then drop by NFA++, the fertilized stimuli on GY could be eliminated by exposure levels of 16 720 nL L^{-1} h for AOT40 and 12.56 mmol m^{-2} PLA for POD6, which though high, are already reached in the Iberian Peninsula (MITERD, 2022).

In Chapter 3, we also calculated AOT40 functions to compare the $\delta^{13}C_{grain}$ response of the first and second assays and to analyze the consistency of the response from both assays. The assays differed in the starting time of O_3-exposure, with the first assay starting at anthesis and the second at tillering. The response was milder in the second assay, even though it had a longer O_3 exposure, which could indicate some O_3-induced plant adaptations in the early stages, such as the observed increase in the number of mesophyll cells mentioned in section 3.

The thesis quantified, for the first time, the O_3 response of Mediterranean spring wheat cultivars, and the great differences caused by the N supplementation, prompting the need for further research into the underlying mechanisms that caused this difference.

9. *Considering Mediterranean cultivars and N-management in ozone risk assessments for Food Security*

This study significantly contributes to addressing knowledge gaps concerning O_3 risk assessment under the Mediterranean perspective. Through a comprehensive investigation of wheat genotypes and nitrogen fertilization effects on O_3 stress, this research sheds light on the underlying mechanisms, enhancing our understanding of the effects of O_3 on crop productivity and quality in the Mediterranean basin to help inform effective policy and crop management interventions (European Commission, 2020a).

The research provides new knowledge about the O_3-sensitivity of Mediterranean wheat cultivars. Up to now, only the O_3-sensitivity of durum wheat Mediterranean varieties was analyzed (Monga *et al.*, 2015), but there was no information about the behavior of spring wheat varieties. Furthermore, the sensitivity of different wheat genotypes needs to be considered to inform breeding programs that aim to increase O_3-tolerance. This needs to consider both the reliance of modern cultivars on antioxidant activity for their O_3-defense (Wang et al., 2014; Feng et al., 2016), as well as the effect that additional N fertilization may have on modulating this process, which prompts further investigation.

O_3-risk methodologies, crop management practices and modeling should consider the effect of O_3 on nitrogen use efficiency. As the response functions showed that O_3-response, as well as the calculated critical levels, depended on the N fertilization regime, this could help improve nitrogen management to avoid losses into the environment, and to follow the objectives of the European Green Deal in the Farm to Fork Strategy (European Commission, 2020a), the

Objective 7 of COP15 for 2030 (United Nations, 2022) and the Biodiversity Strategy (European Commission, 2020b).

Wheat seems to have an early response to O_3 fumigation, which is why pre-anthesis plant responses need to be delved into more to observe if this is a mechanism that differs between different genotypes, as this could provide greater tolerance and decrease senescence and the detrimental effect of ozone on the flag leaf, which is so important for grain filling in modern varieties as stated before. Early fumigation effects could also be studied in landraces to assess the impact of O_3 on the defense of these more tolerant varieties, which rely on resource relocation from plant parts other than the susceptible flag leaf.

Finally, the effect of O_3 on fungal infections also needs to be considered, as returning to pre-industrial O_3 levels could have a detrimental effect on the optimization that has developed between O_3-tolerance and pathogen resistance, as modern wheat cultivars seem to benefit from current O_3 levels. On the other hand, if pre-industrial levels are targeted, future improvement programs should consider increasing the pathogen tolerance of these modern wheat cultivars.

Risk analyses ultimately aim to discover how poor air quality contributes to endangering food security in present and future scenarios. O_3 levels continue to rise and are not expected to decrease by the end of the century (Tai et al., 2014; EEA, 2023). Meanwhile, a meta-modelling analysis by Guarin et al., (2024) indicated that wheat yields could fall by 0.26-1.23% per ppb of additional ozone, for daily concentrations over 25 nL L^{-1}. This is compounded by the projected global population reaching 9.7 billion by 2050 and 10.3 billion by 2100 (UN, 2022), and the act that food security is a growing concern, particularly considering that 9.2% of the global population already suffers from hunger (UN, 2023). Both in Europe and globally, wheat is a significant source of both calories and protein (Erenstein *et al.*, 2022) the expected effects of O_3 to be threatening to food security. It is therefore imperative to consider important agricultural factors such as fertilizer and cultivar use, to accurately understand and mitigate the effects of ozone on this essential crop, and to target a sustainable global food supply amidst growing environmental and demographic pressures.

10. Further research lines

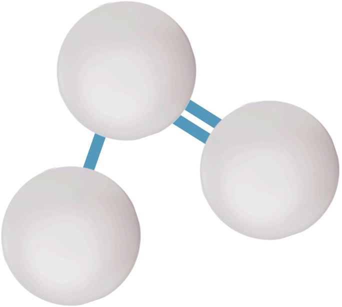
Some future research lines to further delve into the observed results include:

- Continue with ozone sensitivity analyses of Mediterranean wheat varieties. Currently, trials are being conducted in OTC considering varieties from North Africa. The joint analysis of all trials will allow for the determination of ozone tolerance traits of Mediterranean varieties for future varietal selection programs.
- Delving into other factors in interaction with O_3 such as water stress. Water stress has been seen to reduce the impact of O_3 on A, GY and biomass (Broberg *et al.*, 2023) due to the decrease in gas exchange, but some studies have indicated that the interaction can be additive and that water stress does not necessarily protect against the O_3 effect (Alonso *et al.*, 2008). A field assay on this interactive effect has already been performed through the SUSCAP project
- Experimentally study the combination of all 3 factors: O_3, N and water availability, to approach the combined factors under which Mediterranean wheat grows.
- Continue developing the use of C and N isotopes to understand the response mechanisms of ozone in interaction with other factors. Samples from the O3 x water stress assay are already under analysis.
- Delve into the antioxidant response mechanisms of Mediterranean wheat. As wheat seems to invest a part of additional N in defense, future assays could provide further insight into the antioxidant behavior of Mediterranean wheat cultivars, which is

particularly important for Modern cultivars (Fatima et al., 2018) and could explain some of the reductions in NUE.

- Advance new knowledge about the influence of poor air quality on other common wheat diseases, such as Septoria Tritici Blotch (caused by *Septoria tritici*), or Stem rust (caused by *Puccinia graminis)*, including their relationship with agricultural management. Following the methodology of Chapter 4, the evolution of common wheat diseases that spontaneously arise in the OTC trials is currently being monitored.
- Continue optimizing risk analyses for Mediterranean wheat, considering the joint analysis of experimental databases of local varieties, and including the modulation of fertilization and water availability.

CONCLUSIONS



Conclusions

Ozone-sensitivity of Mediterranean wheat genotypes

- Modern commercial wheat varieties (CVs) exhibit a high sensitivity to ozone (O_3) exposure, leading to significant negative impacts on yield and growth parameters. Meanwhile, traditional landraces display a tolerant behavior under increasing O_3 levels. This suggests that genetic diversity could be key in breeding programs aimed at improving O_3 tolerance.

Carbon and Nitrogen isotopic signatures

- $\delta^{13}C$ values in grain correlate strongly both with yield factors and stomatal conductance and present a homogeneous response within each age-type group, nitrogen fertilization regime, and experimental year, making it a strong indicator for the chronic effects of O_3 exposure. This indicator could also be crucial in assessing accumulated O_3 stress and tolerance.
- The response of $\delta^{15}N$ both in leaf and grain showed alterations in nitrogen redistribution and volatilization due to O_3 stress. Better insights are gained when the analyses of $\delta^{15}N$ are coupled with other factors when assessing nitrogen absorption, metabolism, and redistribution.

Effect of pre-anthesis Ozone-exposure

- The importance of the O_3 dose absorbed during the pre-anthesis stages is shown, in contrast to previous studies that emphasize the importance of post-anthesis O_3 exposure. This may indicate unique O_3 exposure patterns and wheat responses under the particular climatic conditions of the Mediterranean, warranting further research.

N-fertilization modulation of the Ozone-response

- Increased nitrogen (N) fertilization generally boosts growth and yield parameters in wheat. However, elevated O_3 levels counteract these benefits limiting N fertilization efficiency and causing more intense effects under high N supplementation.
- Nitrogen fertilization significantly affects O_3 response functions making the slopes steeper, and reducing the CLs derived from them, highlighting the importance of considering N management in O_3 risk assessment methodologies, as it could influence environmental policy decisions.
- An early O_3 exposure to wheat boosted photosynthetic efficiency in preparation for further damage, as shown by resistance to pigment loss, an increase in the number of mesophyll cells, and maintenance of photosynthetic rate. However, this effect did not perdure later into the plant life. This should be considered in breeding programs as it may indicate an increased resistance mechanism in Mediterranean wheat
- The leaf N content that increases in the flag leaf due to additional N does not go towards carbon fixation processes but rather to defense and early protection against ozone.

Ozone and Yellow Rust

- Low O_3 levels mimicking pre-industrial background significantly increase the progression of yellow rust (YR) (*Puccinia striiformis*) in wheat. This reduction was observed regardless of fertilization management.
- Regarding the O_3 and YR combination, maximum wheat production is achieved under current ozone levels, which causes moderate yield losses in O-sensitive wheat but limits the YR infection. The beneficial effects of O_3 on disease control presented a threshold above which the beneficial effects began to reverse. The debate is opened on the implications of returning to pre-industrial ozone levels, as pursued by environmental policies.

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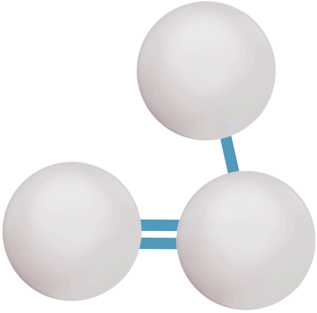

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