



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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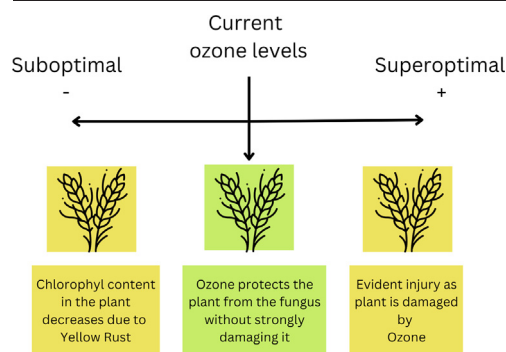
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HIGHLIGHTS

- Pre-industrial O₃ levels favor yellow rust infection in modern Mediterranean wheat.
- Current O₃-polluted Mediterranean atmospheres mitigate wheat rust damage by 22 %.
- Projected O₃-levels induced early senescence decreasing the rust control benefit.
- Nitrogen fertilization increases rust incidence but did not interact with O₃.
- Achieving air quality standards might require improvements in pathogen tolerance.

GRAPHICAL ABSTRACT



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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 O₃ 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 O₃ levels and N-fertilization on spontaneous fungal outbreaks in wheat. Four O₃-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 O₃ treatments; foliar damage, pigment content and gas exchange parameters were measured. Pre-industrial natural background O₃ levels strongly favored the yellow rust infection, where the O₃-polluted levels currently observed at the farm highly benefited the crop, mitigating the presence of rust by 22 %. However, future expected high O₃-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 O₃ exposure. Nitrogen promoted the rust infection by up to 49.5 % without interacting with the O₃-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 O₃-pollution.

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1. Introduction

Due to its high oxidative capacity, high ozone (O₃) ambient levels strongly affect agrosystems (Harmens et al., 2018; Wilkinson et al., 2012). In fact, O₃ 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 O₃-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 O₃-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 O₃ eliminates or highly limits fungal growth and spores formation (Tzortzakis et al., 2008), the effect of O₃ 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; Saroei 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 O₃ levels on fungal infection development throughout the crop-growing cycle are much less known. In this case, O₃ 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 O₃ 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 O₃ 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 O₃ level. Post-exposure wheat inoculations with the necrotrophic gender *Septoria* (*Parastagonospora*) or *Bipolaris* revealed a faster fungal lesion growth on previously O₃-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* spp 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 O₃ 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 O₃ levels. In the earliest studies following this concomitant approach, wheat infected with stem rust (*Puccinia graminis*) was less injured by the infection when peak O₃-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 O₃ 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 O₃ and fungal diseases.

Tiedemann et al. (1991) followed powdery mildew outbreaks caused by *Erysiphe graminis* on wheat during the development of an O₃-exposure assay for 31 days (from seedling until ear emergence) in open-top chambers (OTC) under six different O₃ 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 O₃ compared to control plants. Meanwhile, the opposite response, the reduction of fungal infections due to chronic O₃ 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 *Apiognomonina* gender (Olbrich et al., 2010). However, other studies (e.g. Pflieger et al. (1999)), where wheat was grown in OTC at different densities, found no interaction between O₃ 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 O₃. 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 O₃-response, although the observed enhancement or limitation of N on O₃-induced effects depends on the species, the parameters considered and both the O₃ exposure and N fertilization levels (Sanz et al., 2005, 2015; Wyness et al., 2011). Wheat studies considering the combination of N and O₃ factors have focused on how O₃ 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 O₃. Nonetheless, very few studies have delved into the combined N and O₃ 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 O₃-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, O₃ 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 O₃-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, O₃ exposure (concentration and duration), or N level. Moreover, expanding the knowledge of the complex O₃ 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 O₃-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, O₃ 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 O₃ 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 Higuera”-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 O₃ treatments with 3 repetitions (3 OTC per O₃ treatment) were considered in a complete randomly block design: Filtered Air (FA), reproducing pre-industrial O₃ levels; Non-Filtered Air (NFA), reproducing the ambient O₃ levels at the farm; Non-Filtered air + 20 nL L⁻¹ of added O₃ (NFA+) and Non-Filtered Air + 40 nL L⁻¹ of added O₃ (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 O₃ generator (A2Z Ozone Systems Inc., Louisville, KY, USA) system fed with pure O₂, and diverted to the OTCs through electronic valves to achieve the programmed O₃ 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 (NO₂ and NO; ML9841, 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 O₃-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 O₃ 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 O₃ exposure in the period between the beginning of O₃-fumigation and the YR quantification among the treatments, two O₃ 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 O₃ 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 O₃ 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 O₃ 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 O₃ 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 O₃ 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 O₃ 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-Díaz 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) O₃ 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 O₃ 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 O₃ 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 O₃, 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 O₃ levels on YR can be seen as highly beneficial for later production.

These observed positive effects of O₃ on controlling YR differ from previous assays where a powdery mildew outbreak caused by *Erysiphe graminis* in wheat increased significantly due to all O₃ 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 O₃-enhanced even on undamaged leaves, although O₃ lesions were the starting point of the *Septoria* infection. However, when O₃ 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 O₃ values of 160 µg m⁻³ (80 nL L⁻¹), but significantly decreased at 240 µg m⁻³ (120 nL L⁻¹) although in the case of *Erysiphe* this response also depended on wheat stage and the infection was only limited by O₃ when the inoculations were induced at the younger stages.

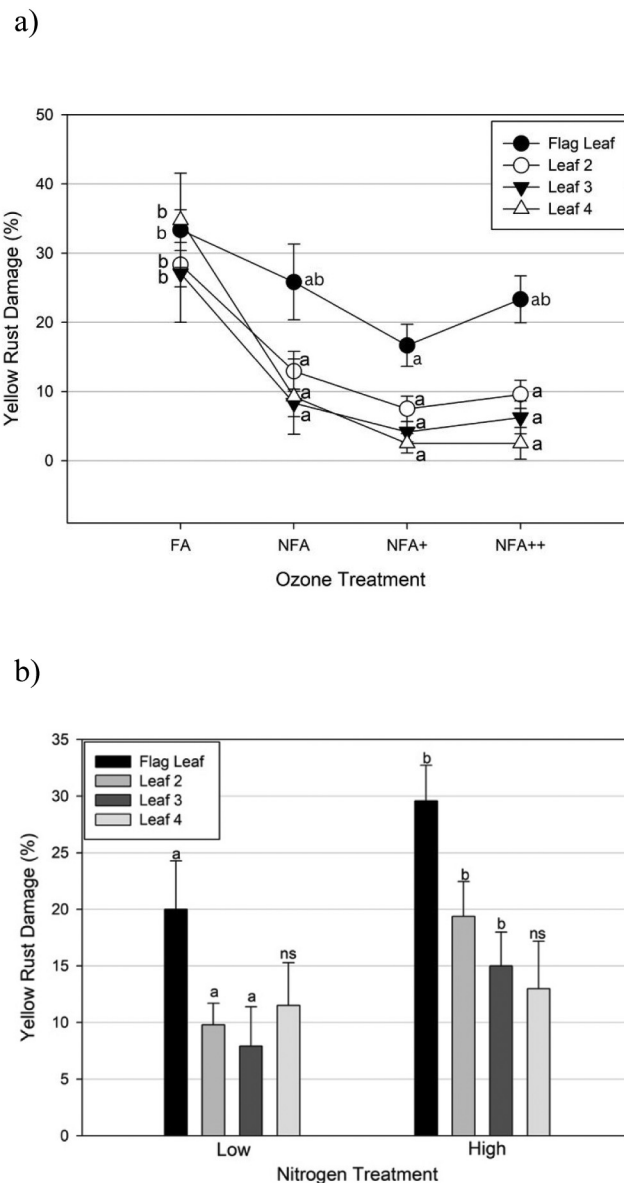


Fig. 1. YR leaf damage (means \pm SE) on leaves of different ages for the O₃ (1a) and N treatments (1b) on June 1 (38 DaE). FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 nL L⁻¹ of O₃) and NFA++ (non-filtered air + 40 nL L⁻¹ of O₃); Low N = 100 kg N ha⁻¹, High N = 200 kg N ha⁻¹. Significant differences among O₃ levels (across N treatment) and significant differences between N levels (across O₃ treatments) ($p < 0.05$) are shown with different letters: p -values for O₃ 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 striiformis* 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 O₃ values reached 60 nL L⁻¹ compared with the filtered treatment which ranged around 20 nL L⁻¹. The O₃ levels in the Artur Nick assay for the 38-day period between the beginning of O₃-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⁻¹) and NFA ++ (non-filtered air + 40 nL L⁻¹). 7-h mean (nL L⁻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⁻¹h. Thus, the control of *Puccinia* by O₃ 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 O₃ 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 O₃-level exposures on the plant should be considered.

3.3. Defense mechanisms

There are different mechanisms by which O₃ can elicit plant reactions comparable to the ones generated by fungal pathogens, which may result in improved resistance to the pathogen as O₃ 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 anti-oxidative cellular system to block the oxidative burst caused by O₃ 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 O₃-induced pathogen resistance. For wheat, several studies showed that ROS-related mechanisms can be highly important in this protection. The O₃-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 O₃ in the earlier phenological stages could grant some defense against pathogens, as the continually accumulating ROS caused by O₃ 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 O₃ 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 O₃ exposure, the NFA ++ treatment (7 h-mean of 87.8 nL L⁻¹ and AOT40 index of 11,762 nL L⁻¹ h.). Thus, O₃ 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 O₃ 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 O₃ threshold for limiting crop diseases. A study by Khan and Khan (1999) revealed that under O₃ 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 O₃ range for fungal crop disease control during crop growth cannot be generalized, and needs to be specifically studied.

The foliar O₃ damage on CV Artur Nick after the exposure period augmented progressively with the increasing pollutant levels, thus healthy leaf surfaces decreased with the progressive O₃ 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 O₃ 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 O₃ that affects fungal infection, but fungal pathogens can also sensitize wheat to the pollutant, and in those assays O₃-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 O₃ symptoms had opposite trends (Fig. 2a; Fig. 2S), where the plant presented more rust symptoms under FA and more O₃ symptoms under NFA ++. Thus, leaves with more damage due to the fungal disease did not show more O₃ 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 O₃ symptoms. The O₃ 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 O₃ 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 O₃ damage, presented a significant chlorosis gradation with increasing O₃ 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 O₃ 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 O₃ damage under NFA + and NFA ++, where senescent leaves caused by the O₃ impact did not boost the progress of the infection. NFA produced plants with the highest foliar pigment content, where its medium O₃ 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 O₃ 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 O₃ 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

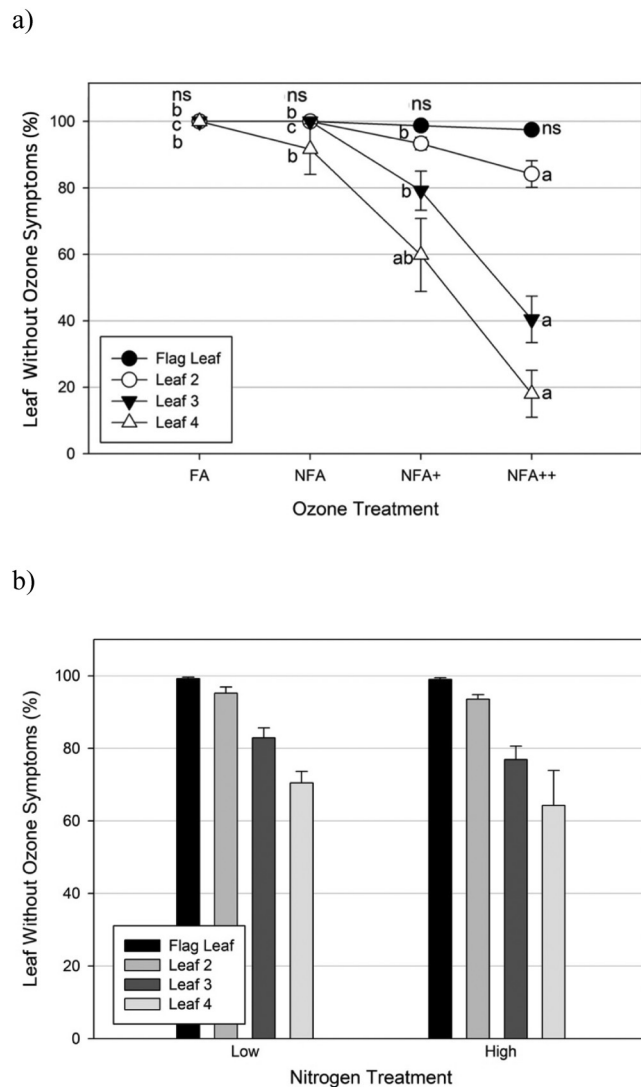


Fig. 2. Foliar O_3 damage on leaves of different ages expressed as a percentage of healthy leaf surface (means \pm SE) for the O_3 (2a) and N treatments (2b) on June 1st (DaE 39). FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 $nL L^{-1}$) and NFA++ (non-filtered air + 40 $nL L^{-1}$); 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.

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 striiformis*; 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

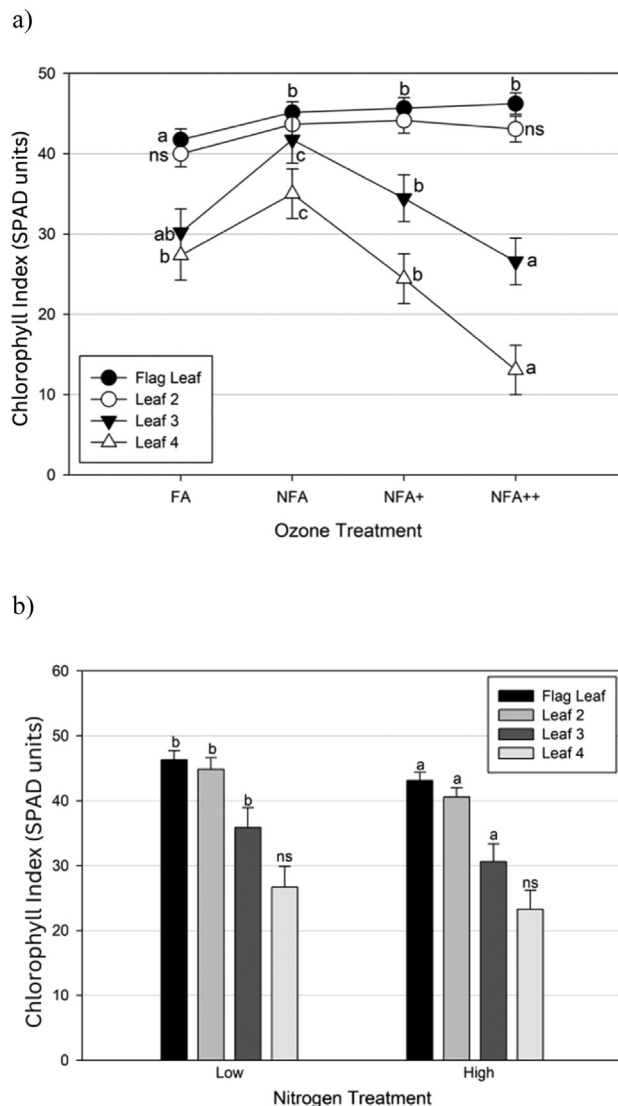


Fig. 3. Chlorophyll Index (SPAD units) (means \pm SE) for the O_3 (3a) and N (3b) treatments on May 30–31. FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 $nL L^{-1}$) and NFA++ (non-filtered air + 40 $nL L^{-1}$); Low N = 100 $kg N ha^{-1}$, High N = 200 $kg N ha^{-1}$. Significant 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.

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.

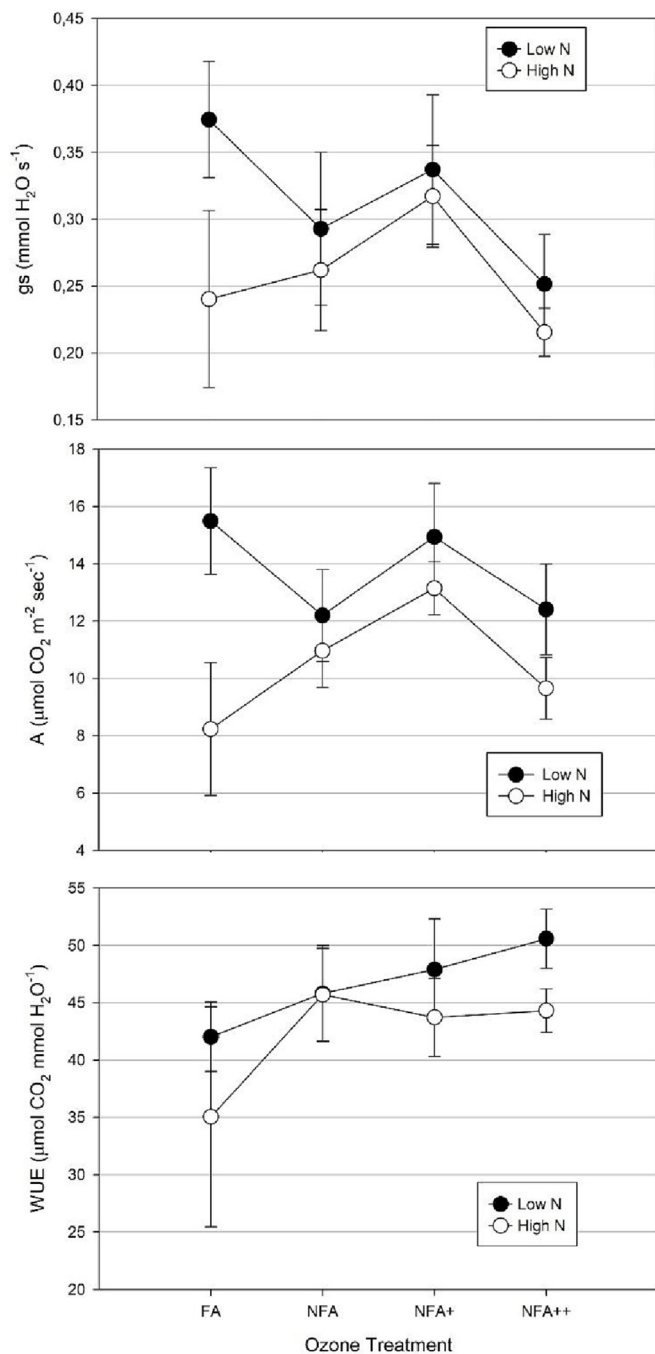


Fig. 4. Gas exchange parameters, stomatal conductance (g_s), photosynthetic activity (A) and water use efficiency (WUE) (means \pm SE), for the different O₃ and N treatments on June 1. FA (filtered air), NFA (non-filtered air), NFA+ (non-filtered air + 20 nL L⁻¹) and NFA++ (non-filtered air + 40 nL L⁻¹). 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 O₃ 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, g_s 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 O₃ 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 O₃-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, O₃-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 O₃ exposure, wheat physiological activity reached its lowest values. Thus, both rust and O₃ play important roles in affecting the photosynthetic activity of the plant, but without interacting; because at low O₃ levels the rust determines plant health and activity, while at high O₃ 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), O₃ levels should not surpass the NFA+ and NFA++ levels, but current O₃ 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 O₃ 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 O₃ 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 O₃ effect seen in the main stem measured on the same dates, a significant O₃-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 O₃ response. Decreasing foliar chlorophyll is a usual response to the pollutant, as it relates to O₃-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 O₃-susceptible stage for wheat (Plejdel et al., 1998).

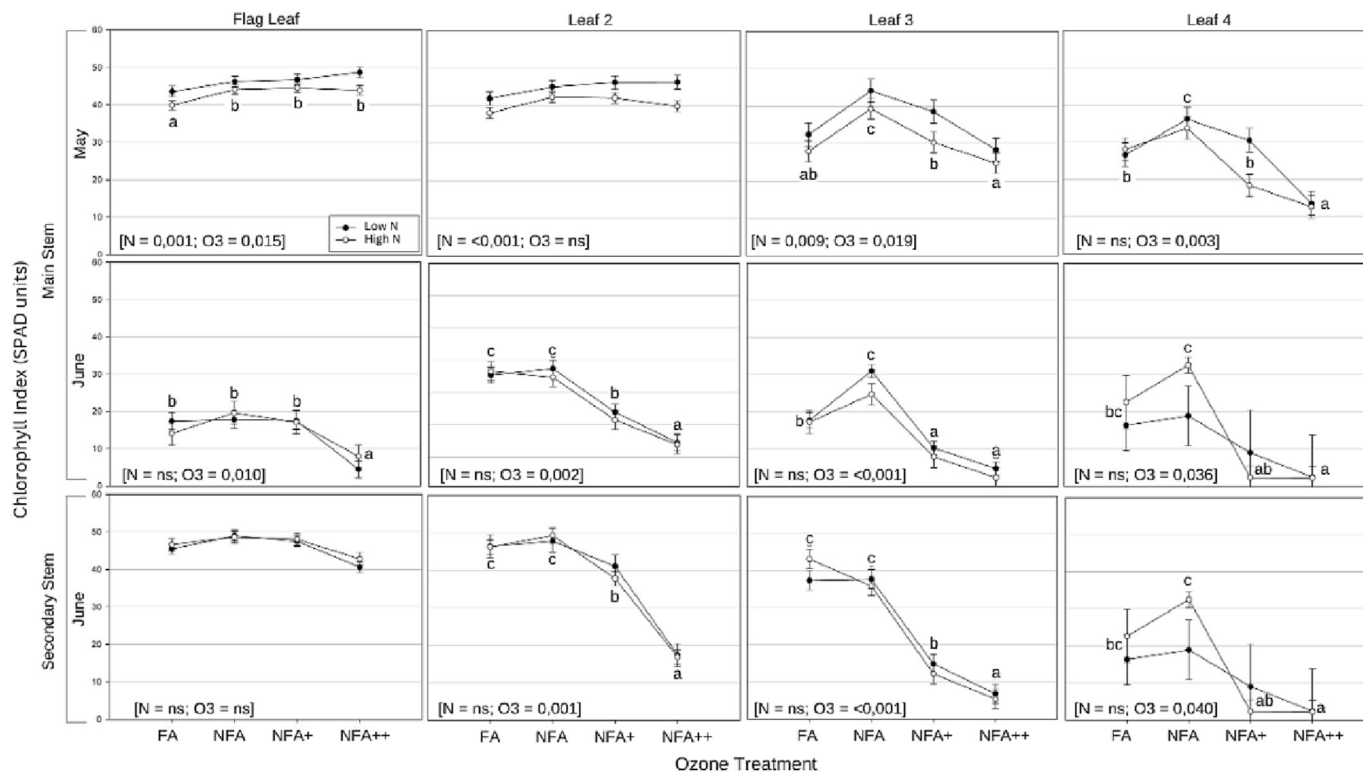


Fig. 5. Chlorophyll Index (SPAD units) (means \pm SE) for the different leaves under O_3 and N treatments on May 30–31 (main stem) and June 12–13 (main and secondary stems). FA (filtered air), NFA (non-filtered air), NFA + (non-filtered air + 20 nL L⁻¹) and NFA ++ (non-filtered air + 40 nL L⁻¹); N-Low = 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 nL L⁻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 -sensitivity 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.

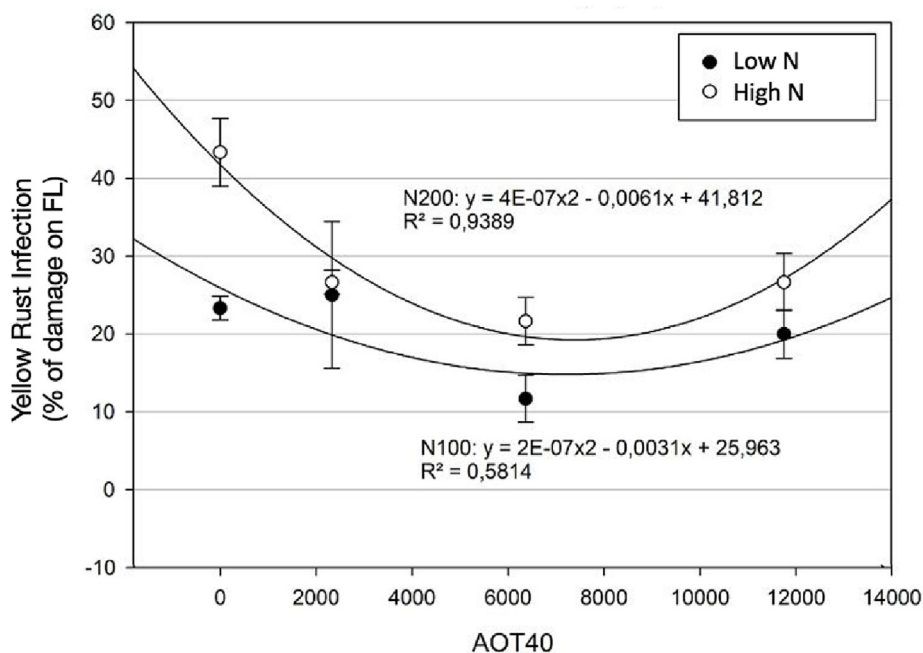


Fig. 6. Response function for YR outbreak based on 1 month-AOT40 index previous to the infection, expressed as a percentage of the affected section of the flag leaf (FL) (means \pm SE) for the different nitrogen treatments: N-Low = added 100 kg N ha⁻¹; N-high = added 200 kg N ha⁻¹.

4. Conclusions

Preindustrial background O₃ 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 O₃ 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, O₃ levels above ambient (NFA + +) switched the O₃ effect in the opposite direction and rebounded the fungal disease. Results of the present assay showed an optimal O₃ 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 O₃ damage, not even under NFA + +, due to its short exposure time, although effects were observed at the physiological scale (A and g_s reduction). Ozone damage caused progressive foliar pigment loss. Nitrogen did not affect O₃ foliar damage. Nonetheless, N and O₃ did not interact in their effects on YR or O₃ 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 O₃ exposure, no N-modulation was found for the YR response due to O₃. However, more experimental effort is needed to generalize these findings.

Despite the undeniable detrimental effect of O₃ 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 O₃.

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.

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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. O₃ and N effect on YR damage and O₃ damage; Table 2S. O₃ and N effect on foliar pigment content; Fig. 1S. Meteorological conditions during the exposure period and Fig. 2S. Yellow rust and O₃ damage relative to total leaf area (%) for the different O₃ 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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