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Ground level ozone and wheat: an exploration of effects on yield, interactions with nitrogen, and potential sources of sensitivity and tolerance

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A thesis submitted for the degree of Doctor of Philosophy

May 2023





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Natural Environment Research Council



UK Centre for Ecology & Hydrology





Declaration

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Rwy'n cadarnhau fy mod yn cyflwyno'r gwaith hwn gyda chytundeb fy Ngoruchwyliwr (Goruchwylwyr)

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

I confirm that I am submitting this work with the agreement of my supervisors.

Signed: Clare Brewster

Date: 25th May 2023

At the time of thesis submission the following research paper had been produced from this body of work and published in a peer-reviewed journal:

Brewster, C., Hayes, F., Fenner, N., (2019). Ozone tolerance found in *Aegilops tauschii* and Primary Synthetic Hexaploid Wheat. Plants, 8, 195, 1 – 12. doi: 10.3390/plants8070195

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Summary

Efforts to maximise wheat yields are being compromised by phytotoxic levels of ground-level ozone which persist in many wheat-growing areas of the world. This research investigated inter-related questions regarding ozone's effect on wheat growth, grain yield and quality, and effects on senescence and interactions with nitrogen, particularly during the critical reproductive growth stages. Throughout the research the key issue of wheat's ozone sensitivity and the potential for breeding more ozone tolerant lines was explored.

During summer plant trials in 2018, 2019, and 2021 several UK spring wheat cultivars and synthetic wheat lines, along with wheat's closest wild relatives, were grown in 6 L pots of soil, and exposed to a realistic range of ozone concentrations, from 30 ppb to 110 ppb, in unheated solardomes in North Wales (UK) over a prolonged (10.5 - 11.5 week) period, under normal nitrogen fertilisation regimes.

In Trial 1 it was found that, of the three closest wild relative genome donors which created hexaploid wheat (*Triticum aestivum* L., AABBDD), *T. urartu* (AA) and *T. dicoccoides* (AABB) were more ozone sensitive and a potential genetic source of wheat's ozone sensitivity, whilst *Aegilops tauschii* (DD) was ozone tolerant, adding to evidence of its useful abiotic stress tolerance properties. In Trial 1 and 2, whilst one line of primary Synthetic Hexaploid Wheat (SHW) was found to be ozone tolerant, another was ozone sensitive, but an F2 line derived from SHW and Paragon had both ozone tolerance and larger grain size. Of the cultivars grown across all three plant trials (cv. Maris Dove (1971), cv. Paragon (1999), and cv. Skyfall (2014)), the more recent the cultivar the more ozone sensitive it appeared to be.

In Trial 2, X-ray microcomputed tomography (μ CT) digital imaging enabled the 3D visualisation of ozoneaffected wheat spikes for the first time, revealing that reductions in grain number were occurring across the middle of the spike, whilst reductions in grain volume were being driven more by reduced width and depth than length.

In Trial 3, ozone triggered earlier visible senescence in all four leaf cohorts (4th, 3rd, 2nd, and flag) of cv. Skyfall, preceded by reduced leaf chlorophyll, particularly in the lower, older leaves, and especially during anthesis/post-anthesis growth stages, with implications for floret fertility and grain fill. Ozone reduced Nitrogen Remobilisation Efficiency between anthesis and harvest, and increased levels of residual nitrogen found at harvest in 'source' plant parts. This increase in residual shoot nitrogen was found in cultivars in all three plant trials despite large variations in each trial's grain yield ('sink'). Measurements of soil nitrate also indicated that ozone can sometimes increase the potential for nitrate leaching from agri-ecosystems. A ¹⁵N trace experiment, with an additional 20 kg ha⁻¹ of nitrogen fertiliser applied at anthesis, revealed that ozone did not affect the uptake of post-anthesis nitrogen, although this extra nitrogen did appear to ameliorate the effect of ozone on other parameters.

Findings from all three plant trials can contribute towards the breeding of ozone tolerant wheat. This research has added to evidence suggesting the more recent the release date of the elite cultivars, the more ozone sensitive they have become, but also identified potential sources of tolerance within one of the main genome donors (DD) and a line of synthetic wheat (BC1). The data relating to the impact of ozone on senescence, leaf chlorophyll, reduced nitrogen remobilisation and increased residual foliar nitrogen, can contribute towards improvements in modelling. The ameliorating effect of the additional nitrogen fertilisation at anthesis highlights the need to ensure that rates of nitrogen fertilisation, and the timing of applications, are taken fully into account in ozone research and modelling.

Contents

Declaration	v
Acknowledgements	vi
Summary	vii
Contents	ix
List of tables	xiv
List of figures	xv
List of acronyms, abbreviations and units of measurement	xxi

1.0 Aims, thesis structure and introduction1 Tropospheric ozone4 1.3.1 1.3.2 1.3.3 Ozone uptake, critical levels and ozone metrics6 1.3.4 Ozone, wheat and food security7 How and when does ozone affect wheat yields?9 1.3.5 1.3.6 Wheat cultivars, wild relatives and synthetic wheat10 Effect of ozone on nitrogen in agro-ecosystems11 1.3.7 1.3.8 Scientific approaches to ozone impact studies11 1.3.9 1.4 Supplementary materials15

2.0 Ozone tolerance found in Aegilops tauschii and Primary Synthetic Hexaploid wheat	17
Abstract	18
2.1 Introduction	
2.1.1 Ozone and wheat	19
2.1.2 Differences in ozone sensitivity and tolerance	19
2.1.3 Role of wild relatives and synthetic wheat in wheat breeding	20
2.1.4 Aims of the study	21
2.2 Materials and methods	22
2.2.1 Plant establishment	22
2.2.2 Ozone treatment	23
2.2.3 Biomass and yield measurements	24
2.2.4 Statistical methodology	24

2.3 Results	25
2.3.1 Shoot biomass	25
2.3.2 Total seedhead biomass	25
2.3.3 1000 grain weight	26
2.3.4 Differences in ozone sensitivity	27
2.4 Discussion	28
2.4.1 Ozone tolerance in the DD genome	28
2.4.2 Ozone tolerance in synthetic wheat	28
2.4.3 Possible reasons for ozone sensitivity in the AA and AABB genomes	29
2.4.4 Differences in ozone sensitivity of the cultivars	30
2.5 Conclusion	30
Supplementary materials	31
3.0 μCT imaging of intact wheat spikes reveals effect of ozone on grain yield components	33
Abstract	34
3.1 Introduction	35
3.1.1 Ground level ozone	35
3.1.2 Ozone's effect on grain	35
3.1.3 Global wheat yields	36
3.1.4 Synthetic wheat	36
3.1.5 Use of μCT scans	36
3.1.6 Aims of the study	37
3.2 Materials and methods	38
3.2.1 Plant establishment	38
3.2.2 Ozone treatment	39
3.2.3 Chlorophyll Index	40
3.2.4 Biomass and yield measurements	40
3.2.5 Grain protein and starch	40
3.2.6 X-ray microcomputed tomography (μCT) imaging	40
3.2.7 Statistical tests	41
3.3 Results	42
3.3.1 Ozone reduces spike number, total grain number and TGW	42
3.3.2 Ozone reduces the number of grains per spikelet	43
3.3.3 Ozone changes grain morphology	45
3.3.4 Ozone increases grain protein and reduces grain starch	47
3.3.5 Ozone reduces chlorophyll levels	48

3.3.6 Ozone reduces shoot, root and seedhead biomass	49
3.4 Discussion	50
3.4.1 Effects on grain number	51
3.4.2 Effects on grain size and quality	51
3.4.3 Potential use of synthetic wheat and μCT digital imaging for plant breeding	53
3.5 Conclusion	53
Supplementary materials	54

4.0 Ozone reduces wheat canopy chlorophyll and Nitrogen Remobilisation Efficiency,	and increases
residual foliar nitrogen, with effects ameliorated by extra nitrogen fertilization	65
Abstract	66
4.1 Introduction	67
4.1.1 Ozone, wheat and nitrogen	67
4.1.2 Senescence and nitrogen partitioning	67
4.1.3 Interactions between ozone, senescence and the C:N balance	70
4.1.4 Aims of the study	70
4.2 Materials and methods	72
4.2.1 Plant establishment	72
4.2.2 Additional nitrogen fertilization, ¹⁵ N tracer experiment, and ozone exposures	72
4.2.3 Senescence and chlorophyll indices	74
4.2.4 Measurements at anthesis and post-harvest	74
4.2.5 C:N and ¹⁵ N anlaysis	74
4.2.6 Nitrogen Remobilisation Efficiency (NRE)	74
4.2.7 15 N trace experiment – calculation of 15 N% recovery	75
4.2.8 Statistical analysis	75
4.3 Results	77
4.3.1 Senescence index	77
4.3.2 Chlorophyll Index	
4.3.3 Nitrogen percentage (N%) in plant parts	81
4.3.4 Carbon percentage (C%) in plant parts	83
4.3.5 C:N ratio in plant parts	84
4.3.6 Total nitrogen in plant parts	86
4.3.7 Nitrogen remobilization efficiency (NRE)	
4.3.8 ¹⁵ N percentage recovery in plant parts	
4.3.9 Root:shoot ratio	90
4.3.10 Grain yield	90

4.4 Discussion	91
4.4.1 Ozone causes earlier chlorophyll reduction and senescence	91
4.4.2 Ozone reduces chlorophyll levels in lower leaf cohorts, especially during grain fill	
4.4.3 Lesser impact on flag leaf compared to lower canopy	92
4.4.4 Senescence is slower in ear/awns during grain fill	93
4.4.5 Ozone effects on root differ with growth stage	
4.4.6 Less effect of ozone on leaf carbon	
4.4.7 C:N partitioning at anthesis and harvest	94
4.4.8 Why is this increase in N% levels occurring?	95
4.4.9 Extra nitrogen fertilization ameliorated effect of ozone	96
4.4.10 ¹⁵ N trace experiment	97
4.5 Conclusion	97
Supplementary materials	
nitrate leaching to groundwater	
Abstract	
5.1 Introduction	
5.1.1 Nitrogen fertilization and nitrogen pollution	
5.1.2 Effects of ozone on residual nitrogen in shoot	101
5.1.3 Effects of ozone on residual soil nitrate	
5.1.4 Aims of the study	102
5.2 Materials and methods	
5.2.1 Plant establishment and ozone treatments	
5.2.2 Total grain weight and grain quality	
5.2.3 C:N ratio and percentage nitrogen	
5.2.4 Nitrate extracted from soil water	
5.2.5 Statistical analysis	104
5.3 Results	105
5.3.1 Total grain weight	105
5.3.2 Grain protein and starch	105
5.3.3 Residual nitrogen in leaf and stem at harvest	
5.3.4 C:N ratios in leaf and stem at harvest	107
5.3.5 Residual nitrogen in soil water	
5.4 Discussion	
5.4.1 Grain yields and residual nitrogen	111

5.4.2 Nitrogen fertilization regimes	
5.4.3 Genotypic variation in sensitivity	112
5.4.4 Additional contributory factors	112
5.5 Conclusion	
Supplementary materials	

6 O Discussion 115
6.1 Discussion of findings in relation to over-arching research questions116
6.2 Effect of ozone and interactions with nitrogen before and at anthesis117
6.2.1 Effects on grain number117
6.2.2 Further research related to ozone and grain number119
6.3 Effect of ozone and interactions with nitrogen after anthesis119
6.3.1 Effects on grain size and grain quality119
6.3.2 Effects on nitrogen translocation121
6.3.3 Effects on residual nitrogen belowground121
6.3.4 Effects on residual nitrogen aboveground122
6.3.5 Additional nitrogen fertilization ameliorates effect of ozone
6.4 What are the possible origins of wheat's ozone sensitivity, and how can wheat's ozone tolerance be
improved?
6.4.1 Genetic origins of wheat's ozone sensitivity123
6.4.2 Can synthetic wheat contribute to yield gains and ozone tolerance?
6.4.3 Elite cultivars, their release date, and factors contributing to ozone tolerance in wheat
6.4.4 Breeding wheat for abiotic stress tolerance125
References

List of Tables

Chapter 1	Chapter	1
-----------	---------	---

Table 1.1. Summary of soil mediums, fertilisation regimes, and ozone exposures for Trials 1, 2, and 314
Table S1.1. Wheat growth stages – decimal code system15
Chapter 2
Table 2.1. Wheat and wild relative species with details of type and source of seed
Table S2.1. Table of p values representing levels of statistical significance of the effect of ozone on each
species and between the different levels of ozone treatment
Chapter 3
Table 3.1. Wheat lines, with details of the 11 lines and origin of the seed
Table S3.1 Applications of ammonium nitrate fertiliser: date, quantity, and growth stage of line: Booting
(GS41-47), Ear emergence (GS51-59), Flowering/Anthesis (GS61-69), Milk development (GS71-77).
Relevant lines in bold
Table S3.2. Timing of fungicide and insecticide treatments applied to maintain plant health, and applied to
all plants in all ozone treatments on each occasion. Extent of greenfly and powdery mildew recorded on 9
July (See Fig. S3.4)
Table S3.3. Difference in total grain number counts between manual counts using 3D images and μ CT
extracted data
Table S3.4. Grain morphology and grain quality parameter p values, representing levels of statistical
significance of the effect of ozone on each line and between the different levels of ozone treatment56
Table S3.5. Chlorophyll index parameter p values, representing levels of statistical significance between
ozone treatment for each line, at pre-anthesis, anthesis, and post-anthesis growth stages
Table S3.6. Grain morphology and grain number percentage differences between the control (30 ppb) and
each elevated ozone concentration for each line59
Chapter 4

Chapter 5
Table S4.2. Parameters which were log transformed in each set and plant part
Table S4.1. Applications of insecticide and fungicide, with date, growth stage and application rate98
narvest (GS93) under low (30 ppb) and very high (85 ppb) ozone treatments
Table 4.3. Root:shoot ratios of NØX plants at anthesis (GS65), and N+ plants, NØ plants and 15N+ plants at
he difference between the low and very high treatment levels88
n low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatment levels, along with
<i>Table 4.2.</i> N remobilisation efficiency between NØX plants at anthesis and both N+ and NØ plants at harvest
NOX, N0, N+ and 15N+) along with growth stage at harvest73
Table 4.1. Summary of amount and timing (growth stage) of nitrogen fertilisation for each subset of plants

Table 5.1. Summary of plant trial growing conditions and ozone treatments in Trials 1, 2 and 3......103

List of Figures

Chapter 1

Fig. 1.3. Estimated production loss for wheat due to ozone, modelled from the POD₃IAM and percentage yield losses and averaged for 2010–2012. Figure and legend reproduced from Mills, Sharps, et al., 20188

Chapter 2

 Fig. S2.1. Daily/weekly ozone concentrations (ppb) achieved over the course of the treatment period (7 June

 - 21 August)
 31

Chapter 3

Fig. S3.4. Rating Index of extent of greenfly and powdery mildew on all lines on 9 July in 30 ppb (a), 55 ppb (b), 80 ppb (c) and 110 ppb (d), where 0 = none, 1 = slight, 2 = moderate, 3 = heavy, 4 = severe......61

Chapter 4

Fig. 4.8. Carbon percentage (C%) in root, 2nd leaf, upper stem, flag leaf, ear and grain in NØX plants at anthesis (A); in N+ plants at harvest (B); in NØ plants at harvest (C), under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n = 6)

Fig. 4.10. C:N ratio in plant parts at harvest in NØ plants under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n = 6)

Chapter 5

Fig. 5.3. Percentage of nitrogen (N%) in plant parts at harvest under low, medium, high and/or very high ozone treatments. Fig. 5.3a. Trial 1: 2nd leaf, upper stem, and flag leaf of Skyfall (n=6); Fig. 5.3b. Trial 2: upper leaves (flag and 2nd leaf combined) and lower leaves (3rd and 4th leaves combined) of Paragon (n=3);*Fig. 5.3c.* Trial 3 (N+ plants): 2nd leaf, upper stem, and flag leaf of Skyfall (n=6), as previously shown in Chapter 4. Error bars show standard error of the mean. See Table 5.1 for ozone treatment levels107

Chapter 6

Fig. 6.1. Graphical representation of wheat's growth stages, the phases of nitrogen uptake and storage, periods when the plant is photosynthesising and senescing; the critical reproductive stages of meiosis, anthesis and grain fill; and the phases when numbers of tillers/ grains and grain size is determined. (Adapted from Sreenivasulu & Schnurbush 2012). See Table S1.1 for a detailed list of wheat's growth stages116

PLEASE NOTE: Supplementary Materials are presented at the end of each chapter

Acronyms and abbreviations

μርΤ	 X-ray micro-computed tomography 	°C	 degrees centigrade
¹⁵ N	- stable isotope of nitrogen	%	- percent
3D	- three-dimensional	μg	- microgram
ANOVA	- Analysis of Variance	μm	- micrometre/micron
AOT40	 accumulated dose of ozone above a threshold of 40 ppb 	cm	- centimetre
С	- carbon	g	- gram - bour
с.	- circa	ha	- hectare
C:N	- ratio of carbon to nitrogen	kg	- kilogram
DNA	- Deoxyribonucleic acid	klux	- 1000 lux
DO₃SE exchan	- Deposition of ozone (O₃) for stomatal ge	L	- litre
HSD	- Honestly Significant Difference	m	- metre
ID	- identification	mg	- milligram
N	- nitrogen	Mg	- Megagram
n	- sample size	ml	- millilitre
NIR	- Near-infrared	mm	- millimetre
NRE	- Nitrogen Remobilisation Efficiency	mmol	- millimole
NUE	- Nitrogen Use Efficiency	nmol	- nanomole
р	- probability value	ppb	- parts per billion
POD	- Phytotoxic Ozone Dose	Tg	- Teragram (1 million tonnes)
рН	- potential of hydrogen	ton	- imperial ton (1,016 kg / 2,240 lbs)
PTFE	- Polytetrafluoroethylene	tonne	- metric tonne (1,000 kg /2,204 lbs)
PWM	- Pulse Width Modulation		

- R² coefficient of determination
- *Rht* Reduced height gene
- RNA Ribonucleic acid
- ROS Reactive Oxygen Species
- SPAD Soil Plant Analysis Development
- TGW Thousand Grain Weight

Units of measurement

1.1 Aims and objectives of the thesis

Overarching research questions

This research aimed to assess several inter-related issues concerning the effects of ozone on wheat:

- Why and when does ozone reduce wheat yields?
- How does ozone affect and interact with nitrogen, before and after anthesis?
- What are the possible origins of wheat's ozone sensitivity?
- How might these factors be interacting to affect modern elite wheat cultivars?
- How can wheat's ozone tolerance be improved?

The research was undertaken using measurements of multiple plant physiological parameters during, and at the conclusion of, three large scale ozone exposure experiments involving elite cultivars of different release dates, the main wild relative genome donors of bread wheat, and multiple lines of synthetic wheat derived from wild relatives and elite cultivars.

The objectives were:

- To assess the relative ozone sensitivity/tolerance of: the main genome donors of bread wheat, three elite cultivars with different release dates, and multiple lines of synthetic wheat (Chapters 2, 3, & 4);
- To measure the impact of ozone on grain formation and morphology using μCT image analysis, and assess the implications of findings in relation to the timing of the effect of ozone on wheat grain yields, and grain quality (Chapter 3);
- To assess ozone's effect on the timing of senescence, and levels of leaf chlorophyll, in all four leaf cohorts of an elite cultivar throughout all key growth stages (Table S1.1, p.15) and to assess the implications of these findings in relation to the timing of ozone's effect on grain number and grain size (Chapter 4);
- To measure ozone's effect on C:N stoichiometry in plant parts at anthesis and at harvest, in order to assess whether ozone affects the uptake, storage and remobilisation of nitrogen (Chapter 4);
- To assess whether ozone affects post-anthesis nitrogen uptake, applied as a late fertiliser addition, and to assess whether this then alters the effect of ozone during grain fill (Chapter 4);
- To assess the effect of ozone on residual nitrogen in shoots, and the relationship between grain yield and residual nitrogen (Chapters 4 & 5);
- To assess the effect of ozone on residual nitrogen in soil water in the form of nitrate (Chapter 5).

1.2 Structure of the thesis

Chapter 1 – Introduction

Contextual information clarifying the reasons for, and purpose of, this research, and setting out the aims, objectives and structure of the PhD thesis.

Chapter 2 - Trial 1 / Paper 1 (published in plants, 2019)

An assessment of the effect of ozone on the genome donors of bread wheat, two cultivars with different release dates (cv. Maris Dove and cv. Skyfall), and a line of primary synthetic wheat, with assessments of relative ozone sensitivity/tolerance based on yield data.

Chapter 3 – Trial 2 / (manuscript in preparation)

An assessment of the impact of ozone on grain formation, grain morphology and grain quality of cv. Paragon, one line of primary synthetic wheat, and multiple derived F2 lines using X-ray micro-computed tomography (μ CT) image analysis; assessment of implications relating to the timing of ozone's effect on wheat yields. μ CT imaging and grain morphology data extraction undertaken by staff at Aberystwyth University National Plant Phenomics Centre. Grain quality analysis undertaken by staff at the John Innes Centre, Norwich.

Chapter 4 – Trial 3 / (manuscript in preparation)

An assessment of ozone's effect on nitrogen dynamics of multiple lines of cv. Skyfall, including chlorophyll levels, senescence, C:N stoichiometry in plant parts at anthesis and harvest, and post-anthesis uptake of nitrogen. *C:N analysis of plant parts undertaken by Bangor University technicians;* ¹⁵N *C:N analysis undertaken by staff at UC Davis Stable Isotope Facility, USA. Grain quality analysis undertaken by staff at the John Innes Centre, Norwich.*

Chapter 5 – Trial 1, 2, and 3

An assessment of the relationship between grain yield and residual nitrogen (Data from Trials 1,2,3); the effect of ozone on residual nitrogen belowground in the form of nitrate (Trial 2 data); the effect of different nitrogen fertilisation regimes on the effect of ozone (Trial 3 data).

Chapter 6 – Discussion

A summation of main findings, structured in the context of the over-arching project aims relating to: how and when ozone affects wheat; the effects on nitrogen dynamics; and factors relating to ozone sensitivity/tolerance in wheat and the breeding of cultivars with greater ozone tolerance.

PLEASE NOTE: Supplementary materials are presented at the end of each chapter

1.3 Introduction

1.3.1 Tropospheric ozone

Unlike stratospheric ozone, which protects the Earth's surface from UV radiation, tropospheric or ground level ozone is a secondary air pollutant formed near the Earth's surface by complex reactions between sunlight and specific precursor gases: nitrogen oxides, methane, carbon monoxide and non-methane volatile organic compounds (VOCs)(Royal Society, 2008). The majority of ground level ozone is formed through these photochemical reactions within the troposphere, with a smaller fraction originating from the stratosphere; ~90% is then lost through further photochemical reactions, and the remaining ~10% is deposited to the Earth's surface (Gaudel et al., 2018). It is estimated to have an average lifetime of 22 days (Young et al., 2013).

Sources of ozone vary geographically and seasonally. In the Northern Hemisphere, fossil fuel and industrial emissions have a strong influence on ozone levels, whilst in tropical mid latitudes and in the Southern Hemisphere ozone is generated more by natural emissions of precursor gases such as VOCs from forest areas (Lelieveld and Dentener, 2000). In both the Northern and Southern Hemispheres there are seasonal maximums of ozone in the spring and summer which coincide with higher levels of sunshine and warmer temperatures, although levels are generally low all year in the lowest latitudes in both hemispheres (Williams et al., 2019). Increases in temperature lead to increases in ozone because a rise in temperature generates higher levels of ozone's precursor gases including nitrogen oxides, but also VOCs from both natural and man-made sources (Archibald et al., 2020).

Data from early observations in Europe and North America suggest that, before 1900, naturally occurring levels of ground level ozone were in the range of 5 - 20 ppb (Vingarzan, 2004). However, after 1900 the substantial increase in use of fossil fuels for transport and industry, and use of artificial fertilisers in agriculture, resulted in steadily increasing levels of ozone's precursor gases and therefore of ozone too, throughout the 20th century (Galloway et al., 2008), with this trend continuing in the first two decades of the 21st century (Archibald et al., 2020). Average summer daytime concentrations currently range from 30 ppb or less in northern Europe to more than 100 ppb in parts of western North America, southern Europe, and parts of Asia (Fig. 1.1., Fleming et al., 2018).

Ozone is especially persistent in the highly populated and industrialised northern hemisphere where many precursor pollutant gases originate (Royal Society, 2008), although some pollution abatement policies, such as those in Europe, have started to reduce source emissions and especially the shorter but more harmful 'peak ozone episodes' (Harmens et al., 2018). Both local and global air movement means that urban areas tend to export ozone's precursor gases to rural areas, and regions with improved pollution regulation, e.g. in Europe and North America, are still experiencing increasing levels of ozone due to an ongoing influx of

precursor gases which originate from areas with higher pollution levels e.g. South and East Asia (Lelieveld and Dentener, 2000).

Modelled forecasts of future ground level ozone concentrations show that future ozone concentrations will be dependent upon changes in emissions of both climate and air pollution gases. With climate change leading to increases in average global temperatures, this may also lead to higher rates of ozone generation in future (Archibald et al., 2020). If there are improvements in pollution abatement, ozone levels are predicted to peak and start reducing between now and 2100 in many regions of the world, but these modelled scenarios vary by region, both spatially and temporally, as they are dependent also upon multiple factors including energy demand and population size (Archibald et al., 2020). In some regions the modelled scenarios indicate a potential for an ongoing increase in ozone up to or even beyond 2100, especially in South and East Asia, whilst there are more likely to be declines in regions with more advanced air pollution control policies such as Europe and North America. However, there are also uncertainties in the modelling, especially beyond 2050, relating to different climate change scenarios, levels of precursor gases, and potential alterations to ozone influx from the stratosphere (Archibald et al., 2020).



Fig.1.1. Current (2010 – 2014) ozone concentrations in non-urban areas in parts per billion (ppb), showing the global distribution of ozone and regional variations, where data was available. These data represent 3,348 data points showing average daytime ozone concentrations during the 6-month warm season, as presented in the Tropospheric Ozone Assessment Report on present day distribution and trends. Figure and legend reproduced from Fleming et al. 2018. 10.1525/elementa.273

1.3.2 How does ozone affect plants?

Ozone is an oxidant that enters the plant through its stomata and quickly reacts with organic substrates in and around the apoplast to generate Reactive Oxygen Species (ROS), including superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Khanna-Chopra, 2012). An increase in ROS is a common response to many forms of abiotic stress and has the same consequences (Taiz et al., 2015, p.734): if ROS levels exceed the capacity of the plant to detoxify them then cell membrane dysfunction results in a cascade of damaging reactions, leading ultimately to reduced photosynthesis and carbon assimilation, increased respiration rates, and premature senescence (Ainsworth et al., 2012; Emberson et al., 2018). With resources also diverted for defence and repair functions (Blokhina et al., 2003) this leads to negative effects on plant growth, with root biomass and the root:shoot ratio also often reduced (Grantz et al., 2006). Plants vary in their ability to tolerate high levels of ROS, and in their ability to reduce ROS via antioxidant systems, which largely take the form of enzymes that scavenge ROS, detoxify lipid peroxidation products, or regenerate antioxidants (Blokhina et al., 2003).

1.3.3 Ozone uptake, critical levels and ozone metrics

A critical factor affecting the extent of ozone damage is the actual amount of stomatal uptake of ozone, which occurs when the plants' stomata are open to allow respiration and gas exchange. Stomatal uptake varies with environmental conditions, including soil moisture levels, vapour pressure deficit, temperature and light intensity. Conditions where stomatal opening is increased e.g. when crops are irrigated, make the plants more susceptible to ozone damage (Harmens et al., 2007; Harmens et al., 2019).

Methods to assess and estimate the level of risk to any type of vegetation resulting from exposure to ground level ozone have been developed based on an estimation of the exceedance of 'critical levels'. These critical levels are defined as "the concentration, cumulative exposure or cumulative stomatal flux of atmospheric pollutants above which direct adverse effects on sensitive vegetation may occur according to present knowledge" (CLRTAP, 2017). Critical levels based on ozone concentrations and 'exposure-response' functions were developed in the 1990s using AOT40 values (accumulated ozone exposure derived from mean hourly ozone concentrations during daylight hours which exceed a threshold of 40 ppb) and based on data from European field studies using open top chambers (Grünhage et al., 2012).

However, for the actual toxicological 'dose-response' relationship of an air pollutant to be determined, the absorbed dose, or flux, of the air pollutant into the plant needs to be estimated (Grünhage et al., 2012). This has led to the development of modelled estimates, based on experimental data, of the Phytotoxic Ozone Dose (POD_y), the cumulative flux of ozone via the stomata above a threshold flux of Y (measured in nmol m⁻² s⁻¹ of the projected leaf area)(Pleijel et al., 2007; Mills et al., 2011; Grünhage et al., 2012). Below this level it is assumed that the plant is able to detoxify any absorbed ozone (Mills et al., 2011). This POD_y

methodology, which uses the DO_3SE^1 model to estimate ozone flux at the leaf level in response to environmental conditions and plant physiology (Emberson et al., 2000) has been found to more accurately reflect the effect of ozone on plants in the field compared to the older exposure-response AOT40 methodology (Mills et al., 2011). Based on experimental data relating to stomatal uptake of ozone by the flag leaf during anthesis and grain fill in multiple European wheat lines, POD₆ was found to generate the strongest relationship between the stomatal flux of ozone and a subsequent 5% reduction in yield i.e. the critical level that was statistically significant and leading to economic impact (Mills et al., 2011; CLRTAP, 2017). An additional metric suitable for large scale 'integrated assessment modelling' (POD_yIAM) has also been developed (CLRTAP, 2017), and has been used, for example, to model the impact of ozone on global wheat yields (Figs. 1.2 & 1.3, Mills, Sharps, et al., 2018).

1.3.4 Ozone, wheat and food security

Ozone is considered one of the primary phytotoxic air pollutants. It is as detrimental to many plants as other forms of abiotic stress such as heat and drought, and affects many of the world's most widely consumed staple food crops, especially wheat and soybean but also maize and rice (Ashmore, 2005; Caporn, 2013; Mills, Frei, et al., 2018). This is a particular concern in the context of the rising pressures on global food security as a consequence of population growth, climate change, energy and fertiliser costs, and the introduction of policies aimed at reducing pollution from agriculture (Wheeler and von Braun, 2013).

Due to its genetic diversity and resulting adaptability, wheat is grown more widely than any other staple crop, ranging from northern temperate regions to elevated areas in the sub/tropics (Shewry, 2009). It is easily harvested, can be stored for long periods, and has unique gluten proteins which enable it to be made into a wide range of food products (Shewry, 2009), providing ~20% of calories for human consumption (Hawkesford et al., 2013). Bread wheat, *Triticum aestivum* L., comprises the vast majority (95%) of all wheat types grown, with pasta wheat, *T. durum*, and additional minority wheat species, contributing just 5% (Shewry, 2009). Wheat's yield potential is high, with maximum yields now exceeding 10 tonnes ha⁻¹, but growth is often constrained by limited water or fertiliser supplies, or effects of either biotic or abiotic stress, meaning average global wheat yields currently reach just 3.54 tonnes ha⁻¹ (Shewry, 2009; USDA, 2022).

In this context, efforts to accurately model ozone's effect on global wheat yield losses (Emberson et al., 2018; Feng et al., 2022), and to develop cultivars which are more tolerant to abiotic stress, including to ozone (Ainsworth, 2017), are more essential than ever.

Depending on the metrics applied, modelled estimates of ozone's impact on global wheat yields have varied, but they suggest ozone has been reducing mean annual wheat yields in different parts of the world

¹ Deposition of Ozone (O₃) for Stomatal Exchange

within the ranges of 7.3% – 12% (Van Dingenen et al., 2009), 3.9% - 15% (Avnery et al., 2011), or by a single global mean of 7.1% (Mills, Frei, et al., 2018), with a greater effect found in the polluted northern hemisphere (9.9%) where the majority of wheat growing also takes place, compared to the less populated southern hemisphere (6.2%; Figs. 1.2 & 1.3; Mills, Sharps, et al., 2018).



Fig. 1.2. The seasonal mean Phytotoxic Ozone Dose above 3 nmol m⁻² sec⁻¹ in wheat growing areas, adapted for largescale Integrated Assessment Modelling (POD₃IAM mmol/m²). All values represent the means per 1° 9 1° grid cell where wheat production exceeds 500 Mg and are averaged for 2010–2012. Values use the POD₃IAM weighted by the proportion of production in the cell that is rain-fed (using POD₃IAM calculated using soil moisture limitation) and irrigated (using POD₃IAM calculated without soil moisture limitation). Figure and legend reproduced from Mills, Sharps, et al., 2018.

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Fig. 1.3. Estimated production loss for wheat due to ozone, modelled from the POD₃IAM and percentage yield losses and averaged for 2010–2012. Figure and legend reproduced from Mills, Sharps, et al., 2018.

1.3.5 How and when does ozone affect wheat yields?

Whilst the ozone concentration, duration of exposure, and extent of ozone uptake by the wheat plant are key factors in yield reductions (Ainsworth, 2017), the timing of ozone exposure in relation to the growth stage may also be critical (Fig. 1.4). Exposure of wheat plants to ozone either before, during, or after anthesis suggests that, whilst stomatal conductance – and therefore total ozone flux – into the plant is greatest at anthesis when there is maximum leaf area (Bassin et al., 2004), the post-anthesis growth stages appear to be the most ozone sensitive, possibly linked to earlier senescence and therefore a shortened grain fill period (Pleijel et al., 1998; Gelang et al., 2000). So, although both the total number of grains, and the size of grains (determined before and after anthesis respectively) affect the final yield, ozone has usually been found to have a greater effect after anthesis on grain size (Feng et al., 2009; Broberg et al., 2015; Pleijel et al., 2018).

Ozone also has an impact on grain quality, in that within each grain the proportion of starch is reduced, and the proportion of protein is increased, although the total harvested yields of both starch and protein are reduced overall (Wang and Frei, 2011; Broberg et al., 2015). Wheat is grown for bread making, milling, seed, or livestock feed, and as each require specific grain quality traits, changes to starch or protein characteristics could affect their value, and the end use of the grain (Nuttall et al., 2017).



Fig. 1.4. Summary of growth stages of wheat (Tottman & Broad, 1987) from early vegetative stages through to final harvest (see Table S1.1, p.15 for full detail), along with the approximate phases when tillers, grain number and grain size are determined. The approximate timing of nitrogen uptake, photosynthetic activity and senescence is also shown, as well as the approximate timing and duration of ozone exposure during the three plant trials. (Adapted from Sreenivasulu & Schnurbush 2012).

1.3.6 Wheat cultivars, wild relatives and synthetic wheat

Differences in ozone sensitivity between wheat cultivars have commonly been found, and more recently released cultivars appear to be more sensitive than older ones (e.g. Pleijel et al., 2006; Singh et al., 2018; Brewster et al., 2019) though in one meta-analysis of 17 wheat cultivars a correlation with release date was not found (Pleijel et al., 2018). Although research has been undertaken to screen some crops for ozone sensitivity, and identify genes connected with tolerance, for example in rice (Frei, 2015) and soybean (Osborne et al., 2016), there is still limited knowledge relating to the genetic background to ozone tolerance in wheat (Begum et al., 2020).

Understanding more about how and why sensitive and tolerant wheat cultivars respond differently to ozone is useful, but assessing the ozone sensitivity of the genome donors of bread wheat (*Triticum aestivum* L.) would also provide genetic information which could be useful to plant breeders. Fig. 1.5 illustrates the phylogenetic history of bread wheat, and the diploid and tetraploid species which are widely accepted as being the genome donors involved (Marcussen et al., 2014). These wild relatives of wheat, all of which are annual grassland species, are useful to plant breeders because they retain much greater genetic diversity than is contained within the hexaploid genome, and this genetic diversity can be exploited to enhance disease and abiotic stress resistance in modern cultivars (Castañeda-Álvarez et al., 2016).



Fig. 1.5. Phylogenetic history of bread wheat (T. aestivum L.) showing original divergence and subsequent hybridization, outlining the origin of the hexaploid AABBDD genome from the diploid donors, and the creation on synthetic wheat from these diploid donor species. Amended from: (Marcussen et al., 2014).

Based on this understanding, CIMMYT created synthetic (or re-synthesised) hexaploid wheat, an artificial cross which replicates the original natural hybridization between *T.turgidum* (AABB) and *Aegilops tauschii (DD)* (Mujeeb-Kazi et al., 1996). At least 1000 lines of synthetic hexaploid wheat have been created using accessions of *Ae.tauschii* and *T. turgidum* stored at CIMMYT (Li et al., 2014). Whilst synthetic hexaploid wheat has much greater genetic diversity, the question remains as to whether or not it has acquired any

ozone sensitivity contained within the genome donor species, especially as *Ae.tauschii* has previously been suggested as the source of bread wheat's ozone sensitivity (Biswas, Xu, Y. G. Li, et al., 2008). Previous work assessing the effect of nitrogen and ozone in combination on the shoot and root of wheat and its wild relatives (Brewster et al., 2018) found differences between wild relative species in both ozone sensitivity and variations in the ozone/nitrogen interaction, suggesting there are genetic differences which could be exploited, and which may have a role to play in effects on nitrogen dynamics.

1.3.7 Effect of ozone on nitrogen in agro-ecosystems

Nitrogen is essential to plant growth, and in agro-ecosystems artificial fertilisers are applied to improve crop yields. However, as much as two-thirds of the nitrogen fertiliser applied to cereal grain crops worldwide is not recovered at harvest, with the average recovery of nitrogen from wheat yield being just 38% (He et al., 2021). Variations in wheat Nitrogen Use Efficiency (NUE) is the result of genetic variation, agronomic practices, and environmental conditions, including both biotic and abiotic stress (Hawkesford and Riche, 2020). A limited number of studies have assessed the effect of ozone on nitrogen use within the wheat plant. For example, in their meta-analysis of 21 studies, Broberg et al. (2017) found that ozone reduced NUE in wheat and also increased levels of residual nitrogen in shoot material at harvest.

Due to these low rates of NUE in crop plants, surplus nitrogen fertiliser contributes significantly to annual anthropogenic additions of reactive nitrogen to terrestrial and marine ecosystems (Fowler et al., 2013). Soil nitrogen is held within organic matter and found in solution in the form of ammonium, nitrite and nitrate, and plants usually take up nitrogen through the soil solution either as ammonium or nitrate. However, nitrate has the potential to be leached from the soil and to accumulate downstream, leading to eutrophication of riverine, estuarine and marine habitats (Cameron et al., 2013). Whilst Cao et al. (2018) found that both nitrate and ammonium levels in the rhizosphere of a pot-grown wheat cultivar were significantly reduced by elevated ozone, levels of ammonium have also been found to increase (W. Chen et al., 2015; Hu et al., 2018), especially under sensitive wheat cultivars (W. Chen et al., 2015; Wu et al., 2016). In addition, surplus nitrogen fertiliser can also lead to gaseous emissions of nitrogen from agriecosystems including nitrous oxide, a powerful greenhouse gas, as well as nitric oxide and ammonia, all of which can contribute to air pollution, including to nitrogen deposition and to ozone formation (Cameron et al., 2013; Van Den Berg et al., 2016).

1.3.8 Scientific approaches to ozone impact studies

There are three broad approaches which are used for assessing the effect of ozone on plants (Paoletti et al., 2017). Firstly, statistical epidemiological studies use large scale spatial data, relating plant parameter data with ozone metrics. Braun et al (2017) have evaluated the use of such studies and how they can be used, for

example, to validate dose-response relationships. This approach has also been applied to assess ozone critical levels for crops; for example one case study used durum wheat production and ozone monitoring data in Italy to review a critical level of ozone for Italian wheat (De Marco et al., 2010).

A second approach is the external application of chemicals to protect control plants from ambient ozone in the field, with ethylenediurea the most commonly used 'antiozonant' for experimental purposes, but other groups of chemicals also used include pesticides, plant growth regulators, micronutrients and antioxidants (Saitanis and Agathokleous, 2020). This approach can only assess the effects of ambient and not elevated ozone levels, but it allows experimentation in areas with problematic ambient ozone and without access to OTCs or FACE technology. This method can establish if ozone is the cause of visible ozone injury, and assess differences in sensitivity to ambient ozone, and has been used, for example, to assess the different ozone sensitivities of multiple wheat cultivars in India (e.g. Fatima *et al.*, 2019). The current and future use of many different forms of plant protectants have been reviewed by Saitanis and Agathokleous (2020).

The third approach simulates natural ozone exposure under experimental conditions, either in closed chambers of varying sizes (e.g. indoor phytotrons and growth chambers, or outdoor solardomes, or greenhouses), or in field-based open-top chambers or free-air fumigation structures. Indoor phytotrons and growth chambers are closed and highly controlled environments with modified light, temperature, humidity, air flow and watering regimes and have been used largely for smaller scale mechanistic experimental purposes using pot-grown plants e.g. a study assessing effects of ozone, carbon dioxide and temperature on European spring wheat cultivars (Hansen et al., 2019). The use of larger outdoor closed chambers, such as solardomes or greenhouses, provide a semi-controlled environment where ozone concentrations and air flow are closely controlled, temperatures can be heated or unheated, and plants receive natural patterns of daylight. These outdoor chambers often have a larger capacity, although plants are usually still pot-grown (Paoletti et al., 2017). Solardomes have been used extensively for research into both crop plants and semi-natural plant species (e.g. Hayes *et al.*, 2020; Mills *et al.*, 2007; Holder *et al.*, 2020).

Field-based experiments, either using open-top chambers or free-air (ozone) concentration elevation (ozone-FACE) have often been used to research ozone's impact on crops in typical field conditions. The majority have used OTCs as they are less costly than the technically more sophisticated ozone-FACE (Z. Feng et al., 2018). Some concerns have been raised relating to the environmental conditions of the OTCs (e.g. Ainsworth *et al.*, 2012): although plants are grown in the field with normal rooting and plant density, and there is a constant and even concentration of ozone in the chambers, the sides of the chamber can cause changes in air flow and air temperature as well as light and humidity. These conditions could lead to both over-estimation of ozone damage (if conditions lower the leaf resistances and increase ozone flux) or underestimation of ozone damage (if warmer air and higher vapour pressure deficit reduces ozone flux)(Z. Feng et al., 2018). Ozone-FACE experiments are considered to be superior – if more costly - than OTCs by

supplying the ozone from an over-head framework, allowing the environmental conditions around the plants to be normal (Z. Feng et al., 2018), although prevailing winds can affect ozone delivery within these systems (Paoletti et al., 2017). However OTCs have the advantage over ozone-FACE experiments of being able to use air filtration to reduce ozone to ambient levels, although more recent ozone-FACE systems are addressing this issue (Paoletti et al., 2017). A comparison of OTCs and ozone-FACE experiments on wheat found that differences between OTCs and ozone-FACE experiments was related to the extent of stomatal conductance, and that OTC experiments appeared to reduce stomatal flux, with the effect of ozone therefore greater in ozone-FACE experiments than in OTCs (Z. Feng et al., 2018).

A meta-analysis of 53 studies on the effect of elevated ozone on wheat found that leaf photosynthesis parameters were more strongly reduced by elevated ozone in field-grown wheat than on plants grown in pots (< 5 L), with a similar greater impact found in OTC experiments compared to indoor growth chambers (Feng et al., 2008).

1.3.9 Description of the solardomes and growing conditions in all three plant trials

All three plant trials were undertaken at the UK Centre for Ecology & Hydrology's air pollution facility at Abergwyngregyn in North Wales where there are eight identical solardomes (Fig 1.6A & 1.6B), five of which provide unheated, temperate climatic conditions. Each solardome is supplied with a computer controlled supply of ozone which enables a regime of specific hourly/daily/weekly ozone concentrations to be set (Fig. 1.6C). Carbon-filtered air is supplied to each dome via an automated fan to ensure no external ambient ozone enters the domes. The air/ozone enters the dome via a circular, plastic, perforated 'donut' tube (Fig. 1.6D) which circulates the air/ozone evenly around the dome. Typically, ozone levels are reduced for two days per week to reflect the normal episodic pattern of ozone exposure, and this also then enables monitoring and sampling to take place safely. Specific details of ozone generation and monitoring of climatic conditions within the solardomes for each of the three plant trials are provided in Chapters 2-4. In all three trials the ozone exposures (ppb) were selected to represent the range of average warm season concentrations currently found in different regions of the world (see 1.3.1) from low ambient levels of 30 ppb (e.g.in northern Europe), through intermediate levels to the high levels above 100 ppb (frequently found in South and East Asia). Although the highest ozone levels in Trial 3 (70 ppb and 85 ppb) were lower than those achieved in Trials 1 and 2 (80 ppb and 110 ppb) due to technical limitations, this still provided a graded increase from ambient to realistically high phytotoxic levels. In the three trials the ozone exposure lasted between 10.5 – 11.5 weeks, starting in mid-vegetative growth stages and ending at harvest (Fig. 1.4, Table 1.1), and represented a long term chronic exposure to ozone. Multiple experiments are run in the solardomes each year meaning space is limited and additional replication is not possible. Previous studies
suggest these un-replicated results are statistically valid (Mills et al., 2009; Hewitt et al., 2016). Growth conditions and fertilisation regimes for all three plant trials are summarised in Table 1.1.



Fig. 1.6. Photographs of the solardomes with (A), and without (B), plant experiments; the computer screen (C) showing computer-controlled concentrations of ozone in each of the eight solardomes; and an example of a wheat experiment showing the perforated donut tube which circulates ozone evenly around the solardome (D).

Table 1.1. Summary of soil mediums	, fertilisation regimes, ar	nd ozone exposures for	Trials 1, 2, and 3
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	Trial 1	Trial 2	Trial 3
Seedlings vernalised	Yes	Yes	No
Soil medium	Sandy loam farm soil	Sandy loam farm soil	Sandy/silt loam
	рН 5.6	pH 6.2	commercial topsoil
			рН 7.2
Total nitrogen fertiliser	Soil N: 77 kg ha	Soil N: 81.5 kg/ha	Soil N: 2.1 kg/ha
(ammonium nitrate)	+50 kg/ha at GS30+	+25 kg ha at GS41+	+70 kg ha at GS13
	+25 kg/ha at GS69+	+50 kg ha at GS55+	+70 kg ha at GS31+
		+25 kg ha at GS70+	+20 kg ha at GS65
	Total = 152 kg/ha	Total = 181.5 kg/ha	Total = 162 kg ha
Ozone treatments	Low (30 ppb)	Low (30 ppb)	Low (30 ppb)
	Medium (55 ppb)	Medium (55 ppb)	Medium (45 ppb)
	High	High (80 ppb)	High (70 ppb)
	Very High (110 ppb)	Very High (110 ppb)	Very High (85 ppb)
Duration of ozone	7 th June – 21 st August	30 th May – 19 th August	3 rd June – 15 th August
treatment	(10.5 weeks) 2018	(11.5 weeks) 2019	(11 weeks) 2021

Supplementary materials

Table S1.1. Wheat growth stages – decimal code system (Tottman and Broad, 198	37).
Source: AHDB Wheat Growth Guide (AHDB, 2021).	

Growth		Growth	
stage	Seedling growth	stage	Ear emergence
GS10	1 st leaf through coleoptile	GS51	1 st spikelet just visible above flag ligule
GS11	1 st leaf unfolded	GS55	Half of ear emerged above flag leaf ligule
GS13	3 leaves unfolded	GS59	Ear completely emerged above flag ligule
GS15	5 leaves unfolded		
GS19	9 or more leaves unfolded		
	Tillering		Flowering/Anthesis
GS20	Main shoot only	GS61	Start of flowering
GS21	Main shoot and 1 tiller	GS65	Flowering half way
GS23	Main shoot and 3 tillers	GS69	Flowering complete
GS25	Main shoot and 5 tillers		
GS29	Main shoot and 9 or more tillers		
	Stem elongation		Milk Development
GS30	Ear at 1cm / jointing	GS71	Grain watery ripe
GS31	1 st node detectable / <i>jointing</i>	GS73	Early milk
GS32	2 nd node detectable	GS75	Medium milk
GS33	3 rd node detectable	GS77	Late milk
GS37	Flag leaf just visible		
GS39	Flag leaf blade all visible		
	Booting		Dough development
GS41	Flag leaf sheath extending	G\$83	Farly dough
GS43	Flag leaf sheath just visibly swollen	GS85	Soft dough
GS45	Flag leaf sheath swollen	GS87	Hard dough
GS47	Flag leaf sheath opening		
			Ripening
		GS91	Grain hard (able to dent)
		GS92	Grain hard (not able to dent)
		GS93	Grain loosening in daytime

CHAPTER 2

Ozone tolerance found in Aegilops tauschii and Primary Synthetic Hexaploid wheat

Declaration of contributions:

Conception, methodology and design: Clare Brewster (CB), Felicity Hayes (FH), Natalie Fenner (NF); Collection and assembly of data: CB; Analysis, interpretation of the data, and drafting of the chapter: CB; Critical revision and final approval of content: CB, FH, NF.

Abstract

Modern wheat cultivars are increasingly sensitive to ground level ozone, with 7–10% mean yield reductions in the northern hemisphere. In this study, three of the genome donors of bread wheat, *Triticum urartu* (AA), *T. dicoccoides* (AABB), and *Aegilops tauschii* (DD) along with a modern wheat cultivar (*T. aestivum* 'Skyfall'), a 1970s cultivar (*T. aestivum* 'Maris Dove'), and a line of primary Synthetic Hexaploid Wheat were grown in 6 L pots of sandy loam soil in solardomes (Bangor, North Wales) and exposed to low (30 ppb), medium (55 ppb), and high (110 ppb) levels of ozone over 3 months. Measurements were made at harvest of shoot biomass and grain yield. *Ae. tauschii* appeared ozone tolerant with no significant effects of ozone on shoot biomass, seed head biomass, or 1000 grain + husk weight, expecially under high ozone (–26%). The older cultivar, 'Maris Dove', had a significant reduction in seed head biomass (–9%) and 1000 grain weight (–11%) but was less sensitive than the more recent cultivar 'Skyfall', which had a highly significant reduction in its seed head biomass (–21%) and 1000 grain weight (–27%) under high ozone. Notably, the line of primary Synthetic Hexaploid Wheat was ozone tolerant, with no effect on total seed head biomass (–1%) and only a 5% reduction in 1000 grain weight under high ozone levels. The potential use of synthetic wheat in breeding ozone tolerant wheat is discussed.

2.1 Introduction

2.1.1 Ozone and wheat

Bread wheat, *Triticum aestivum* L., is a very adaptable crop plant, successfully growing in a wide variety of environments and climatic conditions across the globe. This adaptability is due in part to the complexity of its genome, derived from its progenitor diploid and tetraploid species (Marcussen et al., 2014; Appels et al., 2018) and containing extensive gene replication and diversity (Dubcovsky and Dvorak, 2007). However, the stagnation in the growth of global wheat yields over the last twenty-five years has raised concern because of wheat's central role in global food systems and growing pressures on food security (Curtis and Halford, 2014). Considerable efforts are being made to increase wheat yields but many abiotic stress factors also continue to suppress yields, including ground level ozone pollution (Ashmore, 2005; Emberson et al., 2009; Mills, Frei, et al., 2018; Schauberger et al., 2019).

Ozone is created by photochemical reactions between sunlight and precursor gases including nitrogen oxides and volatile organic compounds (Royal Society, 2008). Background levels of 25–55 ppb often occur, especially in the northern hemisphere where the highest concentrations of anthropogenic emissions of these precursor gases are found (Mills, Pleijel, et al., 2018). Although peak ozone episodes above 100 ppb are now less frequent in Europe and North America, they are still a common occurrence in East Asia (Feng et al., 2015; Li et al., 2019). Wheat is an ozone sensitive species and current levels of ground level ozone suppress mean global wheat yields in the northern hemisphere by 7.1–9.9% per year (Mills, Frei, et al., 2018; Mills, Sharps, et al., 2018).

Ozone enters plants through their stomata, and in common with other types of abiotic stress, leads to oxidative stress through the creation of excess Reactive Oxygen Species (ROS) that oxidize proteins, DNA, RNA and lipids and may also cause the degradation of cell membranes (Taiz et al., 2015; pp.737-738), all of which lead to reduced photosynthesis, early senescence and, ultimately, reduced seed production (Feng et al., 2008).

2.1.2 Differences in ozone sensitivity and tolerance

Spring and winter wheat types are equally affected (Feng et al., 2008), with the growth phases between anthesis and the end of grain-fill found to be the most sensitive (Pleijel et al., 1998; Feng et al., 2016). Studies researching the effects of ozone on different wheat cultivars have found some exhibiting greater tolerance to ozone than others, both above- (Zhu et al., 2011; Pandey et al., 2019) and below-ground (Wu et al., 2016). In both spring wheat (Barnes et al., 1990; Pleijel et al., 2006) and winter wheat (Biswas, Xu, Yong Ging Li, et al., 2008) the more recent the cultivar the more ozone sensitive it has been found to be, though this is not always the case (Pleijel et al., 2018). What causes the different responses between

tolerant and sensitive cultivars, and to what extent this is directly attributable to factors such as higher stomatal conductance increasing ozone flux into the leaf, antioxidant enzymes enabling detoxification, or plant-soil-microbial interactions which act to mitigate negative effects, is still under investigation (Li et al., 2015; Feng et al., 2016; Yadav et al., 2019).

2.1.3 Role of wild relatives and synthetic wheat in wheat breeding

A series of hybridisation events between *Triticum* and *Aegilops* species (Fig. 2.1) led to the creation of hexaploid bread wheat c. 8000 years ago (Marcussen et al., 2014). With the genomes of wheat and its closest wild relatives now sequenced, there is great potential for wheat improvement (Mochida and Shinozaki, 2013; Avni et al., 2017; Appels et al., 2018; Rasheed et al., 2018) as the diversity found within wheat's wild relatives is recognised as a valuable source of biotic and abiotic tolerance (Sasanuma et al., 2002; Schneider et al., 2008).



Fig. 2.1. Phylogenetic history of Triticum aestivum L. showing the hybridisation events between Aegilops and Triticum species which led to the creation of bread wheat (after Marcussen et al. 2014).

Assessments of the ozone sensitivity of some of the close wild relatives of wheat have been made previously in short term experiments (Biswas, Xu, Y. G. Li, et al., 2008; Brewster et al., 2018), with young plants (3–6 weeks old) receiving 3 weeks of ozone fumigation at 100 ppb concentrations. *Ae. tauschii* (diploid DD genome donor) appeared ozone sensitive, with reductions in shoot, root and total biomass and concomitant reductions in physiological performance. The diploid AA genome, *T. monococcum*, was also found to be sensitive, though less so than *Ae. tauschii*, and therefore *Ae. tauschii* was suggested as the likely genetic origin of wheat's ozone sensitivity (Biswas, Xu, Y. G. Li, et al., 2008). Assessments of tetraploid wheats, *Triticum turgidum* ssp. *durum* (Biswas, Xu, Y. G. Li, et al., 2008) and *T. dicoccoides* (Brewster et al., 2018) suggested they had greater ozone tolerance.

Synthetic Hexaploid Wheat, or resynthesized wheat, is a modern artificial re-crossing of the tetraploid AABB and diploid DD species (Kihara and Lilienfeld, 1949) with the majority of recent synthetic wheat lines being created from crosses between lines of *T. turgidum* ssp. *durum*, (AABB genome) and accessions of *Ae. tauschii* (Li et al., 2018). The resulting primary synthetic wheat lines are now used in wheat breeding because they contain a wealth of wild relative genetic diversity lost during wheat domestication, and are also readily crossable with *T. aestivum* L., enabling the transfer of useful traits into elite cultivars (Trethowan and Mujeeb-Kazi, 2008). High yielding synthetic-derived commercial cultivars are being grown extensively in some countries, including, for example, the high yielding Chinese cultivar 'Chuanmai 42' (van Ginkel and Ogbonnaya, 2007).

2.1.4 Aims of the study

As far as the authors are aware, none of the main genome donors of wheat, or a synthetic wheat line have been tested for ozone tolerance to yield bearing stage. This study aimed to test the following hypotheses:

- Ae. tauschii would be more ozone sensitive than T. urartu or T. dicoccoides;
- Synthetic Hexaploid Wheat would have either inherited the ozone sensitivity of the DD genome, or the greater tolerance of the tetraploid AABB genome;
- and the older wheat cultivar, 'Maris Dove', would be less ozone sensitive than the more recent wheat cultivar, 'Skyfall'.

The results suggest that when grown to final yield, *Ae. tauschii* is in fact ozone tolerant, as is primary Synthetic Hexaploid Wheat, with ozone sensitivity found in the AA genome as well as in both the cultivars, though the older cultivar was less sensitive than the more recent one.

2.2 Materials and Methods

2.2.1 Plant establishment

The experiment was undertaken in 2018 at the Centre for Ecology and Hydrology (CEH) air pollution facility at Abergwyngregyn, North Wales (53.2°N, 4.0°W). The species selected for the trial (Table 2.1) included a recent high yielding cultivar released in 2014 (*T. aestivum* L., cv. Skyfall), one older cultivar released in 1971 (*T. aestivum* L., cv. Maris Dove), a primary spring Synthetic Hexaploid Wheat line created from a cross between *T. durum* and an accession of *Ae. tauschii*, and three of the main genome donors of bread wheat: *T. dicoccoides*, *T. urartu*, and *Ae. tauschii*. The accession of *Ae. tauschii* was not the same accession used to create the line of synthetic wheat.

Species/Cultivar	Туре	Seed Supplier	Origin/Date
Triticum aestivum L., Cultivar RAGT Semences		Released 2014	
	(winter/spring)	France	_
Triticum aestivum L.,	Cultivar	John Innes Centre	Released 1971
cv. Maris Dove	(spring)	Seedstore, Norwich UK	
		W0005	_
Synthetic Hexaploid	100% synthetic	NIAB, Cambridge UKNIAB	WISP/Synthetics2011-
Wheat (spring)	wheat	SHW 084, derived	2017
	(spring)	From Hoh-501 (AABB)/Ent- 084(DD)	
Triticum dicoccoides	Primary wild	John Innes Centre	
	relative	Seedstore, Norwich UK	
	AABB genome	T1060020	Israel
			Collection date not
			known
Triticum urartu	Primary wild	John Innes Centre	Turkey
	relative	Seedstore, Norwich UK	Collection date not
	AA genome	T1010004	known
Aegilops tauschii	Secondary wild	John Innes Centre	Afghanistan
(squarrosa)	relative	Seedstore, Norwich UK	Collection date not
	DD genome	T2220019	known

Table 2.1.	Wheat and	wild relative	species u	ised in th	ne trial w	vith detai	ls of type	and source	of seed
10010 2.1.	which and	what i chathe	species a	iscu ini tin		vitii actai	is of type	una source	of secu

De-husked seeds were germinated (11–12 April) in petri dishes in an incubator set at room temperature (21 $^{\circ}$ C). Seedlings were then sown into modular plug trays containing Levington's John Innes No. 1 low nutrient seedling compost and grown in a glasshouse without light or temperature control (13–19 April, Bangor, UK). Vernalisation was started 7 days later, for four weeks, (16 h day length; 1.2 klux light intensity; 5 $^{\circ}$ C) and watered as required. Seedlings were then transplanted (19–21 May) into 6.3 L round plastic pots (25 cm deep, 20 cm diameter at the rim) containing coarsely sieved and well mixed sandy loam soil (originating from Henfaes Research Station, Abergwyngregyn, North Wales), with two seedlings per pot. There were six replicate pots per species and per treatment. Plants were grown in a glasshouse without light or temperature control (22 May–6 June) with pots rotated randomly every 4–5 days. All pots were watered manually, daily or as required, to maintain soil moisture levels throughout the experiment. There was 77 kg N ha⁻¹ equivalent available nitrogen within the topsoil at the start of the trial. Nitrogen fertiliser, in the form of ammonium nitrate, was applied to all species on 21 June after tillering/elongation growth stages with both cultivars and synthetic wheat receiving a rate equivalent to 50 kg N ha⁻¹, and the wild relative species receiving 25 kg N ha⁻¹. After anthesis the cultivars received an additional 25 kg N ha⁻¹ on 1 August.

2.2.2 Ozone treatment

The replicates were distributed randomly within four hemispherical glasshouses (solardomes; 2.1 m high; 3 m diameter). Ozone treatments started on 7th June and continued for 11 weeks until all plants had been harvested (21 August). Using square wave exposures to the maximum levels, ozone concentrations in each solardome provided low (30 ppb), medium (55 ppb), and high (110 ppb) regimes (Fig. 2.2 and Fig. S2.1, p.31), with concentrations reduced at night-time and on two days each week, to reflect natural patterns of ozone exposure. Plants were rotated within the solardomes weekly, and once (20 June) between the four solardomes; environmental conditions were found not to vary between the solardomes (Hewitt et al., 2016).

Ventilation in the solardomes comprised of approximately two air changes per minute with charcoal-filtered air. Ozone was provided through controlled injection using a G11 ozone generator (Ozone Industries, Andover, UK) and Sequel 10 oxygen concentrator (Pure O2, Urmston, UK), with computer-controlled concentrations (Lab VIEW version 2012, National Instruments, Austin, TX, USA). Ozone was supplied via PTFE tubing, and monitored every 30 minutes by two calibrated automatic ozone analysers (400a, Enviro Technology Services, Stroud, UK, and Thermo Scientific Model 49i Electron O3 Analyser, Fischer Scientific, Waltham, MA, USA).



Fig. 2.2. Mean hourly ozone concentrations for the weekly profile achieved over the course of ozone treatment (7 June and 21 August 2018). Seasonal 24-h mean and AOT40 values are also shown.

2.2.3 Biomass and yield measurements

For the cultivars and synthetic wheat, the fully ripened ears were cut from both plants in each pot shortly before harvest. For the wild relatives, seed was either collected by hand prior to shattering (*Ae. tauschii*), or collected through the use of organza bags secured around the seed heads (*T. urartu* and *T. dicoccoides*). As *T. urartu* was still growing at the time of harvest all seed heads were collected, although only ripened seed heads were included in the seed head biomass totals. For all species, both plants from each replicate pot were then harvested by cutting the shoot just above the surface of the substrate. Shoot biomass was obtained by weighing after drying at 65 °C for 14+ days. The ears from each replicate of the cultivars, or the total loose seed collected from each of the wild relative replicates, were weighed to derive the mean total seed head biomass. The ears of the two cultivars and synthetic wheat were threshed using a hand thresher (Minibatt+, Reichhardt Electronic Innovations, Hungen, Germany), and the seeds were weighed. 1000 grain weight was obtained by weighing 100 randomly selected grains from each replicate, then multiplying by ten. For the wild relative species, the seed could not be threshed and 100 grains with husks were selected randomly and weighed, then multiplied by ten to obtain the '1000 grain + husk' weight.

2.2.4 Statistical methodology

The effect of ozone on the total shoot biomass, total seed head biomass, and 1000-grain weight of each species was assessed using linear models (normal error) and post-hoc Tukey tests in R (R Core Team, 2015). Residuals were assessed for normality. Significance was assessed at p < 0.05 (Table S2.1, p.31).

2.3 Results

2.3.1 Shoot biomass

There was no significant overall effect of ozone on the shoot biomass of any species (Fig. 2.3) apart from that of the recently released cultivar 'Skyfall' (p < 0.001), which showed a significant reduction (p < 0.01) in shoot biomass between the low 30 ppb and high 110 ppb ozone levels (-8%) (see Supplementary Materials, Table S2.1, p.31 for all p values.). Whilst there was also a reduction in the shoot biomass of *T. dicoccoides* (-12%) and *T. urartu* (-5%), these effects were not statistically significant. The shoot biomass of the line of primary synthetic hexaploid wheat (+3%) and *Ae. tauschii* (-3%) were less affected by ozone than the other varieties tested.



Fig. 2.3. Mean shoot biomass by species and treatment. Skyfall: T. aestivum 'Skyfall', Maris Dove: T. aestivum 'Maris Dove', SHW: Synthetic Hexaploid Wheat. Low/30 ppb: black bars; medium/55 ppb: dark grey bars; high/110 ppb: light grey bars. Bars show standard error of the mean (n = 6).

2.3.2 Total seedhead biomass

The cultivars 'Skyfall' (p < 0.001) and 'Maris Dove' (p < 0.01) were the only species where ozone had a significant overall effect on total seed head biomass (Fig. 2.4). The recent cultivar, 'Skyfall', showed highly significant reductions in total seed head biomass between the low and high ozone levels (p < 0.001, -21%) and also between the medium and high treatments (p < 0.001). The older cultivar, 'Maris Dove', whilst sensitive to the highest level of ozone, was more tolerant than 'Skyfall', with a smaller but still significant reduction in total seed head biomass (p < 0.05, -9%) between the low and high treatment levels.



Fig. 2.4. Mean total seed head biomass. Skyfall: T. aestivum 'Skyfall', Maris Dove: T. aestivum 'Maris Dove', SHW: Synthetic Hexaploid Wheat. Low/30 ppb: black bars; medium/55 ppb: dark grey bars; high/110 ppb: light grey bars. Bars show standard error of the mean (n = 6).

2.3.3 1000 grain weight

There was a significant overall effect of ozone on the 1000 grain/1000 grain + husk weights of all species apart from *Ae. tauschii* (Table S2.1, Figs. 2.5 & 2.6). There was a highly significant 27% reduction in the 1000 grain weight of 'Skyfall' (Fig. 2.5, p < 0.001) between the low and high ozone treatments. The older cultivar 'Maris Dove' was less sensitive than 'Skyfall', with an 11% reduction between low and high treatments (p < 0.05) leading to the 1000 grain weight of 'Maris Dove' being greater than that of 'Skyfall' under the high ozone treatment. Whilst there was a 5% reduction in the 1000 grain weight of synthetic wheat under both the medium and high levels of ozone compared to the low 30 ppb level, this reduction was only significant between the low and medium levels (p < 0.05).



Fig. 2.5. 1000 grain weight of cultivar and synthetic wheat. Skyfall: T. aestivum 'Skyfall', Maris Dove: T. aestivum 'Maris Dove', SHW: Synthetic Hexaploid Wheat. Low/30 ppb: black bars; medium/55 ppb: dark grey bars; high/110 ppb: light grey bars. Bars show standard error of the mean (n = 6).

Ozone had a significant negative effect overall on the 1000 grain + husk weight of *T. dicoccoides*, (p < 0.05), although the difference between the high and low treatments was not statistically significant (p = 0.055) in this parameter. In comparison, *T. urartu* (AA genome) demonstrated ozone sensitivity in its 1000 grain + husk weight, with a highly significant reduction in the high ozone treatments (p < 0.001) compared to the low ozone treatment.



Fig. 2.6. 1000 grain + husk weight of the wild relatives of wheat. Low/30 ppb: black bars; medium/55 ppb: dark grey bars; high/110 ppb: light grey bars. Bars show standard error of the mean (n = 6).

2.3.4 Differences in ozone sensitivity

These data suggest that *Ae. tauschii* showed ozone tolerance, with ozone having no significant effect on shoot biomass, total seed head biomass or 1000 grain + husk weight (Figs.2.3, 2.4 & 2.6). In contrast, *T. dicoccoides* and *T. urartu* had some degree of sensitivity to ozone, although only *T. urartu* was significantly negatively affected. Across all the parameters measured, the line of primary synthetic wheat appeared more tolerant to ozone than the cultivars, with no significant differences in shoot biomass or total seed head biomass in response to increasing ozone concentration and only a 5% reduction in 1000 grain weight under high ozone (Figs. 2.3, 2.4, & 2.5).

2.4 Discussion

2.4.1 Ozone tolerance in the DD genome

Based on both shoot biomass and yield data, the accession of *Ae. tauschii* in this study was found to be ozone tolerant. This is contrary to previous assessments of the ozone sensitivity of *Ae. tauschii* using young plants in a short ozone exposure during the vegetative stage (Biswas, Xu, Y. G. Li, et al., 2008; Brewster et al., 2018). The previous studies suggested that *Ae. tauschii* may be the source of wheat's ozone sensitivity because it was found to have a significantly reduced photosynthetic rate, as well as reduced relative growth rate and shoot and root biomass in response to elevated ozone compared with the other genome donors tested (Biswas, Xu, Y. G. Li, et al., 2008). However, the photosynthetic rate was not determined during the current study.

Wide genetic variation has been found in the wild populations of many *Aegilops* species (Sasanuma et al., 2002) including *Ae. tauschii* (Rasheed et al., 2018). This variation provides potential for the discovery of novel biotic resistance and abiotic tolerance, but it may also lead to varying responses of different accessions to the same abiotic stress. The discovery that *Ae. tauschii* carries a very high percentage of transposable elements (the DNA sequences that can move within a genome) (Zhao et al., 2017), may explain its ability to adapt to multiple stress factors (Rasheed et al., 2018). The wide geographical range of the *Ae. tauschii* populations means there is potential for multiple novel traits to be accessed, including for a wide range of abiotic stress tolerance (Velissariou et al., 1992; Mujeeb-Kazi et al., 1996; Iehisa and Takumi, 2012; Gorafi et al., 2018).

Prior to the Biswas study, several experiments exposed young wheat plants at the vegetative stage of growth to ozone for 21 days and showed that elevated ozone reduced the mean relative growth rate (Barnes et al., 1990), root:shoot ratios (Velissariou et al., 1992) and photosynthetic rates (Fiscus et al., 2005). Based on these studies it was suggested that the effect seen at the vegetative stage may also be reflected in grain yield (Pleijel et al., 2006), and this was the rationale behind the Biswas study. However, it is preferable to grow crop plants to final yield to assess ozone sensitivity, partly because yield is the primary indicator of productivity, but also because the effect of ozone has been found to increase as wheat progresses through its growth stages (Feng et al., 2008) with the greatest degree of ozone damage found to be between anthesis and grain fill (Pleijel et al., 1998). In this study, no data comparative to the Biswas study were collected at the vegetative stage, so no conclusion can be drawn as to whether this accession of *Ae. tauschii* would have also demonstrated ozone tolerance in the early stages of growth.

2.4.2 Ozone tolerance in synthetic wheat

In this study, the line of primary Synthetic Hexaploid Wheat was found to be ozone tolerant. Whilst there was a yield loss of 5% under the high ozone treatment, this was no greater than that under the medium

ozone treatment and considerably less than the yield losses of both cultivars. To the authors' knowledge, there are no other studies that have assessed the response to ozone of primary synthetic wheat or cultivars derived from synthetic wheat. However, synthetic wheat has previously been found to provide abiotic stress tolerance (Trethowan and Mujeeb-Kazi, 2008), with synthetic lines demonstrating tolerance to drought, heat, waterlogging, salinity and frost damage (van Ginkel and Ogbonnaya, 2007; Pradhan et al., 2012; Bhatta, Morgounov, Belamkar, Poland, et al., 2018).

It is possible that the abiotic stress tolerance found in synthetic wheat in this trial may relate to beneficial root traits (Becker et al., 2016), or potentially to higher abscisic acid (ABA) responsiveness (lehisa and Takumi, 2012). Another causal factor may be that the artificial crossing of the AABB and DD genomes to create synthetic wheat lines has enabled a higher proportion of genetic material from the DD genome of *Ae. tauschii* to be transferred into synthetic wheat compared to hexaploid wheat (Rasheed et al., 2018; Bhatta, Morgounov, Belamkar, Poland, et al., 2018). As *Ae. tauschii* was shown in this trial to be ozone tolerant, this could suggest the DD genome has a role to play in the ozone tolerance of synthetic wheat. A strategic approach to identify the beneficial traits within the *Ae. tauschii* populations has been developed by the National Institute for Agricultural Botany (NIAB) for use in its synthetic wheat breeding programme (Jones et al., 2013). Although some studies suggest the derived lines do not necessarily retain the same level of abiotic tolerance as the primary synthetic (Trethowan and Mujeeb-Kazi, 2008), synthetic lines have been found to have superior abiotic tolerance compared to wheat cultivars, therefore the screening of lines of synthetic wheat for abiotic tolerance is recommended for future research (Sohail et al., 2011; Li et al., 2018). This would be particularly worthwhile in countries that already grow synthetic-derived cultivars and have high levels of ozone, such as India and China.

2.4.3 Possible reasons for ozone sensitivity in the AA and AABB genomes

Based on the 1000 grain + husk data, it can be suggested that *T. urartu*, the AA genome, is ozone sensitive and could have contributed to the genetic origin of wheat's ozone sensitivity. In this study *T. dicoccoides*, the AABB genome was more ozone sensitive than *Ae. tauschii*, but less than *T. urartu*. Higher levels of stomatal conductance can lead to a higher ozone flux and greater sensitivity to ozone if the plant is unable to detoxify (Bassin et al., 2007). Biswas et al. (2008) also found *T. monococcum* (AA genome) to be ozone sensitive, and both *T. monococcum* and *T. urartu* have been found to have high levels of stomatal conductance and photosynthetic rates (Austin et al., 1982; del Blanco et al., 2000; Biswas, Xu, Y. G. Li, et al., 2008; C.Y. Wang et al., 2008). Interestingly, in both *T. monococcum* and *T. urartu*, stomatal frequency on the adaxial surface of flag leaves was found to be nearly as high as on the abaxial surfaces (Austin et al., 1982), which could lead to a greater flux of ozone into the leaf. However, the stomatal conductance of *Ae.tauschii* has also been found to be high (C.Y. Wang et al., 2008) and as *Ae. tauschii* was found to be more ozone tolerant in this trial, this is not necessarily the sole factor involved. *T. monococcum* has been found to have a reduced antioxidant capacity, and subsequently, higher levels of ROS compared to a wheat

cultivar, with cultivated Emmer wheat (*T. dicoccum AABB*) also showing a similar reduced capacity to detoxify (Mao et al., 2018). We suspect that both higher stomatal conductance and lower antioxidant levels may be the reasons why both the AA and AABB genomes appeared more ozone sensitive in this study.

2.4.4 Differences in Ozone Sensitivity of the Cultivars

The seed head biomass and 1000 grain weight of the cultivar 'Maris Dove', released in 1971, suggested that it was less sensitive to ozone than the more recent cultivar 'Skyfall' released in 2014, which had been tested previously and found to be more sensitive than cultivars released in the 1980s and 1990s (Harmens et al., 2018). These data add to the growing body of evidence that indicates that the more recent cultivars are often more ozone sensitive than older ones (Barnes et al., 1990; Pleijel et al., 2006; Biswas, Xu, Yong Ging Li, et al., 2008). The exact cause of this variable response between cultivars is still unclear. As higher levels of ozone damage may be correlated with higher levels of ozone flux there is concern that trait selection to increase stomatal conductance in order to maximise photosynthesis (Fischer et al., 1998) may have led to an inadvertent increase in ozone damage (Mills, Sharps, et al., 2018). However, a plant's ability to detoxify through the production of antioxidant enzymes may also play a significant role in ozone tolerance (Feng et al., 2016), although this factor has not been assessed in relation to the date of release of the cultivar.

The specific genes, or sets of genes, involved in these different ozone tolerance mechanisms are currently unknown but it is worth noting that even though the wheat cultivars and synthetic wheat are both hexaploid wheats, the ozone tolerance trait demonstrated by the line of primary synthetic hexaploid wheat grown in this trial was not present in either of the cultivars. Whilst the AA and BB components of wheat's hexaploid genome have retained c. 30% of the genetic material from their tetrapoloid wild progenitor *T. dicoccoides*, the DD genome component contains just 10% of the diversity found in *Aegilops tauschii* (Borrill et al., 2019). It appears that synthetic wheat, with a greater proportion of the DD genome, has more genetic diversity and therefore has, as this trial would suggest, greater potential for abiotic tolerance.

2.5 Conclusion

Whilst further research is needed to understand the exact causes of wheat's increasing ozone sensitivity, synthetic wheat and its use in plant breeding may offer an opportunity to develop more ozone tolerant cultivars. Screening for ozone tolerant traits in some wheat cultivars has already taken place (Singh et al., 2018). Including synthetic wheat in future screening processes would be a useful next step. The promise that synthetic wheat has shown for ozone tolerance in this trial could be further explored through the assessment of synthetic lines, as well as commercial synthetic wheat cultivars already on the market.

Supplementary materials



Fig. S2.1. Daily/weekly ozone concentrations (ppb) achieved over the course of the treatment period (7 June – 21 August).

Table S2.1. Table of p values representing levels of statistical significance of the effect of ozone on each species and
between the ozone treatment levels.

Shoot biomass	Overall effect	LOW – MED	MED - HIGH	LOW - HIGH
	of ozone			
cv 'Skyfall'	0.0084 **	0.227	0.165	0.0062 **
cv 'Maris Dove'	0.137	0.131	0.839	0.319
SHW	0.209	0.190	0.476	0.796
T. dicoccoides	0.152	0.326	0.868	0.148
Ae. tauschii	0.843	0.987	0.910	0.839
T. urartu	0.114	0.331	0.103	0.750
Total seed head				
biomass				
cv 'Skyfall'	1.017e-05 ***	0.145	0.0003 ***	< 0.0000 ***
cv 'Maris Dove'	0.0018 **	0.341	0.0015 **	0.027 *
SHW	0.733	0.714	0.884	0.946
T. dicoccoides	0.0699	0.998	0.110	0.101
Ae. tauschii	0.949	0.946	0.992	0.978
T. urartu	0.108	0.446	0.572	0.091
1000 grain				
weight				
cv 'Skyfall'	2.574e-6 ***	0.011 *	0.00057 ***	0.0000017 ***
cv 'Maris Dove'	0.0074 **	0.911	0.023 *	0.0102 *
SHW	0.04171 *	0.0475 *	0.901	0.105
T. dicoccoides	0.03746 *	0.962	0.073	0.055
Ae. tauschii	0.1828	0.970	0.204	0.291
T. urartu	0.0001 ***	0.937	0.0004 ***	0.0002 ***
Significance indicated where p < .05 * < .01 ** < .001 ***				

CHAPTER 3

µCT imaging of intact wheat spikes reveals effect of ground level ozone on grain yield components

Declaration of contributions:

Conception, methodology and design: Clare Brewster (CB), Felicity Hayes (FH), Natalie Fenner (NF), Fiona Corke (FC)*, Candida Nibau (CN)*; *Collection and assembly of data*: CB, CN; *Analysis, interpretation of the data, and drafting of the chapter*: CB; *Critical revision and final approval of content*: CB, FH, NF, FC, CN; *Additional technical support*: CN (MATLAB data extraction).

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Abstract

Ground level ozone pollution, an important abiotic stress, is estimated to reduce mean global annual wheat yields by nearly 10% but its impact on the morphology of spikes and grains has never been established. This study exposed an elite cultivar, a primary synthetic wheat, and nine derived F2 lines to four levels of ozone (30 ppb, 55 ppb, 80 ppb, 110 ppb) for three months in solar-domes. X-ray microcomputed tomography (μ CT) digital imaging, which enables 3D visualisation of spikes and in situ grains, revealed for the first time how ozone affects grain formation in each spikelet, as well as its effect on individual grain shape. The μ CT imaging on spikes of the elite cultivar and three F2 lines showed exactly where on the spike ozone reduced grain number and how changes to individual grain morphology parameters lead to reductions in grain size (volume). The ozone-induced losses in grain number occurred primarily across the middle section of the spike. Grain volume reductions were driven more by reductions in width and depth than by reductions in grain length. Yield data revealed differences in ozone sensitivity between the lines, with the elite cultivar and one synthetic line demonstrating tolerance to ozone. Links between grain morphology and grain quality are also explored. These data provide information that could be utilised in future wheat breeding programmes to increase tolerance to ozone of this staple food crop.

3.1 Introduction

3.1.1 Ground level ozone

As a result of increasing emissions of anthropogenic-derived precursor gases leading to the formation of the secondary pollutant ozone within the troposphere (Royal Society, 2008; Fleming et al., 2018) ground level ozone continues to occur in many wheat-growing areas of the world, with a range of concentrations from ambient (20-30 ppb) to phytotoxic (40 – 100+ ppb), (Fleming et al., 2018; Ainsworth et al., 2020). Wheat is ozone sensitive, and ozone is estimated to reduce annual wheat yields by 9.9% (Mills, Sharps, et al., 2018; Pleijel et al., 2018). The current understanding of how plants are affected by ozone have been well documented within reviews (Ainsworth et al., 2012; Emberson et al., 2018; Grulke and Heath, 2020): ozone enters the plant through stomata and, once inside the leaf cells, reacts to form Reactive Oxygen Species (ROS); if these are left unchecked by antioxidants they can ultimately lead to earlier senescence, reduced rates of photosynthesis, and subsequently, reduced assimilation and storage of carbon, with these reduced assimilates allocated to shoots in preference to roots.

3.1.2 Ozone's effect on grain

In wheat the ultimate effect is to reduce grain yields. Meta-analyses of the effect of ozone on wheat (Feng and Kobayashi, 2009; Feng et al., 2009; Broberg et al., 2015; Pleijel et al., 2018) recorded reductions in yield, grain number, and grain weight, with the effect increasing as ozone concentrations increased. Average yield reductions ranged from 8.4% at 36 ppb (Pleijel et al., 2018) to 26% at 77 ppb (Feng et al., 2009); grain number reductions from 1% or 2% at 30-35 ppb (Broberg et al., 2015; Pleijel et al., 2018) to 11% at 77 ppb (Feng et al., 2009); grain weight reductions from 3.6% at 36 ppb (Pleijel et al., 2018) to 18% at 77 ppb (Feng et al., 2009).

Loss of grain yield is thought to result largely from the reductions in assimilates caused by ROS damage and earlier senescence (Ainsworth et al., 2012; Emberson et al., 2018) which can also bring forward anthesis and the timing of grain maturity, thereby shortening the grain fill period (Gelang et al., 2000). Of the four meta-analyses, only Feng at al. (2009) distinguished between exposures at different growth stages (seedling, booting to anthesis, and grain filling). However, exposure to ozone at grain fill has appeared to result in the greatest yield losses, although the exact mechanism is unclear (Pleijel et al., 1998; Soja et al., 2000; Feng et al., 2009). These effects on grain fill have also been associated with a reduction in the proportion of starch and increase in the concentration of protein in the grain (Broberg et al., 2015).

Genetic variation in sensitivity to ozone between wheat cultivars has been noted, with older cultivars sometimes found to be more tolerant than recent ones (Barnes et al., 1990; Pleijel et al., 2006; Sarkar and Agrawal, 2010; Brewster et al., 2019), and some attempts have been made to systematically identify tolerant lines (Singh et al., 2018). Ozone has been found to have a similar effect on both spring and winter

wheat (Feng et al., 2009), and on experimental plants grown either in the field or in pots (Feng and Kobayashi, 2009). Although more winter wheat is grown in Northwest Europe compared to spring wheat, spring wheat still comprises a significant and increasing proportion.

The negative effects on wheat yields of other forms of abiotic stress have been widely studied and reviewed (Barnabás et al., 2008; Ullah et al., 2021). The creation of excess ROS is a common factor, with resulting effects on grain size, similar to those reported for ozone, linked to reductions in photosynthetic activity and assimilate production (Ullah et al., 2021).

3.1.3 Global wheat yields

Global wheat yields have dramatically increased over the past 60 years, from 233 million tons in 1960 at an average yield of 1.15 tons ha⁻¹, to 779 million tons in 2021 at 3.49 tons ha⁻¹ (USDA, 2022). This increased productivity has been generated in large part by an improved harvest index, resulting from an increase in the number of grains per spikelet (Calderini and Ortiz-Monasterio, 2003; Zhou et al., 2021) across the whole of the spike (Philipp et al., 2018), though with reduced average grain size (Flintham et al., 1997; F. Feng et al., 2018). If the predicted future demand for wheat is to be met however, productivity needs to be raised further to 5.9 t ha⁻¹ by 2050 (Reynolds et al., 2021). Increasing the size of these additional grains is now the target (Hawkesford et al., 2013) but achieving this will be challenging, not least in the face of increasing abiotic stress imposed on crops by both climate change and air pollution, including ozone (Reynolds et al., 2011; Knox et al., 2016; Mills, Sharps, et al., 2018; Bailey-Serres et al., 2019).

3.1.4 Synthetic wheat

Synthetic or re-synthesised wheat with its enhanced genetic diversity (Mujeeb-Kazi et al., 1996) is increasingly being used in wheat research and breeding programmes to improve yields (Li et al., 2018; Liu et al., 2018), with potential found to increase grain size (Rasheed et al., 2014) and improve abiotic tolerance (e.g. Bhatta *et al.*, 2018; Gorafi *et al.*, 2018), with one primary synthetic line having previously been found with high ozone tolerance (Brewster et al., 2019).

3.1.5 Use of µCT scans

X-ray micro-computed tomography (μ CT), a form of digital imaging, is being used increasingly within plant science as it enables non-destructive analysis of plant organs, the creation of three-dimensional (3D) images of plant structures, and the possibility of rapid automated phenotyping of desirable traits which would previously have been impossible or too time consuming (Rasheed et al., 2014; Strange et al., 2015). Within wheat grain research it has been used: to compare the grain location and morphology of commercial and landrace types (Strange et al., 2015); to assess the beneficial grain morphology traits and novel alleles within 230+ synthetic hexaploid wheat lines in combination with genome wide association mapping (Rasheed et al., 2014); to reveal differences in the grain morphology of wheat and its wild relatives (Hughes et al., 2019);

and to analyse how the outer and inner tissues of wheat grain morphology change through its growth stages (Le et al., 2019). It has also revealed effects of abiotic stress, such as reduced grain number across the middle of the spike induced by drought (Hughes et al., 2017), and reduced grain volume in the upper and lower spike regions in response to heat and drought (Schmidt et al., 2020). In genetic analysis undertaken in combination with μ CT image analysis of both conventional and synthetic cultivars, most marker trait associations for grain shape and size were found within the D genome (Ali et al., 2020). Synthetic wheat, which carries a higher percentage of the D genome than conventional bread wheat, is therefore a valuable resource for improving grain weight (Rasheed et al., 2018; Ali et al., 2020).

3.1.6 Aims of the study

This study aimed to identify the impact of ozone on grain formation and grain morphology, using μ CT digital imaging to provide a detailed picture of the effects within each spike. It also aimed to investigate the relationship between grain morphology, and grain quality, and in the process, to assess the relative ozone sensitivities of an elite cultivar (Paragon), a line of primary synthetic wheat (a different SHW line to that used in Trial 1), and a set of their derived backcross lines.

The hypotheses being tested were therefore:

- that the µCT digital imaging would reveal that ozone had no effect on the number of grains forming within each spikelet, nor on any of the grain morphology parameters, and with no differences found between the lines;
- that there would also be no relationship found between any changes to grain morphology and to grain quality;
- that no differences in ozone sensitivity (represented by yield parameters) would be found between any
 of the lines.

3.2 Materials and Methods

3.2.1. Plant establishment

The study was conducted in 2019 at the UK Centre for Ecology & Hydrology's air pollution facility, Abergwyngregyn, North Wales ($53.2^{\circ}N$, $4.0^{\circ}W$) using one line of primary synthetic hexaploid spring wheat (NIAB – SHW – 027), one line of the elite spring cultivar Paragon (*T. aestivum L*, cv. Paragon), and nine lines of F2 hybrids derived from them, each with the pedigree Paragon x (Paragon x NIAB SHW 027) (Table 3.1). The seed for all synthetic lines was obtained from the National Institute of Agricultural Botany (NIAB).

Line	Name used in text	Source	Datasets presented
<i>T. aestivum</i> L., cv. Paragon	Daragon (Dar)	DACT Coode	Biomass, yield, μCT scan,
(spring); released 1999	Paragon (Par)	RAGT Seeus	chlorophyll, grain quality
	Primary synthetic		Biomass, yield,
	hexaploid wheat (SHW)	INIAD	chlorophyll, grain quality
	DC1		Biomass, yield, μCT scan,
NIAB – SHVV – BC- 181 - 1	DCI	INIAD	chlorophyll, grain quality
	BC5	NIAB	Biomass, yield, μCT scan,
NIAB – 3HW – BC- 181 - 3			chlorophyll, grain quality
	BC7	NIAB	Biomass, yield, μCT scan,
NIAB – 3HW – BC- 181 - 7			chlorophyll, grain quality
NIAB – SHW – BC- 181 - 3	BC3	NIAB	Shoot, root, seedhead biomass
NIAB – SHW – BC- 181 - 4	BC4	NIAB	Shoot, root, seedhead biomass
NIAB – SHW – BC- 181 - 6	BC6	NIAB	Shoot, root, seedhead biomass
NIAB – SHW – BC- 181 - 8	BC8	NIAB	Shoot, root, seedhead biomass
NIAB – SHW – BC- 181 - 9	BC9	NIAB	Shoot, root, seedhead biomass
NIAB – SHW – BC- 181 - 10	BC10	NIAB	Shoot, root, seedhead biomass

Table 3.1. Wheat lines, with details of the 11 lines and origin of the seed.

Seeds were germinated (29 – 31 March) in petri dishes at room temperature, and germinated seedlings transplanted into modular plug trays of John Innes No.1 (Levington) low nutrient seed compost. Plants were vernalised for 30 days (2 April – 2 May, 16 h day length, 1.2 k lux light intensity, 5°C), then transplanted, two plants per pot, into 6L round, black, plastic pots (25 cm deep, 20 cm diameter at the rim) of sieved and mixed sandy-loam farm soil (Henfaes Research Station, Abergwyngregyn, North Wales) and grown on in a glasshouse for 3 weeks, with pots randomly rotated each 4 - 6 days. Each line had three replicate pots (each with two yield-bearing plants) per treatment. To maintain soil moisture levels, all pots were manually watered, daily or as required, for the duration of the study. Before the start of treatments there was 81.5 kg N ha⁻¹ of available nitrogen in the soil. 100 kg N ha⁻¹ of ammonium nitrate was applied to all lines, in split doses (Table S3.1, p.54). Fungicide and insecticide treatments were applied as necessary to maintain plant health, and applied to all plants in all treatments on each occasion (Table S3.2, p.54). An assessment which rated the extent of greenfly and/or powdery mildew using a simple index score was carried out on all plants on 9 July (Fig. S3.4, p.61).

3.2.2 Ozone treatment

At 'stem elongation' growth stages (GS3-37, Tottman and Broad, 1987) replicates were randomly selected for each treatment and randomly positioned in one of four identical hemispherical glasshouses (solardomes, 2.1 m high, 3 m diameter), on 28 May. During treatments, plants were also randomly redistributed within the domes every 7 - 10 days. Ozone treatments, starting 30 May, were achieved using Pulse Width Modulation (PWM)², with peak concentrations of low (30 ppb / control), medium (55 ppb), high (80 ppb) and very high (110 ppb),(Fig. 3.1), which were maintained for 11.5 weeks until all plants ripened and were harvested (19 Aug). To represent natural ozone exposure these concentrations were all reduced to ~20 ppb at night-time and for two days per week. The mean temperature inside the solardomes during the trial was 23.9°C. The environmental conditions of light, temperature and relative humidity are the same within each solardome (Hewitt et al., 2016; Harmens et al., 2019).

The solardomes were ventilated, with air changes occurring approximately twice per minute. Ozone was added to charcoal filtered air via PTFE tubing from a controlled injection system (ozone supplied by a G11 ozone generator, Ozone Industries, Andover, UK using oxygen from an oxygen concentrator Sequel 10, Pure O2, Urmston, UK), with computer-controlled concentrations (Lab VIEW version 2012, National Instruments, Austin, TX, USA). Two calibrated automatic ozone analysers (400a, Enviro Technology Services, Stroud, UK, and Thermo Scientific Model 49i Electron O3 Analyser, Fischer Scientific, Waltham, MA, USA) monitored the ozone concentrations every 30 minutes in each solardome.



Fig. 3.1. Mean hourly ozone profiles (replicated each week) over the course of ozone treatment (30 May – 19 Aug 2019), along with 24 hour mean and AOT40 values, where 24 hour mean represents the mean ozone concentration across the whole treatment period, and the AOT40 value represents the extent of exposure of plants to ozone concentrations above 40ppb during daylight hours.

² Pulse Width Modulation involves an ozone delivery solenoid valve being opened and closed once per second. It may be opened all that period to deliver 100% or maximum dosage, or 0% to give zero delivery. Regulation is achieved by adjusting the 'on' period within each one second time interval, so a 50% dosage represents a square control waveform ('on' for 500 milliseconds and 'off' for 500 milliseconds).

3.2.3. Chlorophyll Index

Non-destructive measurements of relative chlorophyll concentrations (SPAD units) were made (CCM 200, ADC Biosciences, UK), taking a measurement from the upper surface in the middle section of one flag leaf, one 2nd leaf, and a randomly selected lower leaf of one of the plants in each replicate pot at pre-anthesis, anthesis, and post-anthesis growth stages. Chlorophyll data collected on all lines but presented for Paragon, SHW, BC1, BC5, and BC7 only (Table 3.1).

3.2.4. Biomass and yield measurements

At harvest, fully ripened ears were cut at the base of the spike, and shoots cut just above the substrate. Roots were kept *in situ* in soil for 1 - 3 days before being wet sieved. Roots and shoots were dried in drying cabinets at 65°C for 14+ days then weighed. All ears from each replicate were weighed to obtain total seedhead biomass, and all ears threshed using a hand thresher (Minibatt+, Reichhardt Electronic Innovations, Hungen, Germany) for total grain weight. 1000 grain weight (TGW) was based on the weight of 100 randomly selected grains. Total grain weight and TGW data is presented for Paragon, SHW, BC1, BC5, and BC7 only.

3.2.5. Grain protein and starch

An assessment of grain protein and starch content was made with non-invasive near-infrared (NIR) spectroscopy (Wang and Paliwal, 2007; Caporaso et al., 2018). 10 - 100 + grains per replicate were processed at the John Innes Centre (JIC, Norwich, UK) on a Perten DA 7250 NIR analyser (Perten Instruments AB, Hägersten, Sweden), calibrated by the manufacturer, with additional use of industry standard wheat material from the recommended list varieties to calibrate protein levels for the protein tests. Data presented for Paragon, SHW, BC1, BC5, and BC7.

3.2.6. X-ray microcomputed tomography (μ CT) imaging

Based on seedhead, shoot, and root biomass data which indicated the response of lines to the higher ozone concentrations (Figs. S3.1 - S3.3, p.60), four lines with differing ozone response profiles were selected for μ CT imaging: Paragon and BC1 (more tolerant), and BC5 and BC7 (more sensitive). The SHW line could not be selected for scanning due to a high level of spike shattering, and several synthetic lines had poor growth e.g. BC10 or deformed spikes e.g. BC3. For each of the four selected lines, the three heaviest fully dried spikes from each of the three replicates in each treatment were selected for imaging. Scanning conditions were as described in Hughes et al. (2017). Each spike was imaged by being placed in an individual plastic holder and scanned using a μ CT 100 scanner (Scanco Medical, Switzerland) at the National Plant Phenomics Centre (Aberystwyth, UK). The resulting images were produced at a 0.2 megapixel (512 x512) resolution (68.8 μ m/pixel) in a proprietary ISQ file type format (Scanco Medical, Switzerland).

Previously developed MATLAB software (Hughes et al., 2017), freely available at <u>https://github.com/NPPC-UK/microCT_grain_analyser</u>, was used to extract data on grain morphology using the ISQ images. Grain length (major axis of whole grain), grain width and depth (major and minor axis of a cross-section at midpoint of the grain), grain volume (a count of the complete connected pixels per grain), and surface area (single pixel perimeter calculation mapped in three dimensions) were calculated. Data on grain positions along the spike were also extracted from the 3D images by MATLAB software using XYZ co-ordinates. 3D images for visualisation were created using Fiji 3D viewing plugin (<u>https://imagej.net/Fiji</u>; Jiang *et al.*, 2016) and 'TomViz' (<u>https://tomviz.org/</u>). Numbers of spikelets and grains per spikelet on each spike were also counted manually using these 3D images.

In order to minimise any false positive counts of e.g. rachis or fused grains, a size threshold was set for the segmentation software, and any outliers in the extracted data were identified, using the 0.025 upper and lower percentiles, and removed. In addition, data and grain cross-sections were visually checked for outliers. A comparison with the manually counted grain numbers per spike (using the 3D images) with the μ CT extracted grain number revealed some grains were excluded from the extracted μ CT grain morphology data (Table S3.3, p.55). The cause was either that the grains were under the size threshold settings or that some spikes were slightly taller than the plastic holder and one or two top-most grains were therefore omitted from the μ CT scan data. The greatest differences were seen in the very high (110 ppb) treatment: Paragon 21.5%, BC1 12.8%, BC5 6.1%, and BC7 19.6%. Although in most replicates the difference between extracted data and manual counts was less than 5%, the manually counted grain numbers per spike were used in Fig. 3.2c and in statistics.

3.2.7. Statistical tests

Statistical significance tests to assess the effect of ozone on each individual line (not between lines) were undertaken in R (version 4.0.2, R Core Team, 2020)(Tables S3.4, p.56 & S3.5, p.58). All model residuals and plots were examined and data transformed if necessary. Log transformations were applied to the following data sets: BC1 total grain weight, BC1 TGW, BC1 grain length, Paragon grain width and grain depth. Linear models (package 'stats' version 4.0.2) were used for all parameters apart from those with repeated measures. A linear mixed model (package 'nlme', Pinheiro *et al.*, 2017) was used for the chlorophyll index data, with growth stage included as a factor. Assessments of plots and model fit determined the inclusion of an autoregressive (AR) correlation term, with model fit tested using AIC comparisons. An ANOVA 'type 3' (package 'car', Fox and Weisberg, 2011) was used to summarise model outputs. Where necessary Tukey HSD post-hoc tests were applied (package 'multcomp', Hothorn *et al.*, 2008). Simple linear regression analysis was conducted in Excel (Microsoft Corporation, 2016).

3.3. Results

3.3.1. Ozone reduces spike number, total grain number and TGW

The primary synthetic hexaploid spring wheat (SHW) had more than double the number of spikes compared to all other lines (Fig. 3.2a) and a trend towards spike numbers increasing with higher ozone concentrations. Whilst TGW of SHW was similar to the other synthetic lines, the total grain weight was low. In all other lines there was a trend towards reduced spike numbers with increasing ozone.



Fig. 3.2. Number of spikes per pot (a), total grain weight (b), grain number per spike of μCT scanned lines only (c), and 1000 grain weight (d) for SHW, Paragon, BC1, BC5, and BC7 under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Error bars show standard error of the mean. For statistical P values see Table S3.4, p.56.

Whilst Paragon had the lowest spike number per pot, it had the highest total grain weight compared to the synthetic lines (Fig. 3.2b), as a result of having a much higher grain number per spike (Fig. 3.2c), and despite having the lowest TGW (Fig. 3.2d). Under very high ozone Paragon had a significant (42%, p = 0.0031) reduction in grain number compared to the 30 ppb control. Although BC1 had fewer grains per spike than Paragon, its TGW was greater and it therefore had a similar total grain weight. Whilst BC1 also had a significant 34% reduction in grain number under very high ozone (p = 0.036) the reductions in grain number were less than 2% under the medium and high ozone treatments (Table S3.6, p.59). Both BC5 and BC7 had

fewer grains per spike and lower total grain weights compared to Paragon and BC1, despite having higher TGWs than Paragon.

Under the very high (110 ppb) ozone treatment all lines had fewer grains per spike, reduced TGW, and reduced total grain weights. There was a significant negative effect of ozone on TGW in SHW, Paragon, BC1 and BC7 under the very high (110 ppb) treatment level compared to all other treatment levels (all p values < 0.05), but there were no significant differences between the other treatment levels in any line. The total grain weight reduction under high ozone was only statistically significant for Paragon (p = 0.001) and BC1 (p = 0.003), due to variability within the datasets of BC5 and BC7.

All lines in the low ozone concentration (30 ppb control), and some in the very high (110 ppb) concentration were also subjected to unplanned biotic stress in the form of greenfly and, to a lesser extent, powdery mildew (Fig. S3.4, p.61). Although this affected plant performance in the control plants of BC5 and BC7, the yield parameters of SHW, Paragon and BC1 were much less affected (Figs. 3.2b & 3.2d).

3.3.2. Ozone reduces the number of grains per spikelet

The genetic differences between the lines affected growth and yield parameters, including the number of grains per spikelet (Fig. 3.3), and as just described, the additional unplanned biotic stress also affected some lines (BC5 and BC7) more than others (Paragon and BC1). Of the four lines analysed by μ CT imaging, Paragon had the highest number of spikelets per spike and number of grains per spikelet compared to the other lines. BC1 had fewer spikelets and grain numbers per spike compared to Paragon, but greater grain numbers per spikelet compared to BC5 and BC7. Both Paragon and BC1 maintained higher numbers of grains per spikelet in the low, medium and high ozone treatments compared to BC5 and BC7, but had greatly reduced numbers of grains per spikelet under the very high (110 ppb) ozone treatment, with the largest losses in the middle region of the spike.

Across all treatment levels both Paragon (0.3%) and BC1 (2%) had very few spikelets which had no grains at all, whilst both BC5 (22%) and BC7 (24%) had far more. The complete loss of all grains in spikelets at the top and bottom of the spike rarely occurred in Paragon and BC1, but was frequent in BC5 and BC7. Plots marking the XZ locations of all grains in Paragon's replicates in each treatment (Fig. 3.4) show the reduced density of grains, both in the middle and at the top of the spike, as the ozone concentrations increase. A visual assessment of the 3D spike images showed that under lower ozone concentrations the middle region of the spike usually carried larger grains and higher grain numbers in both the inner and outer florets of each spikelet, whilst under higher ozone levels it carried smaller central grains with either no, or tiny aborted, grains in the outer florets.



Fig. 3.3. Number of grains per spikelet of Paragon (a), BC1 (b), BC5 (c), and BC7 (d) under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. The x axis represents spikelet position from stalk (1) to tip (21). Error bars show standard error from the mean (n = 9). Inset 3.3e: 2D screenshots of the 3D images of representative spikes of Paragon wheat illustrating the μ CT scanned images.



Fig. 3.4. Location (ZX co-ordinates) of every grain on the nine spikes of Paragon replicates in each treatment, 30 ppb (a), 55 ppb (b), 80 ppb (c), and 110 ppb (d). Y axis shows 'Z' co-ordinates (pixels), from stalk (0) to tip of spike. Point size is indicative of grain size.

3.3.3. Ozone changes grain morphology

The data extracted from μ CT grain images of Paragon, BC1, BC5 and BC7 show that elevated ozone also changes individual grain morphology (Fig. 3.5). Both grain volume and grain surface area were significantly reduced under the very high (110 ppb) ozone treatment, compared to the control, in Paragon (p = 0.02, p = 0.02 respectively) and BC1 (p = 0.001, 0.006 respectively), although not in BC5 or BC7 (Table S3.4, p.56). Paragon and BC1 had a grain volume reduction between the control and very high ozone of 33% and 49% respectively (Table S3.6, p.59).

There was a significant reduction in grain length under the very high (110 ppb) ozone treatment only in Paragon (p = 0.002), with a negative trend for BC1 (p = 0.05) and no effect on BC5 or BC7. Grain width was significantly reduced in the very high (110 ppb) ozone treatment in both Paragon and BC1 (p = 0.04 and 0.0002 respectively). The grain width of BC5 was only significantly different between the very high (110 ppb) and medium (55 ppb) treatment levels (p = 0.01), with a strong trend towards a negative effect of ozone on

grain width in BC7 between the low (30 ppb) and very high (110 ppb) treatments (p =0.05). There was a significant negative effect on grain depth in Paragon, BC1, and BC7 under very high (110 ppb) ozone compared to the control (all p values < 0.05), and in all four lines between the medium and very high treatment levels (all p values < 0.05).



Fig. 3.5. μCT extracted data showing grain volume (a), grain surface area (b), grain length (c), grain width (d), and grain depth (e), for Paragon, BC1, BC5, and BC7 under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Error bars show standard error of the mean. For statistical P values see Table S3.4, p.56.

Positive correlations between grain volume and other grain dimension parameters using data from all four μ CT lines suggest that the reductions in grain volume are driven more by reductions in grain width (R² = 0.84, p < 0.0001) and depth (R² = 0.86, p < 0.0001) than grain length (R² = 0.61, p < 0.0001) (Figs.3.6a – 3.6c). There was a strong positive correlation between grain number and grain weight (R² = 0.83, p < 0.0001), but a weak one between grain volume and grain weight (R² = 0.25, p < 0.001) suggesting that grain yield reductions were driven more by reductions in grain number than grain size (Figs. 3.7a & 3.7b).



Fig. 3.6. Simple linear regression analysis assessing relationship between grain volume and grain length (a), grain depth (b), and grain width (c), using combined data for Paragon, BC1, BC5, and BC7 under all ozone treatments. Linear regression trend lines represents data from all four lines.



Fig. 3.7. Simple linear regression analysis assessing relationship between grain weight (μ CT spikes) and grain number (a); and between grain weight (μ CT spikes) and grain volume (b) showing data for Paragon, BC1, BC5, and BC7 under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Linear regression trend lines represents data from all four lines.

3.3.4. Ozone increases grain protein and reduces grain starch

There was a positive effect of ozone on grain protein concentration and a negative effect on grain starch concentration in all lines as the ozone concentrations increased (Figs. 3.8a - 3.8b), although the effects were only statistically significant under the very high ozone concentration (110 ppb) compared to the control in SHW (p = 0.007 and 0.03 respectively), Paragon (p = 0.003, p = 0.01) and BC1 (p = 0.01, p = 0.0001) (*Table S3.4, p.56*). Overall, there were slightly lower protein concentrations in Paragon compared to the synthetic lines.

There was no correlation between grain protein and grain volume (all four μ CT lines, R² = 0.08, p = 0.048, Fig. 3.8c) even though it might have been expected that smaller grains would have a higher concentration of protein. In comparison, there was a strong negative correlation between grain number and grain protein (all four μ CT lines, R² = 0.71, p < 0.0001, Fig. 3.8d) suggesting a higher concentration of protein in a reduced grain yield. Compared to protein, there was a slightly stronger correlation between grain starch and grain volume (R² = 0.25, p = 0.0004) but a slightly weaker one between starch and grain number (R² = 0.44, p < 0.0001) (Fig. S3.5, p.62).



Fig. 3.8. Percentage of grain protein (a) and grain starch (b) found in the grain of SHW, Paragon, BC1, BC5, and BC7 under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Error bars show standard error of the mean. Fig. 3.8c and 3.8d. Simple linear regression analysis assessing relationship between protein percentage and grain volume (c); protein percentage and grain number (d), showing data for Paragon, BC1, BC5, and BC7 under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Linear regression trend lines represents data from all four lines. Inset: Fig. 3.8e. Examples of mid –grain µCT slices of Paragon grains, at 30 ppb (A) and 110 ppb (B).

3.3.5. Ozone reduces chlorophyll levels

Overall, in all lines, the highest chlorophyll levels were found in the flag leaves, and the lowest in the lower leaves at pre-anthesis, anthesis and post-anthesis growth stages (Fig. S3.6, p.63). Paragon had higher chlorophyll levels compared to the other lines. Chlorophyll levels in the fully expanded pre-anthesis flag leaves of all five lines showed no effect of ozone across all four treatment levels (Table S3.5, p.58). At anthesis, the flag leaves of all lines (apart from BC1 under high ozone) maintained their chlorophyll levels in the low, medium and high ozone concentrations but had reductions compared to the control under the very high (110 ppb) treatment; this was significant for SHW (p = 0.01), Paragon (p = 0.006) and BC1 (p = 0.001) but not for BC5 (p = 0.06) or BC7 (p = 0.07). At anthesis both the second and lower leaves were also significantly reduced under the highest ozone concentration (110 ppb), compared to the control, in Paragon (p = 0.02 and 0.007 respectively) and BC1 (p = 0.01 and 0.03 respectively). Chlorophyll levels in post-anthesis flag leaves were substantially reduced in all lines and in all treatments, with no effect of ozone.

3.3.6 Ozone reduces shoot, root and seedhead biomass

Preliminary data collected prior to the selection of the four uCT lines from all 11 lines showed some variation in shoot (Fig. S3.1, p.60) and root biomass (Fig. S3.2, p.60) between the lines, with the line of primary SHW having both smaller shoot and root biomass compared to all the other lines, and Paragon having the largest root. The highest ozone treatment affected both root and shoot growth in all lines, but with a greater effect on root growth.

Seedhead biomass data (spikes and their in-tact grains combined) collected on all 11 lines (Fig. S3.3, p.60) showed that Paragon and BC1 had the highest levels of seedhead biomass compared to SHW and all the other synthetic lines, with a strong effect of the highest ozone treatment in suppressing yield, although the effect of the high (80 ppb) treatment varied by line, with some showing more tolerance e.g. Paragon and BC1, and some more sensitivity e.g. BC5 and BC7.
3.4 Discussion

Ozone caused yield reductions and changes in grain quality, with variations in the magnitude of the response between the different lines. However, the μ CT scans provided new insights and greater detail concerning where on the spike grain number is reduced, and how the grain size and shape changes with elevated ozone.

3.4.1. Effects on grain number

In this study reductions in grain number per spike had a greater influence on yield loss than reductions in grain volume. This is in contrast to previous meta-analyses assessing ozone's effect on wheat grain, where it was found that reduced grain size was the main driver of yield reductions rather than loss of grain number (Broberg et al., 2015; Pleijel et al., 2018).

Total grain number is determined by number of spikes, number of spikelets, and the final number of surviving fertilised florets which form grains within each spikelet. Data from this study support previous findings on ozone's effects on spike numbers, where ozone reduced spike numbers per plant by 5% (Feng et al., 2009). The early vegetative stages of wheat growth determine how many fertile spikes survive from the initial tillers, and the loss of these initial tillers can occur at any time, though usually between GS31 – GS59 (AHDB, 2021). Tiller removal has also been found to lead to higher levels of fertile florets in the remaining spikes in a compensatory mechanism (Guo and Schnurbusch, 2015), and this study provides some evidence to support this as, in Paragon and BC1, grain yields were maintained under high ozone despite reduced spike number.

The number of grains per spike has a major effect on total grain yield (Griffiths et al., 2015). Paragon had both more spikelets per spike, and higher grain numbers per spikelet, than the synthetic wheat lines. Like other modern cultivars Paragon benefits from the *Rht* reduced height gene, with an improved harvest index and increased floret fertility and grain number, as the shorter stem reduces the competition for assimilate between stem growth and reproduction (Fischer, 2011; F. Feng et al., 2018). Although Paragon also demonstrated greater tolerance to ozone compared with the synthetic lines, and the ability to generate the same quantities of grain at 80 ppb as the other lines were producing at lower ozone levels, it also had one of the highest reductions in grain number under the highest ozone level. The μ CT scans revealed that whilst the middle of the spike carried the most grain in all lines, as has been found elsewhere (Hughes et al., 2017; Zhou et al., 2021), this region also saw the greatest reductions in grains per spikelet when under very high ozone, especially in Paragon. Research on Chinese cultivars (F. Feng et al., 2018) has found that the higher yields found in cultivars released in the 2010s, compared to those from the 1950s and 1990s, were achieved partly through an increase in grain number per spikelet, with average grain numbers increasing particularly in the lower and middle regions of the spike. As the μ CT scans revealed, it is these additional grains which are lost in response to high ozone, with the reductions in average grain number per spikelet driving the overall reduction in grain number rather than any reductions in the number of spikelets.

The spike elongation growth stages before anthesis largely determine eventual grain number, with floret development and fertilisation, as well as floret death rates, being controlled in large part by assimilate and nitrogen status and C:N partitioning (Fischer, 2011; González et al., 2011; Sreenivasulu and Schnurbusch, 2012). Pre-anthesis and anthesis chlorophyll measurements covering these growth stages showed ozone reduced associated chlorophyll levels in many lines, particularly in the second and lower leaves, creating potential for a subsequent increase in floret death.

Damage to reproductive organs and processes can also reduce grain number, with research on multiple plant species finding that ozone affected pollen and ovule development, anthesis, and seed development (Black et al., 2000; Leisner and Ainsworth, 2012). Previous research into cold and drought stress in wheat found that the critical factor for floret survival was the plants ability to maintain the supply of sugars to the tapetum which surrounds the microspores (Ji et al., 2010), a structure particularly sensitive to abiotic stress (Parish et al., 2012), especially in self-fertilising crops such as rice, wheat, barley, oil seed rape, and sorghum (Ji et al., 2011; Parish et al., 2012), all of which are known as ozone-sensitive crops. As higher antioxidant levels are associated with tolerance of the tapetum to abiotic stress (Dolferus et al., 2011), and ozone tolerance is also thought to be related to antioxidant levels (e.g. Feng *et al.*, 2016), further research focused on ozone's effect on the tapetum would be beneficial. This issue also has implications for modelling the effects of ozone on wheat, and emphasises the need to take into account the timing of ozone episodes in relation to the sensitive reproductive growth stages, including meiosis as well as anthesis.

3.4.2. Effects on grain size and quality

TGW has been found to correlate with grain size (Millet and Pinthus, 1984; Ali et al., 2020) and in this trial both TGW and individual grain volume were reduced under the highest ozone treatment in all lines, although both Paragon and BC1 showed tolerance to high ozone by maintaining their grain volume. Grain length develops early in grain formation, whilst width and depth gradually increase as the inner part of the grain is filled during the mid-phase of grain development (Le et al., 2019). The very high ozone treatment led to reductions in length, width, and depth in all lines, but the correlations between grain volume and each of these parameters indicated that it was largely the reductions in grain width and depth that were driving the reductions in grain volume.

Although not quantified, examples of the μ CT cross sections of grains showed the effect of high ozone levels both in the shrunken size of grains and in the internal voids within the grain. These internal voids form within the endosperm during normal grain development, then later fill with sucrose and fructans (Ho and Gifford, 1984; Le et al., 2019). Broberg et al (2015) suggest that reduced TGW in ozone affected wheat is likely to be linked to reduced starch formation, and these shrunken grains and internal void spaces under

high ozone would support this. An increase in void spaces has been found in wheat exposed to both heat and drought (Schmidt et al., 2020).

In this trial, the higher ozone levels led to a typical reduction in grain starch and an increase in grain protein concentration. The increase in the proportion of protein in ozone-affected grain (Wang and Frei, 2011; Pleijel and Uddling, 2012; Broberg et al., 2015; Broberg et al., 2020) is thought to be due to ozone having less effect on protein synthesis compared to carbon synthesis with a resulting concentration effect (Pleijel et al., 1999; Emberson et al., 2018). One additional contributory factor may be linked to ozone causing a reduction in both the size and number of grains per floret, as found in this study. The concentration of both macro and micro nutrients found in grains varies with their position on the spike and within the spikelet (Calderini and Ortiz-Monasterio, 2003), so a loss of the smaller, outlier grains which normally have reduced levels of nutrients compared to other grains, both within each spikelet and in the less productive lower and upper regions of the spike, could lead to a higher concentration of protein in the remaining larger more central grains.

Research on other abiotic stress effects on wheat grain size and quality suggest the effects are similar to ozone (Sehgal et al., 2018; Schmidt et al., 2020). Wheat grains develop and fill in stages: early (cell division), mid (cell expansion), and late (desiccation) (Altenbach, 2012). High temperatures or drought have been found to accelerate and shorten these stages, leading to reduced starch content and smaller, lighter grains with a higher proportion of protein (Altenbach, 2012; Tomás et al., 2020; Schmidt et al., 2020; Cohen et al., 2021). As with ozone (e.g. Pleijel *et al.*, 1998) the effect of drought on yields has been found to be most noticeable in the first 14 days after anthesis, in the early stage of grain fill (e.g. Gooding *et al.*, 2003), and greater tolerance shown in the later stages (Altenbach, 2012; Cohen et al., 2021), with any abiotic tolerance at meiosis not necessarily maintained at anthesis or during grain fill (Ji et al., 2010) as these later growth stages have been found to be particularly vulnerable to abiotic stress (Dolferus et al., 2011).

Whilst the reduced starch formation in ozone-affected wheat is thought to be linked to this shortened grain fill period, induced by earlier senescence (Gelang et al., 2000), under optimal conditions Borrill et al. (2015) found final grain weight to be more closely linked to starch synthase activity in later grain fill stages, and not to the timing, or length, of senescence. Although it was noted that in stress environments the earlier senescence still affects yield (Borrill et al., 2015), reductions in starch synthase activity and the size of starch granules have been found in response to abiotic stress (Ullah et al., 2021). This finding suggests the underlying mechanisms affecting the protein and starch components of grain fill in wheat may be separate. The correlations between protein and starch levels and grain morphology data in this study support this, with strong correlations between protein and grain number but not grain volume, and weaker correlations between starch and both grain number and grain volume.

52

3.4.3. Potential use of synthetic wheat and μ CT digital imaging for plant breeding

Increasing grain size, and maintaining it in stress environments, are key to improving future wheat yields (Reynolds et al., 2011). Although some modern cultivars have been found to be more ozone sensitive than older ones (Barnes et al., 1990; Pleijel et al., 2006; Sarkar and Agrawal, 2010) the elite cultivars – as Paragon showed - may still be more productive due to their enhanced grain number (Johnston and Poulton, 2018; Zhou et al., 2021). In this study, the F2 line BC1 demonstrated a similar ozone tolerance to Paragon, whilst also carrying the larger grain size shown by all the synthetic lines, although it carried fewer grains per spike than Paragon. Further research with additional replication in order to confirm the effects of ozone on the synthetic lines found in this study would be beneficial.

The use of μ CT imaging on ozone-affected field-grown wheat would enable further assessment of ozone's effects on grain, and an assessment of whether the tolerance exhibited by some lines in this study also occurs in the field. The increasing availability of μ CT digital imaging now enables *in vivo* non-destructive analysis of grain formation and retention of grain/spikelet positional information. This, in combination with histological analysis on reproductive structures (Tracy et al., 2017) and genetic mapping of target traits (Rasheed et al., 2014; Schmidt et al., 2020), could provide a route to screening for ozone tolerant lines.

3.5 Conclusion

The effects of ozone on wheat yields, grain formation, and grain quality were demonstrated by this study. μ CT digital imaging revealed that the middle section of the spike is the region where the largest reductions in grain number per spikelet occur, with width and depth reductions driving the loss of grain volume. Correlations between yield parameters and grain number/size suggest that in this study reductions in grain number had a greater influence on yield reductions than grain size. The elite cultivar Paragon demonstrated some degree of ozone tolerance by maintaining both high grain number and also grain volume under the high ozone treatment, whilst BC1, with its larger grain size typical of synthetic wheat, had also acquired the ozone tolerance of Paragon and therefore offers potential for abiotic tolerance in combination with larger grain size. The use of μ CT image analysis makes it more feasible to achieve automated screening of large populations to assess lines for improved grain yield components and abiotic tolerance.

Supplementary materials

Table S3.1. Applications of ammonium nitrate fertiliser: date, quantity, and growth stage of line: Booting (GS41-47),Ear emergence (GS51-59), Flowering/Anthesis (GS61-69), Milk development (GS71-77). Relevant lines in bold.

Date	Lines	Doses applied	Approximate growth stage/s of lines at time
		(equivalent kg ha ^{⁻¹})	of N addition (See Table S1.1, p.15)
4 June	All lines	25 kg ha ⁻¹	SHW – Ear emergence;
		.	All other lines – Booting
11 June	SHW , BC3, BC9	50 kg ha ⁻¹	SHW-Flowering/anthesis);
			BC3-Booting; BC9-Booting
12 June	BC1	50 kg ha ⁻¹	BC1-Booting
18 June	Paragon, BC4,	50 kg ha ⁻¹	PAR-Ear emergence; BC4-Ear emergence;
	BC5 , BC6	0	BC5-Ear emergence;
			BC6-Booting/Ear emergence
19 June	BC7 , BC8, BC10	50 kg ha ⁻¹	BC7- Ear emergence; BC8-Booting/Ear
			emergence; BC10-Booting
9 July	SHW	25 kg ha ⁻¹	Ripening
16 July	BC1 , BC9	25 kg ha ⁻¹	Milk development/Dough development
17 July	BC3, BC4, BC7	25 kg ha ⁻¹	Milk development/Dough development
23 July	Paragon, BC5	25 kg ha ⁻¹	Milk development/Dough development
24 July	BC6, BC8, BC10	25 kg ha ⁻¹	Milk development/Dough development

Table S3.2. Timing of fungicide and insecticide treatments applied to maintain plant health, and applied to all plants in all ozone treatments on each occasion. Extent of greenfly and powdery mildew recorded on 9 July (See Fig. S3.4, p.61).

Date	Type / name	Active ingredients	Application method and rate	Approximate growth stage
17 th May	Fungicide: 'Nativo'	Tebuconazole Trifloxystrobin	Spray: 20ml/L	Tillering
19 th May	Fungicide: 'Nativo'	Tebuconazole Trifloxystrobin	Spray: 20ml/L	Tillering
28 th May	Fungicide: 'Fandango'	Fluoxastrobin Prothioconazole	Spray: 5ml/L	Stem elongation
7 th June	Fungicide: 'Corbel'	Fenpropimorph	Spray: 2.5ml/L	Booting
20 th June	Insecticide: Provado	Deltamethrin	Soil drench: 10ml/L	Ear emergence
26 th June	Insecticide: Provado	Deltamethrin	Soil drench: 10ml/L	Flowering
2 nd July	Fungicide: Cyflamid	Cyflufenamid	Spray: 2.5ml/L	Milk/dough dev't
18 th July	Insecticide: Provanto	Thiacloprid	Spray: 1.0 ml/L	Milk/dough dev't

Table S3.3. Difference in total grain number counts between manual counts using 3D images and μ CT extracted data.

μCT error margins		actual	estimated		
		Counted from 3D images	μCT extracted data	Difference	% change
PAR	30	585	535	50	8.5
	55	524	472	52	9.9
	80	542	534	8	1.5
	110	247	194	53	21.5
BC1	30	389	384	5	1.3
	55	383	382	1	0.3
	80	386	374	12	3.1
	110	258	225	33	12.8
BC5	30	191	189	2	1.0
	55	240	237	3	1.3
	80	204	205	-1	-0.5
	110	114	107	7	6.1
BC7	30	152	148	4	2.6
	55	353	353	0	0.0
	80	188	178	10	5.3
	110	189	152	37	19.6

Table S3.4. Grain morphology and grain quality parameter p values, representing levels of statistical significance of the effect of ozone on each line between the different levels of ozone treatment. Significant values (p < 0.05), trend (p > 0.05 and < 0.1).

	LOW - MED	low - High	low- Vhigh	MED - HIGH	MED - VHIGH	high- Vhigh
Grain vol	ume					
Paragon	-	-	0.0245485	-	0.0045044	0.0043407
BC1	-	-	0.0011805	-	0.0125530	0.0063060
BC5	-	-	0.0570816	-	0.0023280	0.0308703
BC7	-	-	-	-	-	-
Grain sur	face area					
Paragon	-	-	0.0222768	-	0.0103565	0.0157267
BC1	-	-	0.0068241	-	0.0605908	0.0257775
BC5	0.0146625	-	-	0.0377334	0.0013222	-
BC7						
Grain cire	rularity					
Paragon	0.0325771	0.0325167	0.0341611	_	-	-
BC1	-	-	-	_	_	
BC1	_	_	_	0.0450210	_	
BCJ BC7	-	-	-	0.0459219	-	-
BC/	-	-	-	-	-	-
Grain len	gtn		0.00000040		0.0000502	0.0010606
Paragon	-	-	0.0023646	-	0.0006503	0.0010686
BC1	-	-	0.0577589	-	0.0569762	0.0995372
(log)						
BC5	-	-	-	-	0.0900567	-
BC7	-	-	-	-	-	-
Grain wid	dth					
Paragon	-	-	0.0461920		0.0208987	0.0148496
(log)						
BC1	0.0167546	-	0.0002849	-	0.0278598	0.0031197
BC5	0.0615379	-	-	-	0.0125659	0.0819766
BC7	-	-	0.0571222	-	-	-
Grain de	pth					
Paragon	-	-	0.0063660		0.0015943	0.0013055
(log)						
BC1		-	0.0001185	-	0.0014269	0.0005110
BC5		-		-	0.0117558	0.0291025
BC7	-	-	0.0434072	-	0.0423167	-
Total gra	in weight					
SHW	0.0467600		0.0005651	0.0533550	0.0000353	0.0005149
Paragon	-	-	0.0010327		0.0002610	0.0002848
BC1		-	0.0036437	-	0.0080384	0.0096053
(log)						
BC5	-	-	-	-	-	-
BC7	-	-	-	-	-	-
1000 grai	in weight					
SHW	0.0506722	-	0.0083483	-	0.0002592	0.0022642
Paragon	-	0.0554387	0.0017695	-	0.0015212	0.0000894
BC1	-	-	0.0016885	-	0.0229337	0.0043452
(log)						
BC5	-	-	0.0836939	-	0.0060885	0.0274004
BC7	-	-	0.0082736	-	0.0030687	0.0102050
Grain nu	mher ner sni	ike	0.0002700		01000000	0.01010000
Paragon	-	-	0.0031343	-	0.0168019	0.0100077
RC1	_	_	0.0360003	_	0.0452/20	0.0421082
BCS	_	_	-	_	-	5.0721302
BC3	-	-	-	-	-	-
Spiles /	-	-	-	-	-	-
эріке/еа	number					
SHW	-	-	-	-	-	-
Paragon	-	-	-	-	-	-
BC1	-	-	-	-	-	-
BC5	-	-	-	-	-	-
BC7	-	-	-	-	-	-

Table S3.4 cont'd. Grain morphology and grain quality parameter p values, representing levels of statistical significance of the effect of ozone on each line between the different levels of ozone treatment. Significant values (p < 0.05), trend (p > 0.05 and < 0.1).

	LOW - MED	LOW - HIGH	LOW - VHIGH	MED - HIGH	MED - VHIGH	HIGH- VHIGH
Protein						
SHW (log) Paragon BC1 BC5	0.0423417	-	0.0079700 0.0036195 0.0187527 -	0.0984529 -	0.0023692 -	0.0174587 0.0119055 -
BC7	-	-	-	-	-	-
Starch						
SHW (log)	-	-	0.0330708	-	-	0.0524502
Paragon	-	-	0.0110209	-	0.0074165	0.0200046
BC1	0.0308426	0.0121916	0.0001336	-	0.0050899	0.0121916
BC5	-	-	-	-	-	-
BC7	-	-	-	-	-	-

Table S3.5. Chlorophyll index parameter p values, representing levels of statistical significance between the different levels of ozone treatment for each line, at pre-anthesis, anthesis, and post-anthesis growth stages. Significant values (p < 0.05), trend (p > 0.05 and < 0.1).

PRE-ANTHESIS FL	AG					
	LOW - MED	LOW - HIGH	LOW - VHIGH	MED - HIGH	MED -VHIGH	HIGH-VHIGH
SHW	-	-	-	-	-	-
Paragon	-	-	-	-	-	-
BC1	-	-	-	-	-	-
BC5	-	-	-	-	-	-
BC7	-	-	-	-	-	-
PRE-ANTHESIS 2 nd	LEAF					
P < 0.05	LOW - MED	LOW - HIGH	LOW - VHIGH	MED - HIGH	MED -VHIGH	HIGH-VHIGH
SHW	-	-	0.081793	-	0.019849	-
Paragon	-	-	-	-	-	-
BC1	-	-	-	-	0.07628	-
BC5	-	-	-	-	-	-
BC7	-	-	-	-	-	-
PRE-ANTHESIS LO	WER LEAF					
P <0.05	LOW - MED	I OW - HIGH	I OW - VHIGH	MFD - HIGH	MFD -VHIGH	нісн-унісн
SHW/	-	-	-	-	-	-
Paragon	_	-	-	-	_	-
BC1	_	-	-	-	_	-
BC5	-	-	-	-	-	-
BC7	-	-	-	-	-	-
ANTHESIS FLAG						
P <0.05	LOW - MED	IOW - HIGH		MED - HIGH	MED -VHIGH	HIGH-VHICH
SHW	-	-	0.010697	-	0.008179	0.006018
Paragon		_	0.006172	_	0.008179	0.022850
		_	0.000172	0.052022	0.023303	0.033833
BCS		_	0.06648	0.033332	0.000813	0.089094
BCJ BC7		_	0.00048	_	0.070113	
	-		0.07324			
P < 0.05	LOW - WED	LOW - HIGH	LOW - VHIGH	NED - HIGH	MED -VHIGH	HIGH-VHIGH
SHW	-	-	-	-	-	-
Paragon	-	-	0.027644	-	-	0.075663
BCI	-	0.037622	0.016254	-	-	-
BCS	-	-	-	-	-	-
ANTHESIS LOW/ER	-	-	0.076408	-	-	-
P < 0.05	LOW - WED	LOW - HIGH		IVIED - HIGH	IVIED -VHIGH	пібп-упібп
SH W	-	-	-	-	-	-
Paragon PC1	-	-	0.007134	-	-	-
BCI	-	0.032408	0.059005	-	-	-
BC3	-	-	-	-	-	-
POST-ANTHESIS F		-	-	-	-	-
Paragon	-	-	-	-	0.026238	-
	-	-	-	-	-	-
BCI	-	-	-	-	-	-
BC7	-	-	-	-	-	-
DOT ANTHERE 3		-	-	-	-	-
	LOW - WIED	LOW - HIGH	LOW - VHIGH	NED - HIGH	NED -VHIGH	пібн-упібн
Paragon	-	-	-	-	-	-
PC1	-	-	-	-	-	-
BC1 BC5	-	-	-	-	-	-
BC3	-	-	-	-	-	-
DOT ANTHESIS I		-	-	-	-	-
				MED JUCH		
	LUW - WIED	LOW - HIGH	LOW - VHIGH	IVIED - HIGH	WED -VHIGH	
Daragen	-	-	-	-	-	-
Falagon	-	-	-	-	-	-
BCI	-	-	-	-	-	-
BC3	-	-	-	-	-	-
DC/	-	-	-	-	-	-

Table S3.6. Grain morphology and grain number percentage differences between the control (30 ppb) and each elevated ozone concentration for each line.

		% diff fr	% diff from 30 ppb control to:		
Parameter	Line	50 ppb	80 ppb	110 ppb	
		44.62	44.00	22.04	
volume	Paragon	11.62	11.89	-33.01	
	BC1	-15.67	-11.43	-49.02	
	BC5	19.62	3.49	-24.67	
	BC7	3.57	-9.95	-28.82	
surface area	Paragon	2 98	1 3/	-19.64	
Surface area	BC1	-9.24	-5 75	-15.04	
	BCI	-5.24	-5.75	-20.73	
	BC3	27.43	4.64	-13.34	
	BC7	1.69	-7.50	-10.51	
circularity	Paragon	9.88	9.88	9.78	
	BC1	-2.57	-4.78	-15.97	
	BC5	-17.36	5.56	-7.62	
	BC7	-3.57	-1.66	-7.40	
length	Paragon	1.52	0.91	-7.06	
	BC1	0.05	-1.62	-13.52	
	BC5	0.53	-8.34	-12.10	
	BC7	5.17	1.01	-4.13	
width	Paragon	3.16	4.15	-15.15	
	BC1	-10.63	-6.09	-20.26	
	BC5	17.62	9.77	-6.67	
	BC7	-3.41	-7.53	-15.64	
depth	Paragon	5.19	5.99	-20.93	
	BC1	-8.11	-5.02	-26.93	
	BC5	10.78	7.93	-7.30	
	BC7	0.11	-4.94	-19.93	
grain number	Paragon	-10.43	-7.35	-41.71	
(manual count)	BC1	-1.54	-0.77	-33.68	
	BC5	25.65	6.81	-40.31	
	BC7	132.24	23.68	24.34	



Fig. S3.1. Dry shoot biomass (g) in all lines under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Error bars show standard error of the mean (n=3).



Fig. S3.2. Dry root biomass (g) in all lines under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Error bars show standard error of the mean (n = 3).



Fig. S3.3. Seedhead biomass (g) in all lines under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Error bars show standard error of the mean (n = 3).



Fig. S3.4. Rating Index of extent of greenfly and powdery mildew on all lines on 9 July in 30 ppb (a), 55 ppb (b), 80 ppb (c) and 110 ppb (d), where 0 = none, 1 = slight, 2 = moderate, 3 = heavy, 4 = severe.



Fig. S3.5. Simple linear regression analysis assessing relationship between grain starch and grain number (a), and grain starch and grain volume (b), using combined data for Paragon, BC1, BC5, and BC7 under all ozone treatments. Linear regression trend lines represents data from all four lines.



Fig. S3.6. Chlorophyll index levels (SPAD units) of flag leaf, 2nd leaf, and one lower leaf sampled at pre-flowering, flowering and post-flowering growth stages in SHW (A), Paragon (B), BC1 (C), BC5 (D), and BC7 (E). Error bars show standard error of the mean (n=3).

Ozone reduces wheat's whole canopy chlorophyll levels, Nitrogen Remobilisation Efficiency, and increases residual foliar nitrogen, with effects ameliorated by additional nitrogen fertilisation

Declaration of contributions:

Conception, methodology and design: Clare Brewster (CB), Felicity Hayes (FH), Natalie Fenner (NF); *Collection and assembly of data*: CB; *Analysis, interpretation of the data, and drafting of the chapter*: CB; *Critical revision and final approval of content*: CB, FH, NF; *Additional technical support*: Ed Rowe* (Stable Isotope calculations).

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Abstract

Nitrogen fertilisation is essential to maintaining and improving wheat yields. The interaction between nitrogen storage and translocation, senescence, and late phase photosynthesis is critical to the postanthesis grain fill period. Ozone's effect on nitrogen storage and translocation, and on C:N dynamics within the wheat plant, is not well understood. This study exposed a widely grown elite spring wheat cultivar, cv. Skyfall to four levels of ozone (30 ppb, 45 ppb, 70 ppb, 85 ppb) and two levels of nitrogen fertilisation, 140 kg ha⁻¹ and 160 kg ha⁻¹, with the higher rate including an addition of 20 kg N ha⁻¹ at anthesis. Ozone triggered earlier senescence in the 4th, 3rd and 2nd leaves but not the flag leaf, with a similar pattern of reduced chlorophyll content in the leaf cohorts taking place before senescence became visible. At anthesis there was no evidence of an effect of ozone on nitrogen storage in upper plant parts. High ozone increased residual nitrogen found within plant parts at harvest, with concomitant reductions in C:N ratios and Nitrogen Remobilisation Efficiency. Nitrogen fertilisation applied at anthesis appeared to ameliorate the effect of ozone on nitrogen content and nitrogen translocation. Despite the grain yield being abnormally low, the application of ¹⁵N ammonium nitrate at anthesis confirmed that the majority of post-anthesis nitrogen uptake went to the ear/grain at harvest, with no effect of ozone, although this late addition of nitrogen was found to ameliorate the effect of ozone on other parameters. These data can inform future modelling of ozone's effect on wheat yields.

4.1 Introduction

4.1.1 Ozone, wheat and nitrogen

Ground level ozone is a secondary air pollutant which continues to have a detrimental effect on global wheat yields. Reports on ozone's effects on wheat in Europe and North America (Pleijel et al., 2019), India (Mukherjee et al., 2021), China (Wang et al., 2021) and Africa (Hayes et al., 2020) suggest ozone causes 4-15% annual yield reductions. Ozone concentrations are highest in the northern hemisphere where higher levels of ozone's precursor gases are emitted and circulate (Fleming et al., 2018). Concentrations range from <30ppb in the northern latitudes of Europe and North America to >100ppb in parts of western USA, southern Europe, and east Asia (Fleming et al., 2018). With ozone concentrations predicted to continue at levels which are phytotoxic to wheat (Revell et al., 2015; Mills, Pleijel, et al., 2018) efforts to more effectively model predicted yield losses (Emberson et al., 2018; Feng et al., 2022), and to develop more ozone tolerant cultivars (e.g. Ainsworth, 2017) are more essential than ever.

Correlations between elevated ozone, earlier senescence, reduced chlorophyll levels and photosynthetic capacity, and subsequent grain yield reductions in wheat, have been extensively researched (Amundson et al., 1987; Pleijel et al., 1997; Gelang et al., 2000; Xu et al., 2009; Osborne et al., 2019; Feng et al., 2022). The same effects are also found in response to many other abiotic stresses (Sade et al., 2018; Sultana et al., 2021), the common cause being oxidative damage within plant cells generated by higher levels of Reactive Oxygen Species (ROS) (Khanna-Chopra, 2012).

Nitrogen fertilisation is essential to plant growth and maintaining higher wheat yields, but the increasing costs of nitrogen fertilisation, and the increasing nitrogen pollution which inevitably arise as a result of wheat's low nitrogen use efficiency (NUE), mean that efforts to improve NUE in wheat – and to avoid reductions in NUE due to abiotic stress – are considered to be essential (Hawkesford, 2017). Ozone's effect on nitrogen storage and translocation, and on C:N partitioning within the plant, is not well understood and is the subject of this study.

4.1.2 Senescence and nitrogen partitioning

Wheat is an annual plant which undergoes monocarpic senescence, systematically dismantling and translocating mineral nutrients to the developing grains whilst the gradually shrinking area of photosynthetically active green shoot continues to generate photosynthate until shortly before the plant completely dies and the surviving grain is harvested; it is a process as highly genetically controlled as any other phase in its development (Gregersen et al., 2013).

Whilst the majority of wheat's grain starch is generated directly by post-anthesis photosynthesis, up to 90% of the grain protein comes from the nitrogen stored prior to anthesis in leaves, stems, ear and root which is then released during senescence and grain fill (Kichey et al., 2007; Bogard et al., 2010; Kong et al., 2016). Because nitrogen is mainly stored in organic form in the plant, mostly as proteins and amino acids within chlorophyll, nitrogen translocation is dependent upon senescence to break down chlorophyll before nitrogen is then translocated via the phloem to the ear and grain (Avila-Ospina et al., 2014; Maillard et al., 2015). A lesser but still important proportion of grain nitrogen can also be taken up via the roots in the post-anthesis growth phases (Kichey et al., 2007; Kong et al., 2016).

In wheat, the leaves, stems and leaf sheaths are the most important source of stored nitrogen (Gaju et al., 2011; Kong et al., 2016), with levels of flag leaf nitrogen remobilisation found to correlate with nitrogen yield parameters (H. Wang et al., 2008). Stem nitrogen is an important nitrogen pool at anthesis and can be an indicator of grain nitrogen (Barraclough et al., 2014), whilst ears can also store large amounts of preanthesis nitrogen before transfer to the grain in early grain fill (Kong et al., 2016; Tambussi et al., 2021). The nitrogen content of plant parts at the start of grain filling is crucial for both efficient nitrogen remobilisation during grain filling as well as ongoing photosynthesis for carbon assimilation (Kong et al., 2016). Wheat root biomass normally peaks during the grain fill growth stages (Andersson et al., 2005), and the roots play a crucial role in this critical phase, with nitrogen being translocated from 'source' leaves both indirectly via the roots, as well as directly to the ear and grain 'sinks' (Fig. 4.1., Simpson *et al.*, 1983).

The rate of senescence affects the rate of nitrogen remobilisation (Gregersen et al., 2008), and accelerated senescence drives a higher rate of nitrogen remobilisation, leading to a higher grain protein content but a lower total grain yield due to the shortened period available for assimilation (Triboi and Triboi-Blondel, 2002). In sub-optimal nitrogen environments, delaying post-anthesis senescence improves final yields (Gaju et al., 2011).

Within ozone research there is a growing consensus that premature and accelerated senescence is one of the primary factors driving yield reductions in ozone-affected field crops, with the triggering of early senescence now a key component within modelling work being undertaken to more accurately estimate the effects of ozone on global wheat yields (Emberson et al., 2018). Whole canopy photosynthesis determines grain yields: in both laminar and non-laminar organs, throughout all growth stages (Araus et al., 2021), and there is acknowledgement of the need to take leaf age and the lifespan of all cohorts of leaves into account more effectively when determining ozone flux thresholds (Emberson et al., 2018; Osborne et al., 2019; Feng et al., 2022).

The reproductive growth phase of wheat appears to be the most sensitive to the effect of ozone (Pleijel et al., 1998; Soja et al., 2000), with reductions in, and changes to the partitioning of, photosynthate during anthesis and grain fill particularly evident (Emberson et al., 2018). Early senescence leads to reduced levels

of available carbon being allocated to ears/grains in preference to other organs, especially roots, commonly leading to a reduced root biomass and root:shoot ratio which may affect nutrient uptake, although ozone's effect on roots has been found to vary considerably (Andersen, 2003; Grantz et al., 2006).



Fig. 4.1. A model of the translocation and utilization of nitrogen in wheat plants (cv. SUN 9E) for one day during the linear phase of grain filling (day 15 after anthesis). White arrows represent translocation of nitrogen in the xylem; black arrows represent translocation of nitrogen via the phloem. The widths of arrows are proportional to the amounts of nitrogen translocated. The amounts of nitrogen are expressed in μ g day⁻¹ plant⁻¹, and each figure is located close to the translocation channel to which it applies. Values in the boxes (representing plant parts which are not associated with a translocation channel) represent the increment or decrement of nitrogen in the plant part on day 15. Figure with original legend reproduced from Simpson et al. 1983.

4.1.3 Interactions between ozone, senescence and the C:N balance

Photosynthesis and plant growth are regulated via the C:N ratio (Wingler et al., 2006). Research on the central role that the C:N balance plays in plant growth emphasises that the carbon and nitrogen metabolisms are very closely linked and inter-dependent (Paul and Foyer, 2001). As senescence plays an important role in balancing carbon and nitrogen within the whole plant, it therefore also interacts with multiple external factors which can generate a C:N or source:sink imbalance, including nutrient supply and abiotic stress (Paul and Foyer, 2001; Wingler et al., 2006). In stress-affected plants accelerated senescence results in both carbon and nitrogen use efficiency being compromised (Gregersen et al., 2013; Sade et al., 2018).

According to Sade et al. (2018) there are therefore two key 're-balancing' processes which affect yields in plants affected by abiotic stress: firstly a reduced 'source' strength which is brought about by premature senescence, chlorophyll degradation, and subsequent reductions in photosynthesis, and secondly a reduced grain 'sink' strength which slows the demand for photosynthate and leads to an accumulation of carbohydrates in photosynthetic tissue, which then triggers a reduction in photosynthetic activity (Albacete et al., 2014; Sade et al., 2018). This accumulation of leaf carbohydrates has been found in ozone affected plants (Grantz and Farrar, 1999; Grantz et al., 2006), and research in tree species has found that ozone can also increase the residual nitrogen concentrations in leaves, stems and roots, with related reductions in C:N ratios (Shang et al., 2018). However, there is a lack of data on C:N stoichiometry in ozone-affected wheat (Emberson et al., 2018).

When assessing the effect of ozone on levels of nutrients in wheat, Broberg et al. (2021) found that ozone reduced the harvest index for the majority of essential nutrients including nitrogen, whilst the total pool of each nutrient was unaffected, suggesting that ozone affected remobilisation and translocation efficiency rather than nutrient uptake. However, there has been limited research into whether, and to what extent, ozone affects levels of nitrogen stored prior to anthesis, its subsequent translocation to the grain, and nitrogen uptake in the post-anthesis period.

4.1.4 Aims of the study

This study therefore aimed to assess the effect of four ozone concentrations on nitrogen partitioning in multiple leaf cohorts throughout the key growth stages of a widely grown elite spring wheat cultivar, cv. Skyfall. This cultivar was selected due to its ozone sensitivity, as found in previous research (Brewster et al., 2019). Due to technical issues the high and very high ozone concentrations in this trial were lower than the two previous trials, although these concentrations are still representative of phytotoxic levels found in many regions, as described in Chapter 1.

The study aimed to assess the effect of ozone on:

- the timing of senescence and levels of chlorophyll in multiple plant parts;
- C:N stoichiometry in plant parts at anthesis and harvest;
- the storage levels of nitrogen at anthesis, and remobilisation of nitrogen from these plant parts to the ear/grain between anthesis and harvest;
- the post-anthesis uptake of nitrogen, applied as a later fertiliser addition at anthesis, and also whether this addition of nitrogen altered the effect of ozone.

4.2 Materials and methods

4.2.1 Plant establishment

The trial took place in 2021 at the UK Centre for Ecology & Hydrology's air pollution facility at Abergwyngregyn North Wales (53.2°N, 4.0°W). One line of a high yielding (winter/spring) cultivar (*T. aestivum* L., cv Skyfall) released in 2014 (RAGT Semences, France) was selected for the trial.

Seeds were germinated (15 - 18 April) in petri dishes at room temperature, and sown into modular plug trays containing low nutrient sandy loam topsoil (J.A. Bowers). Seedlings were grown on in an unheated glasshouse without supplementary lighting or temperature control (19 April – 4 May, Bangor, UK), with one balanced NPK nutrient feed (23 April).

At 3-leaf stage (GS13, 5 May) seedlings were transplanted, into 6.3L round black plastic pots (25 cm deep, 20 cm in diameter at the rim) with individual drip trays, two seedlings per pot, containing well mixed low nutrient sandy loam top soil (J.A. Bowers). The topsoil contained 2.1 kg ha⁻¹ of available nitrogen, 5.6 mg litre⁻¹ of phosphate and 28 mg litre⁻¹ of potassium, at pH 7.2. To ensure adequate plant nutrition for spring wheat grown in sandy loam soil (AHDB RB209, 2022) each pot was fertilised with phosphate (110 kg ha⁻¹ equivalent), and potassium (95 kg ha⁻¹ equivalent) at 3-leaf stage , and with ammonium nitrate (140 kg ha⁻¹ equivalent, in two split doses of 70 kg ha⁻¹) added at 3-leaf and 1st node (GS31, 9 June) growth stages. Micronutrients and soil bacteria were added to all pots via the addition of 30g per pot of farm field sandy loam soil (Henfaes Research Station, Abergwyngregyn, North Wales).

Plants were grown on under glass (5 May – 2 June), with pots rotated randomly every 7 days. All pots were manually watered daily, or as required, to maintain adequate soil moisture levels throughout the trial. To maintain plant health, applications of insecticide (4^{th} June, GS31-32) and fungicide (16^{th} June, GS41-43) treatments were made as required (Table S4.1, p.98).

4.2.2 Additional nitrogen fertilisation, ¹⁵N tracer experiment and ozone exposures

Six replicate pots per treatment were randomly selected, placed in (1 June, GS30), and rotated weekly within, four hemispherical glasshouses (solardomes, 2.1 m high, 3 m diameter). Within each of the four ozone treatments/solardomes there were four nitrogen treatment sub-sets (Table 4.1): NØX plants had no additional ammonium nitrate and were harvested at mid-anthesis (GS65, 2 July); NØ plants also received no additional ammonium nitrate at mid-anthesis but were grown to full term (GS93, 15-17 Aug), whilst the N+ and 15N+ plants were also harvested at full term (GS93, 15-17 Aug) but with the addition of 20 kg ha⁻¹ ammonium nitrate at mid-anthesis. The 15N+ replicates received a solution of unlabelled ammonium nitrate combined with ¹⁵N dual labelled ammonium nitrate (48.23 atom% ¹⁵N) at a combined rate of 20 kg ha⁻¹. In all cases the solution was applied evenly across the pre-wetted soil surface of the pot.

Table 4.1. Summary of amount and timing (growth stage) of nitrogen fertilisation for each subset of plants (NOX, NO, N+ and 15N+) along with growth stage at harvest. (See Table S1.1, p.15 for growth stages).

	NØX	NØ	N+	15N+
Nitrogen (kg ha ⁻¹)	+70 GS13 +70 GS31	+70 GS13 +70 GS31	+70 GS13 +70 GS31 +20 GS65	+70 GS13 +70 GS31 +20 GS65 including ¹⁵ N
Growth stage at harvest:	GS65	GS93	GS93	GS93

Ozone treatments (3 June – 15 Aug, 11 weeks) achieved peak concentrations of 30 ppb (low), 45 ppb (medium), 70 ppb (high), and 85 ppb (very high) (Fig. 4.2) using Pulse Width Modulation (PWM), reduced to ~20 ppb at night-time (~30 ppb in very high ozone treatment dome) and for two days per week to mimic natural ozone fluctuations. Charcoal-filtered air ventilated the solardomes approximately twice per minute, with ozone pumped into the solardomes via PTFE tubing using a controlled injection system (G11 ozone generator, Pacific O3, USA) and AirSep NewLife Intensity 10 Litre oxygen concentrator (CAIRE Inc., USA), with computer-controlled concentrations (Lab VIEW version 2015, National Instruments, USA) which were monitored every half hour by two calibrated automatic ozone analysers (400a, Enviro Technology Services, Stroud, UK, and Thermo Scientific Model 49i Electron O3 Analyser, Fischer Scientific, Waltham, MA, USA).

Ozone concentrations were maintained throughout the plant trial until plants were harvested. The average temperature inside the solardomes was 20.05°C. Environmental conditions within and between the solardomes have been found not to vary (Hewitt et al., 2016; Harmens et al., 2019).



Fig. 4.2. Mean hourly ozone profiles (replicated each week) over the course of ozone treatment (3 June – 15 Aug 2021) with 24 hour mean and AOT40 values, where 24 hour mean represents the mean ozone concentrations (ppb) across the whole treatment period, and the AOT40 value (ppb.h) shows the extent of ozone exposure above 40 ppb during daylight hours.

4.2.3 Senescence and chlorophyll indices

The extent of senescence was assessed weekly in NØ and N+ plants from early stem elongation growth stages (GS31, 9 June) until ripening (GS90s, 10 Aug) using an index of 0-10 (see Fig. 4.3). Relative chlorophyll levels (SPAD units) in the flag, 2nd, 3rd, and 4th leaves of the NØ and N+ plants were measured weekly from stem elongation (GS31, 8 June) until mid-grain-fill (GS80s, 27 July) using non-destructive measurements (CCM 200, ADC Biosciences, UK) of the upper surface of the central section of each leaf from a randomly selected shoot from either of the two plants in each replicate pot. In later stages of senescence readings were taken from an area of the leaf with green pigment, usually a basal section nearer the stem.

4.2.4 Measurements at anthesis and post-harvest

Shoots were cut at the base of the stem and ears cut at the top of the peduncle. NØ, N+ and 15N+ plants had grains extracted from ears manually and grain was then weighed. All shoots, ears and grain were dried in drying cabinets (65°C for 14+ days) before shoots and ears were weighed. Roots of the low and very high treatment plants were kept in situ in pots and left to dry for at least 3 days prior to wet sieving, drying (65°C for 14+ days) and weighing to derive the dry root biomass. After drying NØX, NØ, N+ and 15N+ shoots had flag leaves and 2nd leaves stripped from stems and the segment of upper stem (including leaf sheath), between the flag leaf node and 2nd leaf node was cut just above the 2nd leaf node. Flag, 2nd leaf and upper stem samples from NØX, and N+ plants were weighed to derive actual (and proxy for NØ and 15N+) dry biomass weights for flag leaf, 2nd leaf, and upper stem.

4.2.5 C:N and ¹⁵N analysis

All plant parts (flag leaf, 2nd leaf, upper stem, ear, root and grain) were ground and homogenised using a dry ball mill (Retsch MM200, Haan, Germany), with a dried root sub-sample taken from the upper, middle and lower part of the root. In all cases, 15N+ samples were processed after all others, and in the order of 'least to most' expected ¹⁵N concentration. Samples of NØX, NØ, and N+ plants were then analysed to derive N%, C%, and C:N ratio (Leco TruSpec CN, Michigan, USA). N+ and 15N+ samples were also analysed separately to derive N% and ¹⁵N atom% values (Elementar vario MICRO cube elemental analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany) interfaced to a Sercon Europa 20-20 IRMS (Sercon Ltd., Cheshire, United Kingdom)) using internal standards calibrated against international reference materials. Total nitrogen per plant part was derived from dry biomass and N% values.

4.2.6 Nitrogen Remobilisation Efficiency (NRE)

For NØ and N+ plants, NRE was calculated (Bogard et al., 2010) as follows:

NRE = $\frac{N\% \text{ at anthesis} - N\% \text{ at harvest}}{N\% \text{ at anthesis}}$

4.2.7 15 N trace experiment – calculation of 15 N% recovery

¹⁵N% recovered represents the percentage of ¹⁵N applied (per pot) which was recovered in the plant part (pool), and was calculated (Rowe et al., 2001) as follows:

- 1. 15 N excess atom% = 15 N atom% pool natural abundance
- 2. ¹⁵N excess $\mu g g^{-1} = \frac{N\% \times 15N \text{ excess atom}\%}{10,000} \times 1,000,000$
- 3. ¹⁵N excess μ g (total μ g in pool) = ¹⁵N excess μ g g⁻¹ × pool dry biomass (g)
- 4. ¹⁵N excess g (total g in pool) = $\frac{15N \exp(\text{total } \mu g \ln \text{pool})}{1,000,000}$

5. ¹⁵N% recovered =
$$\frac{15N \operatorname{excess} g (\operatorname{total} g \operatorname{in} \operatorname{pool})}{\operatorname{total} \operatorname{excess} g (15N \operatorname{applied})} \times 100$$

4.2.8 Statistical analysis

R (version 4.0.2, R Core Team, 2020) was used to undertake all tests for statistical significance apart from simple regression analysis which was undertaken in Excel (Microsoft Corporation, 2016). All error margins show standard error. Significance was assessed at p < 0.05.

Linear mixed effect models (package 'nlme', Pinheiro *et al.*, 2017) were used to assess, in each nitrogen fertilisation sub-set, the effect of ozone and plant part, and any interaction between them, on total pool N, N%, 15N% recovered, and C:N ratio, with all plant parts (flag, upper stem, second leaf, ear, and grain) included in the model except root. Ozone treatment level and plant part were used as factors, with replicate ID as a random effect variable.

A linear mixed effect model was also used for repeated measures data (chlorophyll index), with data first separated by each sub-set (NØ, N+) and leaf part (flag, second leaf, third leaf, fourth leaf). Growth phase and ozone were used as factors to assess any effect and interaction, and replicate ID also included as a random effect variable. In all linear mixed effect models Restricted Maximum Likelihood (REML) was used for estimation, and an assessment of plots and model fit determined the suitability of inclusion of an autoregressive (AR) correlation term. Comparisons of Akaike's Information Criteria (AIC, Bozdogan, 1987) was used to assess the best model fit. Results were summarised using ANOVA 'type 3' (package 'car', Fox and Weisberg, 2011) including a Wald chi-square test (χ^2).

In addition, linear models (package 'Ismeans', Lenth, 2016) were used to test for statistically significant differences between ozone treatment levels in each plant part subset within each parameter (total pool nitrogen (Total N), carbon percentage (C%), nitrogen percentage (N%), percentage of ¹⁵N recovered (¹⁵N% recovered), and the C:N ratio).

In all models, residuals and plots were checked for the appropriateness of each model and where necessary data were log transformed (Table S4.2, p.98). Tukey HSD post-hoc tests (package 'multcomp', Hothorn *et al.*, 2008) and pairwise comparisons/contrasts of means (package 'emmeans', Lenth *et al.*, 2020) were carried out where appropriate.

4.3 Results

4.3.1 Senescence index

The higher the ozone, the earlier the senescence started in each leaf cohort

Both NØ and N+ plants senesced progressively from the lower leaves up to the spike until complete senescence of the whole plant took place (Fig. 4.3 & Fig. S4.1, p.98). Differences in the timing and extent of senescence were generated by the different ozone treatments, but the same pattern was found in both NØ and N+ plants, in that senescence was triggered earlier, and therefore lasted longer, in plants exposed to higher ozone, and was also therefore at a more advanced stage during anthesis and grain fill growth stages.



Fig. 4.3. Senescence index: N+ plants. Index ranges from 0 (no senescence) to 10 (fully senesced), mapping the weekly progression of senescence at successive growth stages (see Table S1.1, p.15) under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n=6).

Ozone exposure began at stem elongation, and prior to booting there were almost no visible signs of senescence and no differences between the ozone treatments. After booting the stages of senescence were reached earlier with each increase in ozone concentration. At ear emergence (two weeks after the start of ozone exposure) plants in the very high ozone treatment already had visible senescence in all 4th leaves (Index 3), whilst in the high ozone treatment Index 3 was not reached until the start of anthesis, and not until the end of anthesis in both the low and medium ozone treatments.

At the start of milk development in early grain fill (five weeks after the start of ozone exposure), plants under the very high ozone treatment already had all 2nd leaves senescing (Index 5), those under high ozone were between Index 4 (all 3rd leaves senescing) and Index 5, whilst the plants in both low and medium treatments were still only between Index 3 and Index 4. By dough development, however (7 weeks after

the start of ozone exposure), all plants had reached Index 7 (all flag leaves fully senesced), and all then senesced through to Index 10, although the exact end of ripening was not identified in each treatment level.

4.3.2 Chlorophyll Index

Ozone reduced levels in all leaves apart from the flag, especially in the later growth stages, whilst the addition of nitrogen at anthesis ameliorated the effect of ozone.

Chlorophyll levels varied with leaf, growth stage, and ozone concentration (Figs. 4.4 & 4.5). The decline in chlorophyll content started earliest in the 4th leaf, followed by the 3rd, 2nd and finally the flag leaf. In response to ozone the overall pattern was of lower chlorophyll content and/or an earlier decline occurring with increasing duration and concentration of ozone exposure, particularly under the highest ozone treatment, and particularly in the 2nd, 3rd, and 4th leaves which were exposed to ozone for longer, with little or no effect in the flag leaf. In the NØ plants the differences between low and high treatments were most apparent at ear emergence in the 4th leaf, at the start of anthesis in the 3rd leaf, at the end of anthesis in the 2nd leaf, and at the start of milk development in the flag leaf. The additional nitrogen supplied to the N+ plants at anthesis appeared to maintain higher chlorophyll levels during early grain fill in the flag, 2nd and 3rd leaves, at least in the lower ozone treatments, compared to the NØ plants.

In the 4th leaves chlorophyll levels were lower in the highest ozone treatment but in all treatments the levels of chlorophyll peaked at booting and declined at similar rates so all were below 10 SPAD units at the start of anthesis in both the NØ and N+ plants (Figs. 4.4 & 4.5). In the NØ plants the effect of ozone overall on the fourth leaves was not significant, although there was a significant interaction between ozone and growth stage (p = 0.032), with significant reductions in levels under very high ozone found at ear emergence (low/vhigh p = 0.028) and at the start of anthesis (low/vhigh p = 0.039). In the 4th leaves of the N+ plants there was only a trend towards an effect of ozone (p = 0.069), and no significant interaction between ozone and growth stage, with no significant differences between ozone treatments at any growth stage.



Fig. 4.4. Chlorophyll Index (SPAD units) in the flag, 2^{nd} , 3^{rd} , and 4^{th} leaves of the NØ plants under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments, measured weekly at each growth stage, from stem elongation to ripening, following onset of ozone exposure at stem elongation (orange line). Error bars show standard error of the mean (n = 6).



Fig. 4.5. Chlorophyll Index (SPAD units) in the flag, 2^{nd} , 3^{rd} , and 4^{th} leaves of the N+ plants under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments, measured weekly at each growth stage, from stem elongation to ripening, following onset of ozone exposure at stem elongation (orange line). Error bars show standard error of the mean (n = 6).

In the third leaves of both the NØ and N+ plants (Figs. 4.4 & 4.5) there was a highly significant effect of ozone (p < 0.0001), and interaction between ozone and growth stage (p < 0.0001). In both the NØ and N+ plants, levels of chlorophyll peaked at booting and then declined, dropping below 20 SPAD units in all ozone treatments from the end of anthesis, and below 10 under very high ozone from the start of anthesis. In the NØ plants, although the levels were lower under very high ozone from ear emergence onwards, the only significant difference between treatment levels was found at the start of anthesis (low/vhigh p = 0.02). In the N+ plants there were similar significant reductions under the very high ozone treatments at the start of anthesis (med/vhigh p = 0.032) but also in the early milk development growth stage (low/vhigh p = 0.029; med/vhigh p = 0.025), with a trend towards an ozone effect under high ozone also (med/high p = 0.087), suggesting that the additional nitrogen fertiliser applied at anthesis had maintained chlorophyll levels in the low/medium treatments but not in the higher ozone concentrations.

In the 2nd leaves of the NØ plants (Figs. 4.4 & 4.5) there was a highly significant negative effect of ozone overall on chlorophyll levels (p < 0.0001), and also a significant interaction between ozone and growth stage (p < 0.0001). At the end of anthesis there were significant reductions in chlorophyll levels under the higher ozone treatments (low/vhigh p = 0.0042; med/vhigh p = 0.021), and also in early milk development (low/vhigh p = 0.0054; med/vhigh p = 0.0055). In comparison, in the N+ plants there was no significant effect of ozone overall on the second leaves, although there was a significant interaction between ozone and growth stage (p = 0.0059), with the levels of chlorophyll significantly reduced in the early milk development phase under very high ozone (low/vhigh p = 0.048), with the addition of nitrogen appearing to maintain chlorophyll levels in the lower ozone treatment levels, especially during early grain fill.

In both the NØ and N+ plants, there was no significant effect of ozone on the flag leaf chlorophyll levels at any growth stage, although in the NØ plants there was an non-significant reduction in levels under very high ozone at the start of milk development (high/vhigh p = 0.074).

4.3.3 Nitrogen percentage (N%) in plant parts

No effect of ozone apparent at anthesis, but slight increases in N% under higher ozone treatments at harvest, especially in the NØ plants.

At anthesis in the NØX plants, N% levels were highest in the flag (~3.5%) and 2nd leaves (~3%), followed by the ear (~1.75%) and upper stem (~1.5%), with the lowest levels in the root (~0.75%) (Fig. 4.6). There was no effect of ozone on these plant parts, and no interaction between ozone and plant part, with only a slight and non-significant increase in N% in the upper stem (med/high p = 0.072) and the root (low/vhigh p = 0.075) under the higher ozone treatments. In the roots, similar N% levels were found in the NØX plants at anthesis and in both the NØ and N+ plants at harvest (Figs 4.6 & 4.7).



Fig. 4.6. Nitrogen percentage (N%) in plant parts under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments, at anthesis in NØX plants (left) in root, 2^{nd} leaf, upper stem, flag leaf, and ear; and at harvest in N+ plants (right) in root, 2^{nd} leaf, upper stem, flag leaf, ear and grain. Error bars show standard error of the mean (n = 6).



Fig. 4.7. Nitrogen percentage (N%) in plant parts under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments at harvest in NØ plants, in root, 2^{nd} leaf, upper stem, flag leaf, ear and grain. Error bars show standard error of the mean (n = 6).

At harvest, in the N+ plants (Fig. 4.6), the highest N% levels were found in the grain (~3.5%), followed by the ear (~1.5%), with much lower levels in the flag leaf (~0.75%), 2^{nd} leaf (~0.5%) and upper stem (~0.5%) compared to the levels found at anthesis. The translocation of nitrogen from leaves to ear and grain was similar under the different treatment levels, and there was only a trend towards an effect of ozone overall (p = 0.094). Although mean N% levels in flag, 2^{nd} leaf, upper stem and ear were all slightly higher under the highest ozone exposure compared to the low treatment, there was only one significant difference found, in the 2^{nd} leaf (med/vhigh p = 0.039), with a trend towards an ozone effect also in the 2^{nd} leaf between the low and very high ozone treatments (p = 0.064).

Whilst the NØ plants at harvest (Fig. 4.7) demonstrated a similar re-distribution and level of nitrogen within plant parts to the N+ plants, N% levels overall were significantly affected by ozone (p = 0.032), with a highly significant interaction between ozone and plant part (p = 0.001). Higher N% levels were found under the highest ozone exposures in the flag, 2^{nd} leaf, stem and ear, with significant differences between the lower and higher treatment levels in the 2^{nd} leaves (low/vhigh p = 0.001; med/vhigh p = 0.003), and ears (low/vhigh p = 0.01; med/vhigh p = 0.017), and with trends towards an effect of ozone also in the flag leaf (low/vhigh p = 0.075; med/vhigh p = 0.066) and 2^{nd} leaf (high/vhigh p = 0.06). These data again suggest that the addition of nitrogen at anthesis ameliorated the effect of ozone.

4.3.4 Carbon percentage (C%) in plant parts

C% levels were largely stable, although ozone increased *C%* in roots and stems at anthesis, with slight reductions in stem *C%* at harvest, especially in the *NØ* plants.

In contrast to N%, there was minimal variation in C% levels, both between plant parts and between flowering and harvest, as well as between the different ozone and nitrogen treatment levels (Fig. 4.8), with levels ranging from ~41% in second leaves, ~42-43% in flag leaves and stems, with ~43% in the grain and ~44% in the ears.

Variations in C% levels were most apparent at anthesis. There was a significant increase in C% under the higher ozone treatments in both the root (low/vhigh p < 0.01) and the stem (low/vhigh p < 0.0001; low/high p < 0.0001; med/vhigh p < 0.0001; med/high p < 0.0001) but a significant decrease in C% under the higher ozone treatments, both in the second leaf (low/high p < 0.01; low/vhigh p < 0.05) and the flag leaf (med/vhigh p < 0.05; high/vhigh p < 0.05), with no effect of ozone on C% in the ear.

At harvest, there were no significant differences in the C% levels in any plant parts, in both the NØ and N+ plants, apart from reduced levels in the stems under the higher ozone treatments, with the NØ plants more significantly affected (low/med p < 0.01; low/high p < 0.001; low/vhigh p < 0.0001) than N+ plants (med/high p = 0.01; med/vhigh p < 0.05).





Fig.4.8. Carbon percentage (C%) in root, 2nd leaf, upper stem, flag leaf, ear and grain in NØX plants at anthesis (A); in N+ plants at harvest (B); in NØ plants at harvest (C), under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n = 6).

4.3.5 C:N ratio in plant parts

Ozone had no effect on C:N ratios at anthesis but reduced the C:N ratio at harvest, especially in the NØ plants, driven largely by increased N%.

In the NØX plants at anthesis, C:N ratios were highest in the root (~55/60:1), followed by the upper stem (~30:1) and ear (30:1), and lowest in the 2nd leaf (~15:1) and flag leaf (~10:1) (Fig. 4.9). Both C% and N% levels were relatively stable across all treatments and led to a C:N ratio that showed no effect of ozone overall and no significant differences between treatment levels in the flag, 2nd leaf, ear or root, with only a slight and insignificant increase under very high ozone in the upper stem (low/vhigh p = 0.096).

In the N+ plants at harvest, C:N ratios were highest in the root (~80:1) and upper stem (~75-80:1), followed closely by the 2nd leaf (~70-75:1) and flag leaf (~60:1), with much lower ratios in the ear (~30:1), and the lowest in the grain (~10:1), and ozone significantly reduced the C:N ratio overall (p = 0.015)(Fig 4.9). Under the highest ozone treatment there were significant reductions in the C:N ratio in the 2nd leaves (low/vhigh p = 0.025; med/vhigh p = 0.014) and upper stem (med/vhigh p = 0.047), though not in the flag.



Fig. 4.9. C:N ratio in plant parts at anthesis in NØX plants (left) and at harvest in N+ plants (right) under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n = 6).

In contrast, in the NØ plants at harvest (Fig. 4.10) C:N ratios were highest in the upper stem (~100:1), followed by the 2nd leaf (~75:1), flag leaf (~70:1) and root (~70:1), with lower ratios in the ear (~35:1) and grain (~10:1). Therefore in comparison to the N+ plants, the C:N ratio of the NØ upper stems at harvest was higher, whilst the C:N ratio of the NØ root was lower in comparison to the N+ root. There was also a stronger effect of ozone overall on the C:N ratios of the NØ plants at harvest (p = 0.0014), and a significant interaction between ozone and plant part (p = 0.022). Ozone significantly reduced C:N ratios in the higher ozone treatments at harvest, in the flag leaves (low/vhigh p = 0.003; med/vhigh p = 0.002; high/vhigh p = 0.009), 2^{nd} leaves (low/vhigh p<0.0001; med/vhigh p = 0.0003), and ear (low/vhigh p = 0.023; med/vhigh p = 0.046).
The root C:N increased from \sim 55+ at anthesis to \sim 80+ at harvest in the N+ plants but only to \sim 65+ in the NØ plants at harvest. Although slightly reduced by ozone, there was no significant difference in the root between the low and very high treatments in either the N+ or NØ plants. There was no effect of ozone found on grain C:N due to the very poor grain set.



Fig. 4.10. C:N ratio in plant parts at harvest in NØ plants under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n = 6).

4.3.6 Total nitrogen in plant parts

Ozone increased total root nitrogen at anthesis but decreased it at harvest, with slight increases found in shoot at harvest, especially in the NØ plants.

At anthesis the largest pool of nitrogen in the NØX plants was found in the ears, followed by the roots and then the stems, with the lowest levels in the flag and 2^{nd} leaves (Fig. 4.11). There was only a slight and insignificant increase in total nitrogen in aboveground plant parts in response to increasing ozone (p = 0.078), and analysis of individual parts found no significant differences between treatments, with only a slight and insignificant increase in total nitrogen in the ear in the higher ozone treatments (low/high p = 0.057; med/high p = 0.059; low/vhigh p = 0.092; med/vhigh p = 0.096). There was however a significant increase in total nitrogen found in the root under very high ozone at anthesis (p = 0.0056) driven by a greater root biomass, whilst at harvest total nitrogen in the root was reduced in both N+ (p = 0.084) and NØ plants (p = 0.045), driven by a reduced root biomass.



Fig. 4.11. Total nitrogen (Total N) per plant part pool under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments at anthesis in NØX plants (A), at harvest in N+ plants (B), and at harvest in NØ plants. Error bars show standard error of the mean (n = 6).

At harvest in both N+ and NØ plants (Figs. 4.11) the total nitrogen in the ear had increased from >80 mg at anthesis to > 120 mg, reflecting a greater biomass as well as higher N% levels, with this increase driven by a substantial translocation of nitrogen from both the flag and 2^{nd} leaves and the upper stems in both N+ and NØ plants. In N+ plants there was a significant effect of ozone on total nitrogen (p = 0.013), with a pattern of total nitrogen increasing in the higher ozone treatments. There were significant differences found in the 2^{nd} leaf (med/vhigh p = 0.005) and stem (med/vhigh p = 0.029), and trends in the flag leaf (low/vhigh p = 0.066), 2^{nd} leaf (high/vhigh p = 0.071), stem (low/vhigh p = 0.098), and ear (med/high p = 0.092). Total nitrogen levels in the NØ plants showed a highly significant effect of ozone overall (p = 0.00015), with

significant increases in total nitrogen under higher ozone exposures found in the flag leaf (med/vhigh p = 0.037), 2nd leaf (low/vhigh p = 0.0015; med/vhigh p = 0.0009), and ear (low/vhigh p = 0.041; med/vhigh p = 0.018), and trends in the flag leaf (low/vhigh p = 0.062) and stem (med/vhigh p = 0.093).

Due to poor grain set there was no clear pattern of ozone response in the grain in either NØ or N+ plants and no significant differences between treatments. Although the N+ plants showed an increase in total nitrogen in the grain under very high ozone this was not significant.

4.3.7 Nitrogen remobilisation efficiency (NRE)

Ozone reduced NRE particularly under the highest ozone treatment

In both NØ and N+ plants, NRE was highest in the flag leaf and 2nd leaf, followed by the upper stem, with smaller values for the ear, and very small negative values in the root (Table 4.2). In both NØ and N+ plants, in all plant parts apart from the N+ flag leaf, NRE was lowest under very high ozone exposure compared to the low ozone treatment. The largest reductions in the NRE of each plant part (between low and very high ozone) occurred in the NØ plants, especially in the ear but also in the 2nd leaf and upper stem. In both N+ and NØ plants NRE increased in the flag leaf under both medium and high ozone, before declining again under the highest ozone exposure. The flag leaf in both nitrogen treatments, and the ear in the N+ treatment, showed the least difference between the low and very high treatment suggesting the plant parts with the shortest exposure to ozone were least affected.

Table 4.2. Nitrogen remobilisation efficiency between NØX plants at anthesis and both N+ and NØ plants at harvest in low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatment levels, along with the difference between the low and very high treatment levels.

NØX to N+	root	2nd leaf	upper stem	flag leaf	ear
low	-0.124	2.759	1.204	3.261	0.747
med		2.733	1.154	3.337	0.807
high		2.707	1.230	3.383	0.752
vhigh	-0.089	2.620	1.108	3.263	0.734
difference low-vhigh	0.035	-0.139	-0.096	0.002	-0.013
NØX to NØ					
low	-0.256	2.765	1.294	3.288	0.955
med		2.726	1.252	3.338	0.882
high		2.681	1.336	3.406	0.865
vhigh	-0.227	2.569	1.185	3.238	0.658
difference low-vhigh	0.029	-0.196	-0.109	-0.050	-0.297

4.3.8 ¹⁵N percentage recovery in plant parts

No effect of ozone on post-anthesis nitrogen uptake and translocation

Measurement of the ¹⁵N% levels recovered in the different plant parts of 15N+ plants at harvest (Fig. 4.12), following application of ¹⁵N at mid-anthesis, showed that, as expected, and even with poor grain set, the majority of the recovered nitrogen was translocated to the grain (4-10%) and ear (3-4%), with <1% remaining in the root, <0.4% in the stem, and <0.1% in both flag leaves and 2nd leaves. There was no significant effect of ozone, and there were also no significant differences between ozone treatments in any of the individual plant parts, so although the flag leaf, 2nd leaf, upper stem and ear all reflected a pattern of increasing N% with increasing ozone concentration, none of the differences between treatments were significant. What the trace experiment did show was that the nitrogen added at anthesis travelled to, or through, all parts of the plant and that this translocation was unaffected by ozone.



Fig. 4.12. Percentage of ¹⁵N isotope recovered at harvest in the root, 2nd leaf, upper stem, flag leaf, ear, and grain of 15N+ plants under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error of the mean (n = 6).

4.3.9 Root:Shoot ratio

Ozone increased root:shoot ratio at anthesis but reduced it at harvest.

At anthesis in the NØX plants, an increase in root biomass in response to ozone (data not shown) led to a slightly increased root:shoot ratio (Table 4.3), whilst by final harvest, in the N+, N0, and 15N+ plants, the root:shoot ratio was reduced by the very high ozone treatment.

Table 4.3. Root:shoot ratios of NØX plants at anthesis (GS65), and N+ plants, NØ plants and 15N+ plants at harvest (GS93) under low (30 ppb) and very high (85 ppb) ozone treatments.

	NØX	N+	NØ	N15+
low	0.37	0.38	0.41	0.30
vhigh	0.40	0.29	0.31	0.23
growth stage at harvest	GS65	GS93	GS93	GS93

4.3.10 Grain yield

In all subsets grown to harvest (NØ, N+ and 15N+) there were very low levels of floret fertility (cause unknown) and therefore very low grain yields. Although almost all spikes had some grains, there were zero grains in just one replicate in the NØ plants, and three replicates in the N+ plants across all treatments. The Trial 3 grain yield data (Total grain weight) is presented in Chapter 5 (see Fig. 5.1).

4.4 Discussion

Effective grain fill is determined by multiple factors: whole canopy photosynthesis throughout the life of the plant, the timing and rate of senescence, effective pre-anthesis storage of surplus photosynthate and the efficiency of post-anthesis translocation to the developing grain (Gaju et al., 2014; Araus et al., 2021). This discussion focuses on the extent to which ozone may affect each of these factors.

Effect on whole canopy chlorophyll, photosynthesis, and the timing and rate of senescence

4.4.1 Ozone causes early chlorophyll reduction and senescence

In this study the Senescence Index clearly showed that the higher the ozone concentration, the earlier each successive stage of visible senescence started, and the longer it lasted. As a result visible senescence was already well advanced in the lower half of the plant during anthesis and in the early and mid grain fill growth stages under the higher ozone treatments. These findings conform with those of previous studies which measured senescence either at the whole plant level via measurement of total green leaf area (e.g. Soja and Soja, 1995; Pleijel *et al.*, 1997; Finnan *et al.*, 1998) or used chlorophyll levels in the flag leaf as a proxy for senescence (e.g. Gelang *et al.*, 2000; Osborne *et al.*, 2019). Although previous studies have varied in the length and timing of ozone exposure, in all cases elevated ozone exposure led to reductions in green leaf duration and caused earlier and longer periods of senescence (e.g. Grandjean and Fuhrer, 1989; Finnan *et al.*, 1998), with the resulting reduction in the duration of grain fill being linked to reduced yields (Gelang et al., 2000), which is a common response to early senescence induced by abiotic stress (Sade et al., 2018).

Although chlorophyll levels are often used as a proxy for the progression of senescence (Osborne et al., 2019; Heyneke et al., 2019) with levels decreasing as the plant breaks down these leaf proteins (Avila-Ospina et al., 2014), the concurrent measurements of chlorophyll levels and senescence in all leaf cohorts in this study indicated that ozone-induced reductions in chlorophyll levels began earlier than visible senescence. Under optimal conditions, nitrogen remobilisation in wheat starts immediately after anthesis is completed and before senescence (which normally occurs 8-16 days after anthesis) is visible in the canopy (Kong et al., 2016). This prior onset of nitrogen remobilisation is linked to the subsequent initiation of full senescence (Gregersen et al., 2008; Bogard et al., 2011). The reductions in chlorophyll levels found in this study suggest there is an earlier start to the remobilisation of nitrogen in response to ozone, linked to the earlier start of visible senescence.

4.4.2 Ozone reduces chlorophyll levels in lower leaf cohorts, especially during grain fill

The chlorophyll Index in the lower leaf cohorts revealed a pattern of increasing ozone damage both as the leaf aged, and as the ozone concentration increased, with the effect most apparent during grain fill. Few other studies have reported on the effect of ozone on specific leaf cohorts below the flag leaf but Xu et al.

(2009) also found an increase in visible leaf damage in the 2nd, 3rd and 4th leaves in two wheat cultivars after just three days of elevated ozone exposure applied shortly after the end of anthesis, with damage increasing both with increasing leaf age and with the ozone sensitivity of the cultivar. Reductions in chlorophyll levels inevitably also reduce photosynthetic capacity, and Soja & Soja (1995), measuring the effect of ozone on photosynthesis efficiency via Fv/Fm chlorophyll fluorescence in the flag, 2nd, 3rd and 4th leaves at ear emergence, had similar findings to this trial at this growth stage.

As described in all these data, ozone sensitivity has been found to increase as the age of the leaf increases (Sandelius et al., 1995) making the later growth stages more vulnerable to ozone. This is likely linked to changes in the capacity of plant tissue to detoxify and reduce ROS as the leaf ages and as senescence progresses (Khanna-Chopra, 2012; Bender *et al.*, 1994; Taiz *et al.*, 2015, p.680). An increase in ozone sensitivity at the grain fill growth stage has clear implications for wheat yields, particularly as there is evidence that modern cultivars rely as much on post anthesis flag leaf and ear photosynthesis as on preanthesis stored carbohydrates during grain fill (Maydup et al., 2012; Sanchez-Bragado et al., 2016; Tambussi et al., 2021). This ozone-induced early reduction in total photosynthetic capacity of the whole canopy during grain fill is thought to be the cause of the reduced grain starch levels commonly found in ozone-affected wheat (Broberg et al. 2015), and probably also why the post anthesis, grain fill period appears to be the most sensitive to ozone overall (Pleijel et al., 1998; Gelang et al., 2000; Xu et al., 2009).

4.4.3 Lesser impact of ozone on flag leaf compared to lower canopy

In this study the reductions in chlorophyll levels under the higher ozone treatments were most marked in the 2nd and 3rd leaves but with little effect apparent in the flag leaf which maintained similar levels of chlorophyll in all ozone treatments until normal senescence began at the start of grain fill. Many studies (Soja and Soja, 1995; Finnan et al., 1998; Xu et al., 2009) have found that due to the younger age of the flag leaf compared to the lower leaves there is a lesser impact on the flag leaf compared to the lower leaves due to the shorter exposure to ozone. Although in modelling work carried out by Osborne et al. (2019) a linear relationship was found between reductions in chlorophyll in the flag leaf and the level of ozone flux, it was also found there was a difference between the modelled threshold flux which induced leaf physiology changes and that which triggered yield reductions. In wheat the final yield is affected by the levels of whole canopy photosynthesis in all photosynthesising plant parts (Araus et al., 2021). Research into nitrogen remobilisation in 20 elite wheat cultivars (Barraclough et al 2014) found that, at anthesis, nitrogen was apportioned as follows: stem (28%), ear (23%), flag leaf (15%), leaf sheaths (14%), 2nd leaf (11%), 3rd leaf (6%), and remaining leaves (3%), with 80-85% remobilisation efficiency from each plant part to the grain by harvest. The contributions of the 2nd and 3rd leaves in combination are therefore as important as the flag. It is clear from the chlorophyll data from all four leaf cohorts in this trial that modelling based solely on flag leaf data will obscure the effect of ozone on whole canopy chlorophyll levels and whole canopy

carbon/nitrogen assimilation, confirming previous recommendations that chlorophyll levels in all leaf cohorts need to be incorporated into crop modelling in future (Emberson et al., 2018; Osborne et al., 2019).

4.4.4 Senescence is slower in ear/awns during grain fill

In this study, it was observed that senescence in the peduncle, ear and awns was less rapid than the leaves, leading to the synchronising of the senescence index in the different treatment levels at the start of dough development. Few studies report on the effects of ozone on the photosynthetic capacity of ears of wheat, but Sild et al. (2002) found that ozone had no effect on non-structural carbohydrate levels in ears, and ear photosynthesis has been found to have greater tolerance to abiotic stress than the flag, and to contribute proportionately more to grain fill when the plant is subject to stress (Maydup et al., 2010; Wang et al., 2016; Tambussi et al., 2021). In this study total carbon (structural and non-structural) and C:N ratios in the ear were unaffected by ozone, both at anthesis and at harvest, suggesting that this effect may also have occurred in this study, although non-structural carbohydrates and photosynthetic functioning of the ear were not measured, and could be the focus of further research. The proportion of photosynthate contributed by the ear during grain fill has increased in cultivars throughout the 20th and 21st centuries (Maydup et al., 2012; Wang et al., 2016), possibly linked to a compensation mechanism driven by the reduced stem height, and this may make wheat vulnerable to post-anthesis abiotic stress (Tambussi et al., 2021).

4.4.5 Ozone effects on root differ with growth stage

The earlier senescence and increased loss of chlorophyll in the 2nd, 3rd and 4th leaves is also likely to contribute to yield loss through alterations in partitioning from leaves down to the roots which then subsequently translocate nutrients directly back up to the ear/grain during grain fill (See Fig. 4.1, Simpson *et al.*, 1983; Grantz *et al.*, 2006; Fiscus *et al.*, 2005). In this study, ozone had reduced the root biomass and root:shoot ratio at harvest, conforming with previous studies on a wide range of plants where root biomass, root:shoot ratios, and the carbon allocation to roots were all reduced (Barnes et al., 1995; Grantz et al., 2006). This has repercussions for wheat yield, and modelling studies have shown links between reduced carbon assimilation in shoots and reduced amounts allocated to roots in response to ozone (Grantz et al., 2006). As root activity normally declines earlier and faster than flag leaf chlorophyll levels (Wei et al., 2004) it is possible the loss of chlorophyll in the lower leaves may have been preceded by increased root cortical senescence (Schneider and Lynch, 2018), with implications for nutrient uptake during grain fill.

However, at anthesis the root biomass was found to be larger in response to increased ozone and contained a higher C%. Whilst both Barnes et al. (1995) and Kou et al. (2018) found root biomass suppressed by ozone during pre-anthesis growth stages, Kou et al (2018) found that root length, surface area and volume were significantly increased by ozone during grain fill, and suggest this is due to increased carbon being partitioned to the root. Ozone has been found to reduce soil amino sugars in wheat's rhizosphere at stem elongation but to increase levels during grain fill (Zhang et al., 2014). Research on drought and salt stress

in rice (Mathan et al., 2021) also found that sucrose levels were increased in stressed roots, as well as shoots and phloem sap, and suggest this was linked to sucrose acting in part as an osmolyte to reduce tissue damage. In this study, the higher C% levels found in the root and upper stem at anthesis, under the higher ozone levels, suggest ozone might also lead to increased levels of sucrose. However, further research is needed to clarify whether this is a common response in wheat roots to ozone, and whether it results from remobilised nutrients derived from senescence being triggered earlier in the lower leaves, and/or from higher levels of sucrose generated as a defence mechanism in response to stress (See also Section 4.4.6).

Effects on carbon and nitrogen partitioning

4.4.6 Less effect of ozone on leaf carbon

Although there was minimal variation in leaf carbon found in this study, increased accumulations of carbohydrates in leaves can occur in response to abiotic stress (Albacete et al., 2014; Sade et al., 2018), and has been found previously in some plants in response to ozone (e.g. Grantz and Farrar, 1999), drought (e.g. Chen *et al.*, 2015), and UV light (e.g. Klem *et al.*, 2022). Sild et al. (2002) found that ozone exposure during and after anthesis substantially increased levels of soluble carbohydrates (mostly hexoses and sucrose) and total non-structural carbohydrates accumulating in the flag leaves of wheat, both at mid-anthesis and two weeks after anthesis, but that four weeks after anthesis levels were lower than the control.

These findings, in combination with those on roots described in Section 4.4.5 above (Kou et al., 2018), suggest that ozone stress may increase sucrose levels during anthesis and early grain fill, but this is then followed by a rapid reduction in the latter growth stages. Further research is needed to confirm if sucrose regulated changes in carbon partitioning commonly occur in response to ozone, and to what extent it is implicated in the triggering of early senescence. A measure of the non-structural soluble carbohydrates in this study might have provided an improved assessment of the effects of ozone on carbon assimilation in leaf, stem and ear, both pre- and post-anthesis.

4.4.7 C:N partitioning at anthesis and harvest

N% and total nitrogen levels varied considerably between plant parts and growth stages, as might be expected in pre- and post-senescent wheat as nitrogen translocation took place. At anthesis, the stable C:N ratios in all plant parts across all treatments suggested that C% and N% levels in the upper half of the plant, represented by the 2nd leaf, upper stem, flag and ear, were still yet to be noticeably affected by ozone. Had C:N data been collected on the 3rd and 4th leaves however, it seems probable that a difference between the treatments at anthesis may have been found, given the reductions found in chlorophyll content. Further research is required to clarify if changes to the C:N ratio do occur in the lower leaves of wheat, and if C:N changes are linked to the triggering of early senescence.

In this study, the slight increase in residual N% and total nitrogen at harvest, and related reductions in C:N ratios, especially in the leaves and upper stem in response to the highest ozone treatment, are similar to the effects of ozone on C:N stoichiometry found in other studies, undertaken mostly on tree leaf material. These studies revealed that whilst 'total C' levels largely remained stable, nitrogen increased by ~5%, resulting in reduced C:N ratios (Wittig et al., 2009; Cao et al., 2016). Few studies have previously assessed this issue in crop plants but a meta-analysis of the effects of various abiotic stress on a wide range of rice cultivars also found an increase in residual N% in rice straw (Yulong et al., 2021).

4.4.8 Why is this increase in N% levels occurring?

Several explanations have been proposed previously for the increase in residual nitrogen concentrations. Shang et al. (2018) noted that, as the increase in residual N% was occurring in both the leaves and stems of poplar, with genetic variations in tolerance to ozone affecting the response, one possible cause may be linked to reductions in NUE. In a study of ozone's effect on the remobilization of both macro and micronutrients in wheat Broberg et al (2021) found that ozone did not affect the total uptake of nitrogen but did reduce NUE, reducing the rate of remobilisation of nitrogen, and concluded that this was caused by the shortened grain fill period due to the early onset of senescence. This shorter grain fill effect on NUE could lead to a higher residual nitrogen remaining in 'source' plant parts, and also to a higher N% in proportion to reduced C%. The efficiency of nitrogen remobilisation (NRE) measured in the current study, based on N% values at anthesis and harvest, suggests that the translocation of nitrogen from aboveground plant parts were slightly reduced by very high ozone, particularly from the 2nd leaf and upper stem, whilst NRE from the flag leaf was largely unaffected. However, the increased residual N% levels in the flag leaf as well as the 2nd leaf and stem, especially in the NØ plants, suggest that reduced NRE is not the only cause of the increase in residual nitrogen. Equally, as the chlorophyll levels were unaffected by ozone in the flag leaf, but were affected in the 2nd leaf, this might have indicated a link with a localised defence response, but the elevated N% levels in both suggest the response may be at the whole plant level. Even though there is a caveat for this study, that due to the poor grain set these data may not reflect a typical wheat plant, N% data previously collected in wheat where yields were normal, suggest that the same pattern occurs irrespective of the sink demand from the grain. (See Chapter 5).

It has been suggested that the increase in residual nitrogen found in leaves and stems could also be linked to more nitrogen being allocated to structural proteins to protect cell membranes and ensure effective translocation of photosynthates (Shang et al., 2018). High levels of ROS resulting from abiotic stress can damage cell membranes, with evidence of this found in ozone-affected wheat (e.g. Biswas *et al.*, 2008; Ojanperä *et al.*, 1992). Higher concentrations of the amino acid, proline, are thought to stabilize membranes and act as a scavenger of ROS in order to limit oxidative stress (Hayat et al., 2012; Boublin et al., 2022), as well as to act as temporary nitrogen storage until the stress reduces (Nikolaeva et al., 2010). Proline has been found to accumulate in plant tissues in response to many abiotic stress including ozone (Verbruggen

and Hermans, 2008; Sarkar et al., 2010), and increased levels of proline have been found in wheat exposed to ozone (Li et al 2016). Flavonols, linked to a plants defence/stress response, have also been found to increase in plant tissue in response to ozone stress (Boublin et al., 2022), with an interaction found between nitrogen fertiliser levels and ozone which affected leaf chlorophyll and flavonol levels (Chang-Espino et al., 2022). However, further research is needed to identify exactly which substances make up these increased levels of residual nitrogen found in in ozone-affected wheat, and the underlying mechanisms which cause them.

Effect on post-anthesis nitrogen uptake and translocation, and interactions with nitrogen fertilisation

4.4.9 Extra nitrogen fertilisation ameliorated effect of ozone

Although the poor grain set in this study prevents a comparison of how the addition of nitrogen at mid anthesis affected yield and grain quality, there were many noticeable differences between the NØ and N+ plants which reveal important issues relating to how nitrogen levels affected the response to ozone. Extra, readily available nitrogen - applied at anthesis - protected chlorophyll levels during grain fill in the flag, and in the 2nd and 3rd leaves in the lower ozone treatments, thereby delaying senescence and maintaining photosynthesis in these leaves at a critical growth stage. This addition of nitrogen reduced the levels of residual nitrogen in the leaves and stem, and ameliorated the effect of ozone on C:N ratios and on the efficiency of translocation of stored nitrogen in the 2nd leaf, upper stem and ear. Whilst abiotic stress is known to decrease levels of cytokinin, the plant hormone involved in delaying senescence in plants, increasing the levels of nitrogen available to a plant can lead to an increase in both cytokinins (Taiz *et al.*, 2015, p.682-3) and antioxidants (Ma et al., 2022), enabling the plant to reduce levels of ROS and oxidative damage. Whilst antioxidant levels were not measured in this study, increased antioxidant levels in response to higher nitrogen fertilisation have been found in ozone-affected wheat (Pandey et al., 2018).

Further research would be needed to assess the extent to which different levels of nitrogen fertilisation ameliorate the effect of ozone on wheat, but these results serve to highlight the fact that fertilisation rates do affect the response. The timing and triggering of early senescence is dependent upon nitrogen supply, and Heyneke et al. (2019) found that nitrogen application rates had a significant effect on total chlorophyll in leaves, with chlorophyll levels being maintained longer and at higher levels under adequate fertilisation, and declining later compared to low nitrogen levels. Previous studies assessing ozone's effect on chlorophyll and senescence have not always provided clear data on levels of nitrogen fertilisation, and future experimental work and modelling needs to investigate the effect of different levels of nitrogen on ozone sensitivity.

4.4.10¹⁵N trace experiment

Interestingly, despite the differences between the nitrogen treatments noted above, the ¹⁵N trace experiment revealed that only a small amount of nitrogen applied at anthesis was taken up by the plants. It has been found in cereal crops that post-anthesis nitrogen uptake via the root can be favoured over remobilised stored nitrogen, with a preference shown for the transport of nitrogen direct to the spike (Kong et al., 2016), with anything between 5% and 50% of grain nitrogen coming from this root-derived post-anthesis nitrogen uptake (Gaju et al., 2014).

Although the partial sterility and very low grain yields in Trial 3 plants (see Chapter 5) will have drastically reduced the sink strength, the majority of the nitrogen was still translocated either to the ear or the grain, and only small residual amounts were left in the root, upper stem, 2nd leaf and flag leaf, suggesting a fraction of the nitrogen did not go directly to the ear, and may have played a role in delaying senescence during grain fill. However, there were no differences found between any of the treatments, indicating that the uptake by the root and subsequent translocation of nitrogen to the shoot was unaffected by ozone.

4.5 Conclusion

This study found that when a modern spring wheat cultivar was exposed to ozone for 11 weeks it led to an earlier and extended senescence under the higher ozone treatments, and also to an earlier start to nitrogen translocation, represented by reductions in chlorophyll prior to visible senescence. The reductions in chlorophyll were particularly evident in the older leaves of the lower leaf cohorts during anthesis and grain fill. Notably, the flag leaf was largely unaffected by ozone, and therefore not representative of lower – and total - canopy chlorophyll levels, suggesting that lower leaf cohorts need to be accounted for in ozone modelling work. These reductions in total canopy chlorophyll will inevitably reduce photosynthetic capacity, and the duration and potential of grain fill. This is a critical issue, especially due to modern wheat's greater reliance on post-anthesis photosynthesis to maximise grain fill and starch content.

Whilst nitrogen storage prior to anthesis was unaffected by ozone, nitrogen remobilisation efficiency between anthesis and harvest, was reduced by the highest ozone treatment, particularly in the 2nd leaf and upper stem but not the flag. In addition, N% and total nitrogen found in plant parts at harvest increased under the highest ozone treatment, leading to reduced C:N ratios. A protective effect of the additional nitrogen fertilisation applied at anthesis was revealed by the chlorophyll, N%, C:N and NRE data, highlighting the role that nitrogen fertilisation may play in tolerance mechanisms in ozone-affected wheat.

Further research is needed to clarify the effect of ozone on nitrogen dynamics within the wheat plant, in terms of whole plant nitrogen storage prior to anthesis, nutrient partitioning between the shoot and root, especially during anthesis and grain fill, and the role that levels of nitrogen fertilisation may play in any ozone tolerance linked to delaying senescence and maintaining chlorophyll levels and photosynthetic capacity during anthesis and grain fill in all leaf cohorts.

Supplementary materials

Date	Type/name	Active ingredients	Application method and rate
4 th June GS31-32	Insecticide Bug Clear Ultra	Acetamiprid	Spray: 10ml/L
16 th June GS41-43	Fungicide: Cyflamid	Cyflufenamid	Spray: 2.5ml/L

Table S4.1. Applications of insecticide and fungicide, with date, growth stage and application rate.

Table S4.2. Parameters which were log transformed in each subset and plant part.

Model	Subset	Parameter / part	
LME	N+	Total N	
LME	NØ	Total N	
LME	NØ	C:N	
LM	N+	Total N - flag	
LM	N+	N% - root	
LM	N+	N% - flag	



Fig. S4.1. Senescence index: NØ plants. Index ranges from 0 (no senescence) to 10 (fully senesced), mapping the weekly progression of senescence by growth stage (see Table S1.1, p.15) under low (30 ppb), medium (45 ppb), high (70 ppb) and very high (85 ppb) ozone treatments. Error bars show standard error from the mean (n=6).

CHAPTER 5

Ozone increases residual nitrogen in wheat shoots irrespective of grain yield, and causes an additional increase of nitrate leaching to groundwater

Declaration of contributions:

Conception, methodology and design: Clare Brewster (CB), Felicity Hayes (FH), Natalie Fenner (NF); *Collection and assembly of data*: CB; *Analysis, interpretation of the data, and drafting of the chapter*: CB; *Critical revision and final approval of content*: CB, FH, NF.

Abstract

Approximately one third of applied nitrogen fertiliser is captured within the grains of cereal crops such as wheat, with this low nitrogen use efficiency leading to a reduced yield potential and increased nitrogen pollution. Understanding the effect of ozone on the relationship between yields and nitrogen use could help to maximise yields but also reduce nitrogen pollution. This study used data from three plant trials where elite cultivars were exposed to multiple levels of ozone, and where total grain yields varied from high (Trial 1) to very low (Trial 3). This enabled an assessment of the effect of yield size and ozone treatments on residual nitrogen, found both in various plant parts at harvest (Trial 1, 2, 3), and in the form of nitrate in extracted soil water (Trial 2). Despite wide variation in grain yields and therefore in sink capacity, the residual nitrogen found in laminar plant parts at harvest increased under the highest ozone treatment, with a related decrease in C:N ratios. In addition, a late application of nitrogen fertiliser during grain fill led to an increase in extracted nitrate levels, but only under the highest ozone treatment and not in all lines, and with no clear correlation found with root or shoot biomass at harvest.

5.1 Introduction

5.1.1 Nitrogen fertilisation and nitrogen pollution

Feeding the ever-increasing global human population, now over eight billion, has required ongoing intensive use of synthetic nitrogen fertilisers in order to maintain and increase yields of staple food crops such as wheat. However, estimates suggest only one third of applied nitrogen fertiliser is captured within the grains of global grain crops, with this low nitrogen use efficiency (NUE) affected by genetic differences within the plant, by abiotic and biotic stresses, and by variations in agronomic management practice (Hawkesford and Riche, 2020). This has led to significant losses of nitrogen to both water and air, including increased emissions of the precursor gases which can generate phytotoxic levels of tropospheric ozone (Zhang et al., 2015). Improving NUE in wheat, through improvements to uptake and translocation of nitrogen, has therefore become a target for wheat breeders, both to maximise yields but also to reduce nitrogen pollution (Xing et al., 2019).

5.1.2 Effects of ozone on residual nitrogen in shoot

NUE in wheat is particularly affected by nitrogen availability and uptake during the grain filling growth stages, in that if post-anthesis uptake via the roots is possible it leads to a reduction in the remobilisation of nitrogen already stored within the plant (Barbottin et al., 2005). The timing of abiotic and biotic stress therefore is important to NUE: if it reduces post-anthesis uptake it can improve NUE by maximising the remobilisation of stored nitrogen, with wheat cultivars that were tolerant to biotic stress found to maintain nitrogen remobilisation efficiency during grain fill (Barbottin et al., 2005). However, in a meta-analysis of 21 studies Broberg et al. (2017) found that increasing ozone concentrations were correlated with reduced NUE in ozone-affected wheat. In a related experiment on wheat plants exposed to ozone for four weeks during and after anthesis (Broberg et al., 2017) a reduction in nitrogen remobilisation from shoot to grain was also found, with the percentage of nitrogen remaining in leaf and stem - residual shoot nitrogen - increasing as ozone concentrations increased. This suggests that an increase in the residual nitrogen found in plant parts at harvest could be used as an indicator of reduced nitrogen remobilisation in the post-anthesis period, and of reduced NUE. Notably, Broberg et al. (2017) also found that the reductions in grain nitrogen were larger than the increase in residual shoot nitrogen, suggesting additional reductions in nitrogen uptake or translocation were taking place in response to ozone. The exact mechanisms behind this increase in residual nitrogen remain unclear, but this response has been found before in both semi-natural vegetation (e.g. Wittig et al., 2009) and other crop plants such as rice (e.g. Yulong et al., 2021). See Chapter 4 for a discussion of possible causes.

5.1.3 Effects of ozone on residual soil nitrate

In most plants, nitrogen is taken up by roots in the form of nitrate and translocated to the leaf where it is then reduced to ammonium and rapidly processed into amino acids, using sugars produced in the leaves (Bindraban et al., 2015). Whilst both nitrite and nitrate are negatively charged anions and therefore prone to being leached from the soil system, nitrite is usually rapidly transformed into nitrate, and it is nitrate that is largely responsible for eutrophication of water environments. The highest losses take place between late autumn and early spring when plant uptake of nitrogen is low and, in temperate regions, when rainfall exceeds evapotranspiration, especially when inputs of fertiliser exceed plant uptake (Cameron et al., 2013).

The effect of ozone on wheat shoot and root growth, and therefore on nitrogen uptake, has the potential to affect losses of nitrogen from the agri-ecosystem (Andersen, 2003). Increases in nitrate levels in the rhizosphere of ozone-affected wheat have been found in several studies (W. Chen et al., 2015; Wu et al., 2016; Hu et al., 2018), linked both to increases (W. Chen et al., 2015) and decreases (Wu et al., 2016) in nitrifying enzyme activity. A meta-analysis of 41 studies covering a wide range of terrestrial ecosystems including croplands found that ozone increased levels of soil nitrates and rates of denitrification, with reductions also shown in soil carbon parameters (Hu et al., 2022).

5.1.4 Aims of the study

The data in this chapter is derived from all three plant trials (see Chapters 2, 3, and 4), and uses data relating to grain yields, grain quality, nitrogen content of plant parts, and nitrate levels in soil water, to explore the effect of ozone and other contributory factors on residual nitrogen within the plant and in soil water.

The hypotheses being tested are:

- that the size of grain yield does not influence the effect of ozone on levels of laminar residual nitrogen;
- that the level of nitrogen fertilisation does not influence the effect of ozone on levels of residual nitrogen;
- that the different ozone exposure regimes made no difference to the levels of residual nitrogen.

5.2 Materials and Methods

5.2.1 Plant establishment and ozone treatments

A summary of plant establishment, nitrogen fertilisation and ozone treatments in all three plant trials is listed in Table 5.1. For full details of seed, plant growth and ozone treatments see Chapter 2 (Trial 1, 2018), Chapter 3 (Trial 2, 2019), and Chapter 4 (Trial 3, 2021).

Table 5.1. Summar	v of plant trial	arowina conditions	and ozone treatments	in Trials 1. 2 and 3
rabie origination	<i>y</i> oj pranc cinan	growing contaitions		· · · · · · · · · · · · · · · · · · ·

	Trial 1	Trial 2	Trial 3
Seedlings vernalised	Yes	Yes	No
Soil medium	Sandy loam farm soil	Sandy loam farm soil	Sandy/silt loam
	pH 5.6	pH 6.2	commercial topsoil
			рН 7.2
Total nitrogen	Soil N: 77 kg ha	Soil N: 81.5 kg/ha	Soil N: 2.1 kg/ha
fertiliser (ammonium	+50 kg/ha at GS30+	+25 kg ha at GS41+	+70 kg ha at GS13
nitrate)	+25 kg/ha at GS69+	+50 kg ha at GS55+	+70 kg ha at GS31+
		+25 kg ha at GS70+	+20 kg ha at GS65
	Total = 152 kg/ha	Total = 181.5 kg/ha	Total = 162 kg ha
Ozone treatments	Low (30 ppb)	Low (30 ppb)	Low (30 ppb)
	Medium (55 ppb)	Medium (55 ppb)	Medium (45 ppb)
	High	High (80 ppb)	High (70 ppb)
	Very High (110 ppb)	Very High (110 ppb)	Very High (85 ppb)
Duration of ozone	7 th June – 21 st August	30 th May – 19 th August	3 rd June – 15 th August
treatment	(10.5 weeks) 2018	(11.5 weeks) 2019	(11 weeks) 2021
Lines/data included in	cv. Skyfall	cv. Paragon	cv. Skyfall (N+)
Chapter 5		[SHW, BC1, BC5, BC7	
		nitrate level data only]	

5.2.2 Total grain weight and grain quality

Total grain weight was determined as described in Chapter 2 (Trial 1, 2018), Chapter 3 (Trial 2, 2019), and Chapter 4 (Trial 3, 2021). Percentages of grain protein and grain starch were obtained as described in Chapter 3 (Trial 2, 2019).

5.2.3 C:N ratio and percentage nitrogen

Shoot and root samples were collected as described in Chapters 2-4, with subsequent C:N analysis to derive percentages of nitrogen and carbon, as described in Chapter 4.

5.2.4 Nitrate extracted from soil water

To assess levels of nitrates in soil water in Trial 2 (2019), samples of soil solution were extracted using rhizon MOM samplers (5 cm porous section with 0.15 µm pore size and 2.8 mm bulb tip, and 12 cm PVC/PE tubing; Rhizosphere Research Products, The Netherlands) connected to 25 mm x 0.6 mm BD microlance stainless steel sterilised needles (Fisher Scientific, Loughborough UK). Rhizon samplers were inserted between the

two plants within each pot when plants were at 3-leaf stage (GS13+) at an angle of approximately 45°, with the tip reaching 12-15 cm depth. After the two applications of ammonium nitrate, 50 kg ha⁻¹ equivalent at ear emergence (GS51+) and 25 kg ha⁻¹ equivalent at mid grain fill (GS70+), soil solution samples were collected overnight (5pm to 9am), using 9 ml sterilised vacuum tubes (Elkay Laboratory Products, Basingstoke UK). Samples were frozen within an hour of collection and stored at -20°C until ion chromatography analysis (930 Compact IC Flex, Metrohm AG, Switzerland).

5.2.5 Statistical analysis

All statistical significance tests were conducted using R programming (version 4.0.2 R Core Team, 2020), except for simple regression analysis where R^2 and related significance values were generated using Excel (Microsoft Corporation, 2016). Error margins represented on all figures show standard error. Significance was assessed at p < 0.05.

Linear models (package 'Ismeans', Lenth, 2016) and Tukey HSD post-hoc tests (package 'multicomp', Hothorn *et al.*, 2008) were used to assess each line (Skyfall in Trial 1, Paragon in Trial 2, and Skyfall N+ plants in Trial 3) in each parameter (total grain weight, grain protein and starch percentages, nitrogen percentage, carbon percentage, C:N ratio, and levels of nitrate in soil water) for statistically significant differences between the ozone treatment levels. Residuals and plots were checked to assess data for normality, and data were log transformed where necessary (Trial 2 Paragon C:N ratio, upper leaf).

Linear mixed effect models (package 'nlme', Pinheiro *et al.*, 2017) were applied to assess the effect of ozone and line on levels of nitrate in soil water in Trial 2, after the two applications of ammonium nitrate (50 kg ha⁻¹ and 25 kg ha⁻¹) to lines of SHW, Paragon, BC1, BC5 and BC7. Replicate ID was used as a random effect variable, with ozone treatment and line as factors. Estimation, assessment and model fit were completed as described in Chapter 4 for Trial 3.

5.3 Results

5.3.1 Total grain weight

The total grain weight of the elite cultivars varied across all three plant trials (Figs.5.1a – 5.1c). Skyfall in Trial 1 had the highest total grain weight, but there was also a clear effect of ozone, with yields significantly reduced as ozone increased (low/med p < 0.01; low/vhigh p < 0.0001; med/vhigh p < 0.0001). In Trial 2, Paragon's total grain weight was lower compared to Skyfall in Trial 1, but was only significantly affected by the highest ozone treatment (low/vhigh p = 0.001; med/vhigh p < 0.001; high/vhigh p < 0.001). In Trial 3 the very poor grain set in all plants resulted in extremely low total grain weights with no significant differences between the ozone treatments.



Fig. 5.1. Total grain weight (g) under low, medium, high and/or very high ozone concentrations. Fig. 5.1a. Trial 1, Skyfall (n=6)); Fig. 5.1b. Trial 2, Paragon (n=3); Fig. 5.1c. Trial 3, Skyfall (N+ plants)(n=6). Error bars show standard error of the mean. See Table 5.1 for ozone treatment levels in each trial.

5.3.2 Grain protein and starch

In Trial 1, whilst Skyfall's total grain weight was significantly reduced, its grain protein concentrations (Fig. 5.2a) were significantly increased, from ~11% to 14%, under the highest ozone treatment (low/vhigh p < 0.0001; med/vhigh p < 0.0001), whilst starch levels (Fig. 5.2b) were significantly reduced by increasing ozone, ranging from ~75% in low ozone to ~70% under the highest ozone treatment (low/med p < 0.001; low/vhigh p < 0.0001; med/vhigh p < 0.0001).

In Trial 2, protein concentrations in Paragon's grain (Fig.5.2c) were also significantly increased under the highest ozone treatment, rising from ~14% to ~18% (low/vhigh p < 0.01; med/vhigh p < 0.01; high/vhigh p < 0.05), but there were no significant differences between the other treatment levels. There was a similar pattern for starch concentration (Fig. 5.2d), with a significant reduction (~69% to ~64%) only under the highest ozone treatment (low/vigh p = 0.01; med/vhigh p < 0.01; high/vhigh p < 0.05).

In Trial 3, with the poor grain set in all Skyfall plants, protein levels in the few grains that did form were higher than normal across all treatment levels (~20%, Fig. 5.2e), with just one significant difference (low/med p < 0.05). Starch (Fig. 5.2f) was lower than normal across all treatments, ranging from ~59% to~64%, and with no significant differences between the ozone treatments.



Fig. 5.2. Percentage of grain protein and grain starch in grains of Skyfall in Trial 1 (a, b), Paragon in Trial 2 (c, d), and Skyfall N+ plants in Trial 3 (e, f). Error bars show standard error of the mean (Trials 1 & 3, n = 6, Trial 2, n = 3). See Table 5.1 for ozone treatment levels in each trial.

5.3.3 Residual nitrogen in leaf and stem at harvest

The residual nitrogen found in leaves and stems at harvest (Figs. 5.3a - 5.3c) showed similar patterns across all three trials, with an increase in nitrogen under the highest ozone concentration, but with no differences at the lower treatment levels.

In Trial 1 (Fig. 5.3a), residual nitrogen at harvest was significantly higher under the highest ozone treatment in Skyfall's second leaf (low/vhigh p < 0.0001; med/vhigh p < 0.0001) and flag leaf (low/vhigh p < 0.0001; med/vhigh p < 0.0001), with just a trend in the upper stem (low/vhigh p < 0.1).



Fig. 5.3. Percentage of nitrogen (N%) in plant parts at harvest under low, medium, high and/or very high ozone treatments. Fig. 5.3a. Trial 1: 2nd leaf, upper stem, and flag leaf of Skyfall (n=6); Fig. 5.3b. Trial 2: upper leaves (flag and 2nd leaf combined) and lower leaves (3rd and 4th leaves combined) of Paragon (n=3); Fig. 5.3c. Trial 3 (N+ plants): 2nd leaf, upper stem, and flag leaf of Skyfall (n=6), as previously shown in Chapter 4. Error bars show standard error of the mean. See Table 5.1 for ozone treatment levels.

Similarly, in Trial 2 (Fig. 5.3b), there were significant increases in residual nitrogen found under the highest ozone treatments in Paragon's lower leaves (low/vhigh p < 0.01; med/vhigh p < 0.01; high/vhigh p < 0.01) and upper leaves (low/vhigh p < 0.05; med/vhigh p < 0.05; high/vhigh p < 0.05).

In Trial 3 (Fig. 5.3c), as reported in Chapter 4, the levels of nitrogen overall, and the increase in residual nitrogen under the highest ozone treatment, were not as great compared to Trials 1 and 2, with significant increases/trends found only in the 2^{nd} leaf (med/vhigh p < 0.05; low/vhigh p < 0.1), and no significant differences between treatments found in flag leaves, or stems.

5.3.4 C:N ratios in leaf and stem at harvest

A comparison of C:N ratios in stems and leaves across all three trials (Figs. 5.4a – 5.4c) suggests that at harvest the stems have the highest C:N ratio, whilst the flag (Trial 1 and Trial 3) or combined upper leaves (Trial 2) have the lowest C:N ratio, reflecting their higher nitrogen levels. In all three trials, all the C:N ratios were reduced under the high/very high ozone concentrations, driven predominantly by increases in nitrogen.

In Trial 1 (Fig. 5.4a), C:N ratios were significantly reduced under the highest ozone treatment in Skyfall's second leaf (low/vhigh p < 0.0001; med/vhigh p < 0.0001), flag leaf (low/vhigh p < 0.0001; med/vhigh p < 0.0001), and stem (low/vhigh p < 0.05). In Trial 2 (Fig. 5.4b), there was also a significant reduction in the C:N ratio of Paragon's lower leaf under the highest ozone treatment (med/vhigh p < 0.05), with trends also

found in the lower leaf (high/vhigh p < 0.1), and the upper leaf (med/vhigh p < 0.1; high/vhigh p < 0.1). In Trial 3 (Fig. 5.4c), as reported in Chapter 4, the highest ozone treatment again led to significant reductions in the C:N ratio of Skyfall's stem (med/vhigh p < 0.05) and second leaf (low/vhigh p < 0.05; med/vhigh p < 0.05), with a trend also in the flag leaf (med/vhigh p < 0.1).

The comparison of Skyfall's C:N ratios in Trial 1 and Trial 3 indicates that, across all ozone treatments, there were higher C:N ratios in the 2nd leaf, flag leaf and stem in Trial 3 when the grain failed to set, suggesting an accumulation of carbohydrates in all plant parts, although under higher ozone, levels of carbon were reduced and residual nitrogen increased.



Fig. 5.4. C:N ratio in plant parts at harvest under low, medium, high and/or very high ozone treatments. Fig. 5.4a. Trial 1: 2nd leaf, upper stem, flag leaf of Skyfall; Fig. 5.4b. Trial 2: upper leaves (flag and 2nd leaf combined), lower leaves of Paragon; Fig. 5.4c. Trial 3: 2nd leaf, upper stem, and flag leaf of Skyfall (N+), as previously shown in Chapter 4. Error bars show standard error of the mean (Trial 1 and Trial 3, n = 6; Trial 2, n = 3). See Table 5.1 for ozone treatment levels.

5.3.5 Residual nitrogen in soil water

Nitrate levels found in extracted soil water after applications of 50 kg ha⁻¹ of ammonium nitrate at ear emergence (GS55+, Fig. 5.5a) varied significantly between the different lines (p < 0.0001), with the lowest nitrate levels found in Paragon's soil water, and the highest in the primary SHW line, suggesting differing nitrate demands from each line, likely due, at least in part, to variations in plant biomass, both root and shoot, which was evident at harvest (Figs. S5.1 & S5.2, p.114). Although there was a significant effect of ozone overall on nitrate levels across all lines at this growth stage (p < 0.05) the only significant effect of ozone within each line was found in BC1 (low/med p < 0.01; med/high p < 0.05), with a negative trend under the highest ozone treatment in BC7 (high/vhigh p < 0.1).

Following the application of 25 kg ha⁻¹ during grain fill (GS70+, Fig. 5.5b), there were no significant differences in nitrate levels in extracted soil water between the different wheat lines, whilst there was a highly significant effect of ozone overall (p < 0.0001). Nitrate levels remaining in soil water significantly increased in Paragon under the highest ozone treatment (low/vhigh p < 0.05; med/vhigh p < 0.05; high/vhigh p < 0.05), with a strong positive trend also found in BC7 (low/vhigh p = 0.058) under the highest ozone exposure. There were no significant differences between treatments in the line of primary SHW, BC1 and BC5, and notably, no effect on uptake of nitrogen/nitrates at the lower ozone concentrations.



Fig.5.5. Nitrate concentrations in extracted soil water (mg/L) in Trial 2, in lines of primary SHW, Paragon (PAR), BC1, BC5 and BC7, under low (30 ppb), medium (55 ppb), high (80 ppb) and very high (110 ppb) ozone concentrations; Fig.5a: Nitrate concentrations after 50 kg N application at ear emergence (GS51+); Fig. 5b. Nitrate concentrations after 25 kg N application at mid grain fill (GS70+). Error bars show standard error of the mean (n = 3).

Simple linear regression analysis (Figs. 5.6 & 5.7) found no correlation between the residual nitrate in soil water and either the reduced root biomass ($R^2 = 0.16$, p = 0.078) or the reduced shoot biomass ($R^2 = 0.16$, p = 0.076) found under the higher ozone treatments, although the variability in the nitrate data may have contributed to this lack of correlation.



Fig. 5.6. Simple linear regression analysis assessing relationship between nitrate levels found in extracted soil water and root biomass at mid grain fill in Trial 2 following application of 25 kg N ha (equivalent) during grain fill, showing data by ozone level for SHW, Paragon, BC1, BC5, and BC7, under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Linear regression trend line represents the data from all five lines.



Fig. 5.7. Simple linear regression analysis assessing relationships between nitrate levels found in extracted water and shoot biomass at mid grain fill in Trial 2 following application of 25 kg N ha⁻¹ during grain fill, showing data by ozone level for SHW, Paragon, BC1, BC5 and BC7, under low (30 ppb), medium (55 ppb), high (80 ppb), and very high (110 ppb) ozone treatments. Linear regression trend line represents data from all five lines.

5.4 Discussion

Nitrogen use efficiency is a complex trait and is affected by interactions between genotype, environment, the amount and timing of nitrogen fertilisation, and subsequent grain yield (Hawkesford and Riche, 2020; Sieling and Kage, 2021). Across all three plant trials there was a clear pattern in the residual nitrogen data, indicating that the highest ozone treatment led to an increased proportion of residual nitrogen in leaves and stems, and of nitrate levels within the rhizosphere/bulk soil, both of which suggest reductions in NUE. The possible contributory factors generating these increases in residual nitrogen, in the context of the differences between the three trials, including variations in yield, nitrogen fertilisation, and genotype, are the focus of this discussion.

5.4.1 Grain yields and residual nitrogen

The ear and grain normally form the major sink for approximately 90% of the nitrogen found within the plant at maturity, which is largely remobilised from shoots during senescence, and partly taken up via roots in the post-anthesis period (Barraclough et al., 2014). In both Trial 1 and Trial 2 the sharp drop in grain yield under the highest ozone was also accompanied by a sharp increase in residual nitrogen within the stem and/or leaves, and a related drop in the C:N ratio. Whilst this increase in residual nitrogen also occurred in Trial 3 under the highest ozone levels (though was less marked), this took place in the context of extremely low grain yield, such that the normal sink for nitrogen was substantially reduced. Grain protein levels also increased in Trial 1 and Trial 2, which is a commonly found response to higher ozone when grain yields are normal (Broberg et al., 2015), whilst in Trial 3, in the few grains that did form, protein levels were much higher, and starch levels much lower than normal in all treatments.

Together, these data suggest that the increase in the proportion of residual foliar nitrogen may be linked to factors other than grain yield and sink strength. It may simply be related to the reduced proportions of carbon due to reductions in photosynthesis. However, as discussed in more detail in Chapter 4, this residual nitrogen needs further investigation in order to identify the exact components. It is possible that it is linked to reduced capacity to remobilise nitrogen from source tissues, and/or to an increase in cell membrane thickness in response to high Reactive Oxygen Species (ROS) levels (Le Gall et al., 2015), possibly in the form of a build-up of the amino acid, proline, which accumulates in response to stress (Verbruggen and Hermans, 2008). Ozone tolerance at the lower ozone concentrations, demonstrated in multiple parameters across all trials but especially by Paragon in Trial 2, suggest that residual nitrogen levels could also be used as an indicator of ozone sensitivity.

5.4.2 Nitrogen fertilisation regimes

The differences in the nitrogen fertilisation regimes in the three trials (Table 5.1), both in total amounts of nitrogen applied and in the timing of applications, may have contributed to differences in nitrogen uptake and use. For example, the residual nitrogen levels in leaves under the very high ozone treatments was

highest in Trial 2, which might be expected as this trial also had the highest nitrogen fertilisation rate, with the final nitrogen application also at the latest growth stage (GS70+) when plant growth under high ozone was already severely affected and had the least capacity to either use or take up the additional nitrogen. The higher and later nitrogen fertilisation in Trial 2 may also have contributed to the high soil nitrate levels found in several lines under the highest ozone treatments. However, the increased levels under the highest ozone treatment still support the suggestion by Broberg et al. (2017) that ozone reduces nitrogen uptake in wheat in the post-anthesis period. In the meta-analysis by Hu et al. (2022) similar increases in nitrate levels in response to higher ozone were found in multiple terrestrial ecosystems. Reductions in growth, and therefore demand for nitrogen, could have been the cause of reduced uptake, although in Trial 2, regression analysis suggests the smaller root and shoot biomass in the higher ozone treatments were not directly correlated with nitrate levels, and therefore the reduced capacity of the plant to take up nitrogen is not necessarily the direct, or only, cause of the increase in nitrates.

5.4.3 Genotypic variation in sensitivity

There are examples across all three trials of genotypic variation in response to ozone. Despite differing environmental conditions and fertilisation regimes, Skyfall, which was grown in both Trial 1 and Trial 3, demonstrated greater ozone sensitivity with increasing ozone than Paragon, grown in Trial 2, which showed more tolerance at the lower ozone levels. In addition, nitrate levels, monitored in Trial 2 only, also revealed genotypic variation, with the primary SHW taking up less nitrate than all the other lines, especially the elite cultivar Paragon which had the lowest levels of residual nitrate and therefore the highest nitrogen uptake. Further research is required to understand whether there is any relationship between genotypic variation in NUE and ozone sensitivity/tolerance which can be exploited in future wheat breeding programmes.

5.4.4 Additional contributory factors

The elevated ozone concentrations were lower in Trial 3 compared to Trial 1 and Trial 2. In particular the very high ozone level was 25 ppb higher in Trial 1 (110 ppb) than Trial 3 (85 ppb) and this may have contributed to the smaller differences in residual nitrogen in leaves between treatments that were found in Trial 3.

The slightly lower residual nitrogen levels found in the leaves and stems In Trial 3, compared to plants in Trial 1 and Trial 2, may be linked to the poor grain set at anthesis which led to additional late spikes forming, and therefore very likely to nitrogen being translocated from older senescing leaves to these emerging green shoots.

In addition, as different soil pH affects the availability of nutrients, the low pH in Trial 1 (pH 5.6) may have slightly limited nitrogen availability, whilst the high pH in Trial 3 (pH 7.2) may have led to a slightly reduced availability of iron (needed for chlorophyll synthesis) and boron (needed for cell wall synthesis) in all plants (Taiz *et al.*, 2015, p130-132).

5.5 Conclusion

These findings across all three wheat trials suggest that ozone-affected wheat is likely to be both lower yielding and less effective at using available nitrogen, leading to increased levels of nitrate leaching. In addition the ozone-induced stress also leads to a higher proportion of residual nitrogen in the shoot material, irrespective of final grain yield, although the exact cause and specific compounds of this residual nitrogen needs further research. Whilst this will all contribute to lower NUE within the crop cycle, it will also lead to higher nitrogen levels in the unharvested wheat straw, which is baled for low nutrient feed or animal bedding, or ploughed back into the soil. This additional residual soil nitrogen may be used by the next crop, but it may also lead to additional nitrate leaching.

Supplementary materials



Fig. S5.1. Dry shoot biomass (g) in Trial 2, in lines of primary SHW, Paragon (PAR), BC1, BC5 and BC7, under low (30 ppb), medium (55 ppb), high (80 ppb) and very high (110 ppb) ozone concentrations. Error bars show standard error of the mean (n = 3).



Fig. S5.2. Dry root biomass (g) in Trial 2, in lines of primary SHW, Paragon (PAR), BC1, BC5 and BC7, under low (30 ppb), medium (55 ppb), high (80 ppb) and very high (110 ppb) ozone concentrations. Error bars show standard error of the mean (n = 3).

CHAPTER 6



6.1 Discussion of findings in relation to over-arching research questions

This research - based on three experimental plant trials - has explored several aspects of ozone's effect on wheat, and added greater detail to our understanding in relation to several key inter-related questions:

- How and when does ozone reduce wheat yields?
- How does ozone affect and interact with nitrogen, before and after anthesis?
- What are the possible origins of wheat's ozone sensitivity?
- How might these factors be interacting to affect modern elite wheat cultivars?
- How can wheat's ozone tolerance be improved?

The findings outlined in this thesis which help to address these questions are highlighted in this chapter, framed within the context of the key growth stages of wheat. Recommendations for further research are also made, and limitations to the studies acknowledged.

Fig. 6.1 summarises the growth stages and the main factors which contribute to the growth and yield of wheat, any of which have potential to be affected by ozone. The number of grains which form on the spike are controlled by effects which occur both before, and at, anthesis, whilst the period after anthesis largely controls the size of grains and concomitant changes to the proportions of grain protein and starch which alter grain quality.



Fig. 6.1. Graphical representation of wheat's growth stages, the phases of nitrogen uptake and storage, periods when the plant is photosynthesising and senescing; the critical reproductive stages of meiosis, anthesis and grain fill; and the phases when numbers of tillers/ grains and grain size is determined. Adapted from Sreenivasulu & Schnurbush, 2012. See Chapter 1 Table S1.1, p.15 for a detailed list of wheat's growth stages.

Each of the three plant trials contributed evidence to different aspects of this process, and these are reviewed below, firstly in terms of how they relate to the effect of ozone on grain formation, both before and after anthesis, and secondly in the context of wheat's ozone sensitivity/tolerance and the breeding of ozone tolerant cultivars.

6.2 Effect of ozone, and interactions with nitrogen, before and at anthesis

6.2.1 Effects on grain number

During the growth stages before anthesis, nitrogen status, assimilate levels, and C:N partitioning are the primary factors influencing the final number of mature spikes and fertile florets within each spike, both of which contribute to final grain number (Fischer, 2011; Sreenivasulu and Schnurbusch, 2012). In Trial 2 ozone reduced the number of mature spikes in most lines under the higher ozone levels, and reduced grain numbers in multiple lines in both Trial 1 and 2, adding to the large body of evidence that ozone reduces grain number (e.g. Broberg et al., 2015; Pleijel et al., 2018). These data indicate that effects must have occurred prior to and/or during anthesis. The 3D μ CT images of the wheat spikes in Trial 2 also revealed for the first time that the increased number of infertile florets were occurring primarily across the middle section of the spike, the region which normally holds the highest number of grains per spikelet, especially in elite cultivars (Zhou et al., 2021), as illustrated by Paragon in Trial 2, with the grains usually being lost from the outer-most floret positions within each spikelet.

The triggering of early senescence is now thought to be one of the main mechanisms by which yields are reduced by ozone (Emberson et al., 2018), but this can affect yields both before and after anthesis. If senescence occurs before anthesis any reduction in the production and partitioning of photosynthate to the ear increases the potential for floret death (González et al., 2011). Chlorophyll levels provide a proxy for nitrogen status, and there was clear evidence in Trial 2 and 3 of reductions in chlorophyll content of ozone treated plants before and at anthesis. Trial 2 revealed ozone-induced reductions before anthesis during booting and ear emergence in the 2nd leaf and lower leaves, and at anthesis ozone-induced reductions in chlorophyll were found in the flag as well as the 2nd and lower leaves. Trial 3's more detailed assessment of all four leaf cohorts confirmed that chronic ozone exposure led to reductions in chlorophyll levels during booting and ear emergence. Chlorophyll levels were reduced under higher ozone in the 2nd, 3rd and 4th leaves, though not the flag, whilst at anthesis the effect of ozone was again most marked in the 2nd and 3rd leaves. Under the highest ozone treatment visible senescence was also already evident in lower leaves before, and at, anthesis, with chlorophyll reductions and nitrogen translocation being initiated before senescence became visible.

These data provide additional evidence that this is an important mechanism for reductions in grain number in ozone-affected wheat, confirming previous findings relating to reduced chlorophyll in the whole canopy prior to, and at, anthesis (e.g. Finnan et al., 1998), but they provide more detail specific to each leaf cohort. These data add further evidence that the age of the leaf and the level and duration of ozone exposure influence the degree of chlorophyll reduction. Therefore the timing and duration of any ozone exposure, and the degree of ozone flux, prior to and at anthesis, will be important factors determining the effect on grain number in the field. In addition the raised carbon levels found in the root and stem at anthesis in Trial 3, which possibly indicated a stress-induced defence response (Mathan et al., 2021), also suggest carbon could have been diverted away from the ear.

These data also clearly demonstrated that the lower, older leaf cohorts exhibited greater and earlier ozoneinduced reductions in chlorophyll compared to the flag leaf, which has traditionally been used to monitor and model ozone effects on chlorophyll and photosynthesis. The contribution of nitrogen by the lower leaves in combination is greater than the flag leaf, and confirms the previously identified need for the effect of ozone on all leaf cohorts to be accounted for in future modelling of the effects of ozone on wheat (Emberson et al., 2018; Osborne et al., 2019). These data can contribute to that modelling work.

The Senescence Index developed to map the progress of senescence during Trial 3 proved successful in illustrating the effect of ozone on bringing forward the initiation of senescence in each leaf cohort. There were some slight inconsistencies within the Index due to its creation as the plants grew. A revised version is shown here for future use.

- 0 No senesced leaves
- 1 1 or more small basal leaves senescing
- 2 All 4th leaves senescing
- 3 All 3rd leaves senescing
- 4 All 2nd leaves senescing
- 5 All flag leaves senescing
- 6 All flag leaves fully senesced
- 7 All stems and peduncles senescing
- 8 All ears and awns senescing
- 9 All leaves, stems, spikes, awns fully senesced

6.2.2 Further research related to grain number

C:N ratios in the upper leaves, stem and ear showed no effect of ozone at anthesis, with nitrogen levels unaffected by ozone in the flag and 2nd leaves (Trial 3). However, as it was clear from chlorophyll data that ozone affected nitrogen status and photosynthetic capacity to a greater extent in the lower canopy, further research would be beneficial to assess the exact interaction between ozone, phenology, reduced chlorophyll levels in the whole canopy, and the subsequent effects on reduced photosynthate and floret fertility rates.

Ozone appears to have the same effect on wheat grain formation/floret fertility as other forms of abiotic stress such as heat and drought. In related abiotic stress research it has been found that specific components of the reproductive structures of self-fertilising crops like wheat, rice and sorghum are particularly sensitive to high Reactive Oxygen Species (ROS) levels induced by abiotic stress, both at meiosis and at anthesis, and tolerance appears to be related to the plants' antioxidant defence capacity (Ji et al., 2011; Parish et al., 2012). Interestingly, these are all also ozone-sensitive crops, therefore, further research which screens wheat lines for ozone tolerant reproductive structures is needed to clarify if this is an important potential source of ozone sensitivity/tolerance.

Although each of the three plant trials exposed plants to ozone from early vegetative growth stages until harvest, providing an assessment of the effect of chronic ozone exposure on multiple lines of wheat, wild relative species and synthetic lines, this experimental design prevented an analysis of the direct effects of ozone on meiosis and anthesis. There is a need for studies which limit ozone exposure to these key reproductive stages of meiosis and anthesis in order to separate – and quantify - these direct effects on reproductive processes and grain formation from those related to reduced chlorophyll and photosynthetic capacity, and early senescence.

6.3 Effect of ozone and interactions with nitrogen after anthesis

6.3.1 Effects on grain size and grain quality

Once florets are fertilised at anthesis, the period from anthesis to the end of grain fill largely determines eventual grain size, and grain quality (Sreenivasulu and Schnurbusch, 2012). In Trial 2, the grain morphology data extracted from the μ CT images of individual grains revealed that ozone-induced reductions in grain volume were being driven by reductions in grain width and depth rather than grain length. Ozone has often been found to reduce the proportion of grain starch with a concomitant increase in the proportion of grain protein (e.g. Pleijel et al., 1999). Broberg et al. (2015) suggest that these grain volume reductions are very likely the result of a decrease in starch which makes up the majority of wheat grain mass. This effect of ozone on grain quality, commonly found in wheat in response to other forms of abiotic stress (Wang and

Frei, 2011; Altenbach, 2012), was confirmed in cultivars grown in Trial 1 and Trial 2, alongside concomitant reductions in thousand grain weight (TGW). A detailed analysis of grain quality, such as changes to the characteristics of the proteins and starches within the grains, or to milling and baking qualities, was not undertaken as part of this research, but it is important to note that any changes to grain size and quality have implications for the end use and the value of the grain (Nuttall et al., 2017).

Previous research suggests that the post-anthesis, early grain fill growth stages are the most sensitive to ozone, and this is generally considered to be due to the reduced grain fill period brought about by the earlier start to senescence (Pleijel et al., 1998; Gelang et al., 2000), with resulting reductions in photosynthate and subsequent reduced starch yields. As discussed in Section 6.2.1, Trial 3 chlorophyll data confirmed that chronic ozone exposure starting during early vegetative growth stages is leading to earlier senescence and significant reductions in 'whole canopy' chlorophyll. This was especially evident during early grain fill in all leaf cohorts below the flag, especially in the older leaves in the canopy. In the more recently released cultivars this loss of photosynthetic capacity during this critical growth stage is a particular concern, as they contribute the majority of starch for grain fill in the post-anthesis period (Maydup et al., 2012; Tambussi et al., 2021). This may be an additional reason why modern cultivars have often been found to be more ozone sensitive than older lines (e.g. Pleijel et al., 2006; Singh et al., 2018), and why the post-anthesis period is increasingly vulnerable to ozone.

As the lower leaves also divert photosynthate down to the root which controls water and nutrient uptake these reduced levels of lower leaf chlorophyll may also have a secondary negative impact on yield formation during these critical anthesis and grain fill growth stages (Simpson et al., 1983. See Fig. 4.1.). Root biomass was significantly reduced by ozone at harvest in multiple lines in Trials 2 and 3, implying rapid root senescence during the latter stages of grain fill. In addition, both root biomass and carbon levels in the root and stem were increased by ozone at anthesis, supporting similar findings in both wheat and rice elsewhere, and possibly indicating a stress defence response (Zhang et al., 2014; Kou et al., 2018; Mathan et al., 2021) and a further diversion of resources from grain fill. One limitation of Trial 3 was the lack of assessment of the soluble non-structural carbohydrates present in plant parts. Further research would be needed to assess ozone's effect on the exact make up and partitioning of soluble carbohydrates both to root and shoot during anthesis and grain fill, and subsequent related changes to grain size and grain fill period would be beneficial, as other research suggests that abiotic stress reduces activity (Keeling et al., 1993), and even under optimal conditions final grain weight may be linked more to rates of starch synthase activity during grain fill than to the duration of the grain fill period (Borrill et al., 2015).

Together, all these post-anthesis ozone effects will be contributing to reduced grain fill, smaller grains, and therefore the suppression of yields in wheat growing areas affected either by persistent high levels of ozone or by peak ozone episodes during grain fill.

6.3.2 Effects on nitrogen translocation

Trial 3 tracked the effect of ozone on the translocation of nitrogen which took place between anthesis and harvest from photosynthesising plant parts to the ear/grain. Even though Nitrogen Remobilisation Efficiency (NRE) was reduced by the highest ozone level, especially in lower leaves where chlorophyll levels were reduced by ozone, the majority of nitrogen was still translocated to the ear/grain in all treatment levels. This suggests that the mechanism of translocation was mostly unaffected by ozone. Equally, the ¹⁵N trace experiment, involving the application of labelled ammonium nitrate fertiliser at anthesis, indicated that whilst only small amounts of nitrogen were taken up by all plants in the post-anthesis period, with the majority translocated to the ear/grain, there was no effect of ozone on uptake or translocation. These data, therefore, support previous suggestions that whilst ozone does affect the production of photosynthate in the post-anthesis period and the timing of senescence, the actual process of dismantling and translocating nitrogen to the ear is largely unaffected (Ojanperä et al., 1992), and therefore leads to the relatively higher proportions of protein in ozone-affected grain (Pleijel et al., 1999; Wang and Frei, 2011). However, as the grain set was abnormally low in Trial 3, a repeat of this experiment would be needed to confirm these results.

6.3.3 Effects on residual nitrogen belowground

Increases in nitrogen pollution had been predicted by Broberg et al. (2017) as a likely consequence of ozoneinduced reductions in growth and NUE. Although NUE could not be measured in Trial 3, NRE was reduced under the highest ozone treatment. In addition, an indication that ozone can also reduce nitrogen uptake came from measurements of soil nitrates from soil water in Trial 2, with increases in some lines under the highest ozone treatment when a late application of ammonium nitrate was made during grain fill. These data support previous findings where increased levels of both nitrate and ammonium were found in ozoneaffected wheat rhizospheres (e.g. Hu et al., 2018). However, in Trial 2 this effect did not occur when the nitrogen fertiliser was applied at the earlier pre-anthesis growth stage, nor after either application at lower ozone concentrations, nor in all lines under the late application, and did not appear to be correlated with root biomass at harvest.

Any increase in soil nitrate levels has clear implications for nitrogen pollution from agri-ecosystems, via both nitrate leaching and also potentially from volatilisation of nitrous oxide, as a by-product of soil nitrification and denitrification processes (Cameron et al., 2013; Hu et al., 2018). These data, therefore, contribute to evidence highlighting the impact of abiotic stress on nitrogen pollution from agri-ecosystems, and the need
CHAPTER 6

to increase both wheat's NUE and its abiotic tolerance in order to reduce costs to the farmer and to the environment.

6.3.4 Effects on residual nitrogen aboveground

Analysis of plant parts at harvest in Trial 3 found that high concentrations of ozone had led to reductions in the C:N ratio in leaves, driven largely by increases in levels of residual nitrogen, a response which has also been found in other plants (e.g. Wittig et al., 2009) and some crops, including rice (Yulong et al., 2021). This increase in residual nitrogen could be linked to grain yield reductions and reduced demand for nitrogen, but increases in residual nitrogen in leaves were found in ozone-affected Trial 3 plants where grain set largely failed, as well as in the Trial 1 and Trial 2 cultivars whose grain yields were normal. This suggests that this response occurs irrespective of the size of the grain sink. However, the exact cause, and the exact constituents, of this increased residual nitrogen were not assessed in Trial 3, and remain unknown.

Several explanations for this effect on residual nitrogen levels have previously been proposed and would provide a starting point for further research. Reductions in nitrogen use efficiency (NUE) as a result of the shortened grain fill period could mean less nitrogen is dis-assembled and translocated from photosynthesising plant parts (Shang et al., 2018; Broberg et al., 2021). Alternatively a defence response at the whole plant level could be leading to increased structural proteins to protect cell membranes, including the amino acid proline and/or flavonols which have been found to accumulate in plant tissues in response to abiotic stress , including ozone (Verbruggen and Hermans, 2008; Sarkar and Agrawal, 2010; Boublin et al., 2022).

Whatever the cause, the resulting increase in residual nitrogen left in the shoot has implications for NUE and additional nitrogen being lost from agri-ecosystems, either within harvested straw, or when unharvested root/shoot material is ploughed back into farm soil where it may either be beneficial, if used by a subsequent crop, or detrimental if leached, depending on environmental conditions.

6.3.5 Additional nitrogen fertilisation ameliorates effect of ozone

In Trial 3, the differing responses of the N+ plants, which received extra nitrogen fertilisation at anthesis, compared with the NØ plants which did not, raise important issues related to assessing the impact of ozone on crop plants. The relatively small addition of nitrogen at anthesis (20 kg N ha⁻¹ equivalent) reduced the negative impact of ozone on chlorophyll levels, to a limited extent in the flag leaf, but clearly delaying senescence and maintaining chlorophyll levels at this critical growth stage in the 2nd and 3rd leaf cohorts, whose combined photosynthate output make an important contribution to plant growth (Simpson et al., 1983). The impact of ozone on residual nitrogen in leaves, on C:N ratios, as well as on NRE, were also moderated by higher nitrogen fertilisation. Although effects on final yield and grain quality could not be

made in Trial 3, these data support findings elsewhere that suggest an increase in nitrogen fertilisation can protect plants from abiotic stress-induced ROS, such as via increased levels of antioxidants (e.g. Pandey et al., 2018).

Further research assessing the defence responses in ozone-affected wheat under different nitrogen fertilisation regimes would be beneficial. In addition, further experimental work is needed to quantify the extent to which different nitrogen fertilisation regimes ameliorate the effect of ozone on stomatal conductance, chlorophyll and photosynthesis, and ultimately on grain yield parameters, as previous research has often not taken this issue fully into consideration. In order to mitigate against ozone damage to wheat crops it could be inferred that increasing nitrogen fertilisation, especially at times when (if monitored) ozone pollution occurs at critical growth stages, would be beneficial, but there would also be an obvious risk of increasing levels of nitrogen pollution.

6.4 What are the possible origins of wheat's ozone sensitivity, and how can wheat's ozone tolerance be improved?

Wheat is an ozone sensitive crop, but there are variations in sensitivity between different lines which could be exploited in future to promote lines with greater ozone tolerance. In Trials 1 and 2 several of wheat's closest wild relatives were exposed to ozone, along with lines of synthetic wheat derived from the original wild relatives, and three different elite cultivars varying in release date. A comparison of multiple parameters allowed an assessment of differences in their ozone sensitivity. These, combined with the assessment of interactions with nitrogen explored in Trial 3, enables some recommendations to be made in relation to further research and the breeding of wheat with greater ozone tolerance.

6.4.1 Genetic origins of wheat's ozone sensitivity

Very few studies have assessed the potential genetic origins of wheat's ozone sensitivity (Biswas, Xu, Y. G. Li, et al., 2008; Brewster et al., 2018), and fewer still have attempted to identify the specific genes associated with ozone sensitivity/tolerance, which could in future form the basis for the breeding of ozone tolerant wheat (Begum et al., 2020). Although genetic analysis was beyond the scope of this research, an assessment of ozone sensitivity of wheat's closest wild relatives can inform future genetic research into ozone tolerance.

Triticum aestivum L., bread wheat, is a hexaploid wheat, with a large genome comprising components of each of its wild ancestors (AABBDD). Contrary to a previous study, which had exposed the main genome donors of bread wheat to ozone only at the vegetative growth stages and found *Aegilops tauschii* (diploid, DD) to be ozone sensitive and a tetraploid (T. durum, AABB) line to be more tolerant (Biswas, Xu, Y. G. Li, et

CHAPTER 6

al., 2008), Trial 1 exposed plants to yield bearing stage and found that, a line of *Ae. tauschii*, was tolerant of ozone, whilst, *T. urartu*, (diploid, AA) and to a lesser extent *T. dicoccoides*, (AABB) were more sensitive. These sensitive *Triticum* wild relatives could therefore be proposed as the possible genetic source of wheat's ozone sensitivity, whilst *Ae. tauschii* could be a source of tolerance.

The physiological causes of the differences in sensitivity/tolerance of the wild relative species were not examined in Trial 1, therefore further research would be needed to identify the specific underlying mechanisms associated with these differences. In addition, a further assessment of the effect of ozone on multiple lines of all the progenitor species is needed to confirm how much variation in sensitivity is present between lines of the same species, whether there is a consistent, genetically controlled response to ozone, and if so, which sets of genes are linked to their tolerance. The ability of *Ae. tauschii* to tolerate abiotic stress has been highlighted previously, and attributed in part to its wide geographical range (Gorafi et al., 2018). It may therefore provide a rich source of ozone tolerance genes which, once identified, could be exploited in breeding programmes and contribute to efforts to ensure wheat can continue to be grown widely despite ongoing ozone pollution.

6.4.2 Can synthetic wheat contribute to yield gains and ozone tolerance?

Synthetic, or re-synthesised wheat, has been created by artificially re-crossing the original wild relative diploid (DD) and tetraploid (AABB) species which created wheat 10,000 years ago. Synthetic lines are being used increasingly within plant breeding, partly due to their genetic diversity (Li et al., 2018), and have been found to offer multiple abiotic stress tolerance traits (Trethowan and Mujeeb-Kazi, 2008). There was no evidence in the literature to suggest that synthetic lines had ever been assessed for ozone sensitivity before and, based on the physiological, biomass, and yield data collected in Trial 1 and 2, it appears that the synthetic lines are affected by ozone in the same way as normal hexaploid wheat, and are as genetically diverse and varied in their capacity to tolerate ozone. For example, whilst in Trial 1 a line of primary synthetic hexaploid wheat (SHW) was found to have strong ozone tolerance, in Trial 2 a different line of primary SHW proved to be ozone sensitive. Differences in ozone sensitivity were also found between the F2 lines derived from primary SHW and the elite cultivar Paragon, but one line (BC1) exhibited similar levels of ozone tolerance as Paragon but with the larger grain size typical of synthetic lines. This makes the primary SHW from Trial 1 and the BC1 line from Trial 2 potentially useful to wheat breeders as a source of both larger grains and abiotic tolerance, traits which could be exploited to support the urgently required increase in wheat's yield potential in the face of increasing abiotic stress related to climate change and air pollution (Mills et al., 2016). However, further research would be needed to identify the physiological and genetic origins of ozone tolerance found in these synthetic lines. An assessment of the ozone sensitivity of commercial synthetic lines already being grown in countries where high ozone pollution still occurs, such as China (Liu et al., 2018), would also be beneficial in order to assess their relative tolerance compared to traditional cultivars.

6.4.3 Elite cultivars, their release date, and factors contributing to ozone tolerance in wheat

Multiple small-scale experiments (e.g. Barnes et al., 1990), and large-scale screening of cultivars (e.g. Singh et al., 2018), have identified lines with greater or lesser ozone sensitivity, as well as regional differences between cultivars commonly grown in different parts of the world (e.g. Emberson et al., 2009; Pleijel et al., 2019). In the process, ozone sensitivity has often been found to correlate with the cultivar's release date (e.g. Pleijel et al., 2006), suggesting that the more recent the cultivar, the greater is its ozone sensitivity. Based on total yield and TGW in Trial 1 and 2, the older cultivar Maris Dove, (released 1971) proved to have greater ozone tolerance than the more recent cultivar, Skyfall (released 2014), whilst Paragon (released 1999) also showed greater tolerance to ozone compared to Skyfall. These therefore provide additional examples of how a later release date appears to correlate with greater ozone sensitivity. Physiological and genetic analyses to explain the exact mechanisms behind these differences would be needed to identify why these differences in sensitivity between these lines occurred.

Despite the fact that both Paragon and Maris Dove had lower average yields than Skyfall, meaning that Skyfall could still outperform these older varieties in the field even under higher ozone concentrations, it is still a cause for concern that recent cultivars are often more ozone sensitive, especially in the context of the pressure to increase global wheat yields. This tendency towards greater ozone sensitivity has been attributed to the selection of cultivars with increasingly higher rates of stomatal conductance, which generate higher photosynthetic rates and yields, but inadvertently also leads to higher ozone flux (Mills, Sharps, et al., 2018). However, differences in ozone sensitivity have also been found to be linked more to variation in antioxidant defence capacity (Feng et al., 2016). The introduction of the Rht gene has led these short-stemmed cultivars to be more dependent on post-anthesis photosynthesis to generate sugars for grain filling rather than pre-anthesis stored photosynthate (Tambussi et al., 2021), reinforcing the suggestion that differences between sensitive and tolerant wheat cultivars may be related to antioxidant defence systems and the ability to prevent ozone from triggering early senescence (Feng et al., 2016). The requirement to increase wheat yields in future will, in part, be dependent upon the supply of adequate levels of photosynthate during grain fill to achieve the increases in grain yields (Hawkesford et al., 2013). Identifying ozone tolerance genes linked to maintaining chlorophyll levels and photosynthetic capacity after anthesis may therefore be crucial.

6.4.4 Breeding wheat for abiotic stress tolerance

Throughout this thesis there have been many examples of the similarities between wheat's response to ozone and its response to other forms of abiotic stress, with the common link often related to increased

CHAPTER 6

ROS and the plant's antioxidant defence capacity. With the increasing threat to wheat yields posed by climate change, plant breeders are working to improve wheat's abiotic stress tolerance, especially to heat and drought (Reynolds et al., 2021), often using the genetic resources now more readily available within wheat's wild relatives and synthetic lines (Trethowan and Mujeeb-Kazi, 2008). Although ozone is rarely taken into consideration in wheat breeding (Mills, Sharps, et al., 2018), if this work leads to improvements in antioxidant defence systems which delay the onset of earlier senescence, particularly during anthesis and grain fill growth stages, this could also improve wheat's tolerance to ozone.

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