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Research paper

Ozone exposure consistently increases δ13C in wheat grain

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ABSTRACT

Tropospheric ozone (O_3) is a regional air pollutant, formed by solar radiation from nitrogen oxides and volatile organic compounds. It is known to impair crop yields. The mechanisms of O_3 damage to plants are linked to gas exchange and carbon metabolism. The carbon isotopic signature in plant tissues represented by $\delta^{13}C$ offers a time-integrating approach to assess the performance of plant gas exchange. We combined wheat grain $\delta^{13}C$ data from seven O_3 experiments performed in four countries (Switzerland, Spain, Sweden, United Kingdom). For one experiment $\delta^{13}C$ data for stems were available. There was a significant positive relationship between grain $\delta^{13}C$ and O_3 exposure (R^2=0.37). Using a relative scale to account for variation in the $\delta^{13}C$ level among experiments, a stronger linear relationship was obtained (R^2=0.77). Furthermore, the relative yield loss from O_3 was negatively linked to the relative effect on $\delta^{13}C$ (R^2=0.72). Stems were more depleted in ^{13}C than grain but also showed a significant, less steep, positive $\delta^{13}C$ relationship with O_3 exposure. The most important conclusion from the positive relationship between $\delta^{13}C$ and O_3 exposure is that the O_3 effect on stomatal conductance dominates over the impairment of CO_2 fixation by Rubisco. However, also discrimination associated with redistribution of carbohydrates from non-reproductive plant parts to grains can contribute to the O_3 effect on $\delta^{13}C$. Based on the unified pattern of $\delta^{13}C$ response over a range of experiments performed in different sites, we conclude that the mechanisms of O_3 damage in wheat with respect to gas exchange are highly consistent.

1. Introduction

Tropospheric (ground-level) ozone (O_3) is a regional air pollutant formed by photochemical reactions of nitrogen oxides and volatile organic compounds, including methane, and carbon monoxide. Due to anthropogenic emissions of O_3 precursors, background O_3 concentrations have risen from pre-industrial levels of around 10 ppb to current 35–40 ppb (Cooper et al., 2014; Tarasick et al., 2019). Elevated O_3 concentrations are today found over wide geographical areas of the Earth (Mills et al., 2018a) and are known to negatively affect yields of important crops (Mills et al., 2018b). Wheat is considered one of the most O_3 sensitive crops and is at the same time one of the most important in terms of agricultural production (Mills et al., 2018b; Erenstein et al., 2022). A global meta-analysis of the effect of air filtration to remove O_3 showed an average negative effect on wheat yield of 8.4 % from current ambient O_3 concentrations compared to pre-industrial levels (Pleijel et al., 2018).

O_3 enters plant leaves through stomata and then acts as an oxidizing agent that initiates stress response pathways, resulting in stomatal closure (lower stomatal conductance, g_s) and reduced net carbon dioxide (CO_2) assimilation (A_n) by the enzyme Rubisco (Ainsworth, 2016). Exposure to O_3 is also well known to cause pre-mature senescence, which shortens the photosynthetic period (Grandjean and Fuhrer, 1989; Gelang et al., 2000). Stomatal gas exchange controls the leaf exchange of CO_2, the transpiration loss of water vapour to the atmosphere as well as O_3 uptake. An important question in this context is if the negative O_3 effect on g_s is more important than the effect on carbon (C) assimilation by Rubisco and thus if there is a positive or negative effect on water use efficiency (WUE, which is the amount of carbon assimilated per unit water transpired). If the effect by O_3 on g_s is larger than that on A_n, WUE will be higher even if the absolute level of C assimilation is reduced. Thus, an increase in WUE does not necessarily mean that growth and yield will also increase. As an additional research question, it is significant to know whether the pattern of O_3 effects on plant gas exchange is

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consistent in different wheat genotypes and under variable environmental conditions such as different geographical areas and years.

While active, cuvette-based measurements provide snapshots of gas exchange (g_s and A_n) the study of δ ^{13}C of plant tissues provides a time integrated signature of gas exchange characteristics (Sanchez -Bragado et al., 2014; Araus et al., 2021). This approach is based on the discrimination of the heavier stable C isotope ^{13}C in relation to the quantitatively dominating isotope ^{12}C (Farquhar et al., 1989). $\delta^{13}C$ is defined as:

$$\delta^{13}C = \left(\frac{\left(^{13}C/^{12}C\right)_{sample}}{\left(^{13}C/^{12}C\right)_{standard}} - 1\right) \tag{1}$$

where the standard is the Vienna Pee-Dee Belemnite (V-PDB) based on a cretaceous marine fossil from the Peedee Formation of South Carolina (Tscherkez et al., 2011). In the case of C assimilation by plants, the discrimination of the heavier ^{13}C isotope in the C uptake by plants has two components related to its larger mass: the slower diffusion of ^{13}C through the stomatal pores and the lower reactivity of $^{13}CO_2$ with the carbon-fixing enzyme Rubisco. This can be quantitatively expressed as (Farquhar et al., 1989):

$$\delta^{13}C_{\text{plant}} = \delta^{13}C_{air} - a\frac{p_a - p_i}{p_a} - b\frac{p_i}{p_a} = \delta^{13}C_{air} - a - (b-a)\frac{p_i}{p_a} \tag{2}$$

where a represents the fractionation of the two C isotopes occurring during diffusion through the stomatal pores (≈4.4 ‰), b is the net fractionation of C isotopes by Rubisco carboxylation (≈27 ‰), p_a is the partial pressure of CO_2 in the ambient air surrounding the plants, and p_i is the partial pressure of CO_2 in the mesophyll of the leaf. It can be inferred from Eq. (2) that $\delta^{13}C$ becomes less negative if the negative impact of O_3 on g_s is larger than the O_3 induced impairment of A_n.

Further fractionation of ^{13}C vs. ^{12}C in the plant can also occur during metabolic processes converting C compounds (Tscherkez et al., 2011) and redistribution of C within the plant (Badeck et al., 2005). For example, starch is known to be enriched in ^{13}C vs. ^{12}C, in comparison to C assimilated in the photosynthesis, by ~2 ‰ as a consequence of ^{13}C being favoured in the enzymatic activity of a biochemical step of starch formation (Tscherkez et al., 2011). Thus, one can expect wheat grain, which consists to ~60–75 % of starch (Shevkandi et al., 2017), to have a higher $\delta^{13}C$ than leaves and stems. In line with this, Saurer et al. (1991) found $\delta^{13}C$ to be higher in wheat grains compared to leaves. Wheat stems are of large importance to consider since they store considerable amounts of photosynthates that are redistributed and contribute to grain filling along with current photosynthates distributed directly to the grain (Schnyder, 1993).

To evaluate the efficiency of a seed crop like wheat in converting above-ground biomass to grain it is commonplace to estimate the harvest index (HI), i.e. the fraction of above-ground biomass in the grain at harvest (Luo et al., 2015). Improved yield in wheat during the 20th century was to a substantial extent the result of breeding for higher HI (Austin et al., 1980). Although a crop trait with high heritability, HI can be negatively affected by stress factors like heat (Bergkamp et al., 2018), drought (Thapa et al., 2019) and ground-level O_3 (Pleijel et al., 2014). Apart from biomass yield, HI can be evaluated for different elements such as nitrogen (N, e.g. Hawkesford, 2014), but also for other essential or non-essential elements (Broberg et al., 2021). Of particular interest when evaluating $\delta^{13}C$ is the carbon harvest index (C-HI). Since the allocation of carbon from non-reproductive to reproductive parts of the wheat plant affects C-HI, and $\delta^{13}C$ is likely to be affected by such redistribution leading to different isotopic signals of individual plant organs (Badeck et al., 2005), it can be assumed that there is link between $\delta^{13}C$ and C-HI, e.g. under the influence of O_3 stress.

Another stable isotope commonly used to study processes within plants is ^{15}N, which reflects uptake and redistribution of this isotope compared to the dominating ^{14}N (Evans, 2001). $\delta^{15}N$ (defined analogously to Eq. 1 but using the N isotopic composition of air as reference) is strongly dependent on the soil N source, not directly affected by O_3, and has been used to a lesser extent than $\delta^{13}C$ in O_3 effect research and when investigated, patterns of O_3 effects for $\delta^{15}N$ have been less conclusive compared to $\delta^{13}C$ (Möcker et al., 1996; Chang Espino et al., 2021; Brewster et al., 2024).

We used data from seven different experiments performed in four countries to investigate the relationship of the C (all seven experiments) and N (a subset of four experiments) isotopic signatures of wheat with O_3 exposure. Our hypotheses were:

1. $\delta^{15}N$ is less useful as an indicator of O_3 stress in wheat compared to $\delta^{13}C$.
2. $\delta^{13}C$ is positively affected, i.e. WUE is promoted, by O_3 exposure.
3. Grain yield reductions from O_3 are correlated with the O_3 effect on $\delta^{13}C$.
4. $\delta^{13}C$ is higher in the starch-rich grain than in stems.
5. $\delta^{13}C$ is affected by the degree of C redistribution from the shoot to grains, represented by C-HI, under O_3 exposure.

2. Methods

2.1. Data

Data regarding $\delta^{13}C$, grain yield and the daytime average ozone concentration, $[O_3]$, required for the analysis were available for four different countries: Switzerland, Spain, Sweden and the United Kingdom as specified in Table 1. The stable isotope data of three open-top chamber experiments from Switzerland have been published earlier (Saurer et al., 1991). Likewise, the data for the Spanish open-top chamber experiment, including $\delta^{13}C$, $\delta^{15}N$, $[O_3]$ and grain yield data, were already published by Chang Espino et al. (2021, only data for post-green revolution cultivars included, four spring wheat and three winter wheat). In the case of the Swedish open-top chamber experiment and the two UK solardome experiments the stable isotope data have not been published earlier, but the results regarding $[O_3]$, grain yield and other agronomic variables have been published where experimental design and sampling are described in detail (Gelang et al., 2000; Broberg et al., 2021, 2023; Hayes et al., 2020). For these three experiments, C and N isotope ratios were obtained using an elemental analyzer coupled to an Isotope Ratio Mass Spectrometer (Automated Nitrogen Carbon Analyser ANCA TGII and 20–22 isotope ratio mass spectrometer, SerCon Ltd., Crewe, UK). Only the well-watered ambient air temperature O_3 treatments were used for the experiment described in Broberg et al. (2023). Elevated temperature and drought could in themselves have impacts on $\delta^{13}C$. In Broberg et al. (2023) drought was shown to significantly reduce the negative impacts of O_3 exposure on both gas-exchange and yield variables, suggesting that there are also potential interactions with O_3 exposure for effects on $\delta^{13}C$. In this study the focus was to compare the O_3 effects as such in all included experiments without consideration of interaction of the O_3 effect with other environmental variables. The Swedish data set contained data for both grain and stems at the final harvest. For the present study, averages for the seven cultivars investigated in the Spanish study (Chang Espino et al., 2021), as well as for the five African cultivars explored by Hayes et al. (2020), were used. The Swiss and Swedish experiments were conducted with field grown wheat, while the experiments in Spain and UK were performed with potted plants (Table 1). The supplementary file contains the data used that have not been published earlier.

For the four experiments performed in Spain, Sweden and the United Kingdom, but not in the three experiments from Switzerland, $\delta^{15}N$ data were available in addition to $\delta^{13}C$.

2.2. Derivation of response functions and statistical analysis

Linear regression was used to analyse the associations of biological

Table 1
Basic information about the seven experiments from which data were obtained for the present study regarding country, year of experiment, location of the experimental site, cultivars, ozone exposure system and root environment. OTC, open-top chamber.

ID (country year)	Experiment site	References	Cultivar	Exposure system	Root environment
CH 1986	Oeschberg, Bern, Switzerland	Saurer et al. (1991); Fuhrer et al. (1989)	Albis	OTC	Field
CH 1987	Oeschberg, Bern, Switzerland	Saurer et al. (1991); Fuhrer et al. (1989)	Albis	OTC	Field
CH 1988	Oeschberg, Bern, Switzerland	Saurer et al. (1991); Fuhrer et al. (1989)	Albis	OTC	Field
SE 1997	Östad, Gothenburg, Sweden	Gelang et al. (2000); 2001; Broberg et al. (2021)	Dragon	OTC	Field
ES 2015	La Higueruela, Toledo, Spain	Chang-Espino et al. (2021)	7 Spanish cultivars[a]	OTC	Pots
UK 2016	Abergwyngregyn, Bangor, UK	Osborne et al. 2019; Broberg et al. (2023)	Skyfall	Solardome	Pots
UK 2017	Abergwyngregyn, Bangor, UK	Hayes et al. 2020	5 African cultivars[b]	Solardome	Pots

[a] Ablaca, Artur Nick, Berdun, Califa Sur, Marius, Nogal, Yécora
[b] Eagle, Hawk, Njoro, Wren, Korongo

variables with $[O_3]$ and among biological variables. Thus, response functions were derived between absolute $\delta^{13}C$ and $[O_3]$, the relative effect by O_3 on $\delta^{13}C$ and $[O_3]$, and between the relative O_3 effect on grain yield and the relative O_3 effect on $\delta^{13}C$ with $[O_3]$. The relative effects were obtained by scaling with respect to the value obtained of the biological variables for zero O_3 exposure by regression in each individual experiment, i.e. at zero exposure, the effect on the biological variables was assumed to take the value zero (no O_3 effect associated with zero exposure, Fuhrer, 1994; Grünhage et al., 2012). For grain yield this transformation is necessary to make the observed values from potted vs. field grown, as well as different magnitudes of yield in different sites and years, comparable. Similarly, the magnitude of $\delta^{13}C$ (regardless of O_3 exposure) in different experiments differs for biological or environmental reasons, as well as the change over time in the $\delta^{13}C$ of atmospheric CO_2 (Canadell et al., 2021); accounting for this variation allows the direct comparison of O_3 effects on $\delta^{13}C$ in the different experiments. Statistical analyses were made in Excel; comparison of the slopes for $\delta^{13}C$ relationships with $[O_3]$ concentration for grains and stems was made with GraphPad Prism. Values for the carbon harvest index (C-HI, the fraction of aboveground carbon found in the grain at harvest) for the Swedish experiment were based on the sampling described in detail by Broberg et al. (2021). The C content was determined using a CHNS-O analyser (model EA 1108, Fison Instruments, Rodano, Italy).

3. Results

3.1. $\delta^{15}N$ and $\delta^{13}C$ in wheat grain under O_3 exposure

For the four experiments for which both $\delta^{15}N$ and $\delta^{13}C$ were available, these two variables were plotted against each other to investigate if there was a general relationship between them, as well as if there was a separation among O_3 treatments of the individual experiments with respect to $\delta^{15}N$ and $\delta^{13}C$. The overall response pattern showed a positive relationship between $\delta^{15}N$ and $\delta^{13}C$, where a high discrimination of $\delta^{13}C$ was strongly associated with a high discrimination of $\delta^{15}N$. Thus, there were clear differences between experiments with respect to both $\delta^{15}N$ and $\delta^{13}C$ (Fig. 1) with a higher $\delta^{15}N$ associated with a higher $\delta^{13}C$. The variation among O_3 treatments within experiments, which could be attributed to O_3 exposure, was much larger for $\delta^{13}C$ compared to $\delta^{15}N$ (Fig. 1). From this pattern it was concluded that for the present data set the variation in $\delta^{15}N$ was not useful as an indicator of the O_3 response of wheat and the focus of the remaining analysis was on $\delta^{13}C$ only.

3.2. Relationships of $\delta^{13}C$ with O_3 exposure

A highly significant positive relationship ($R^2 = 0.77$; $p < 0.0001$) between the relative O_3 effect on $\delta^{13}C$ in wheat grains and $[O_3]$ was

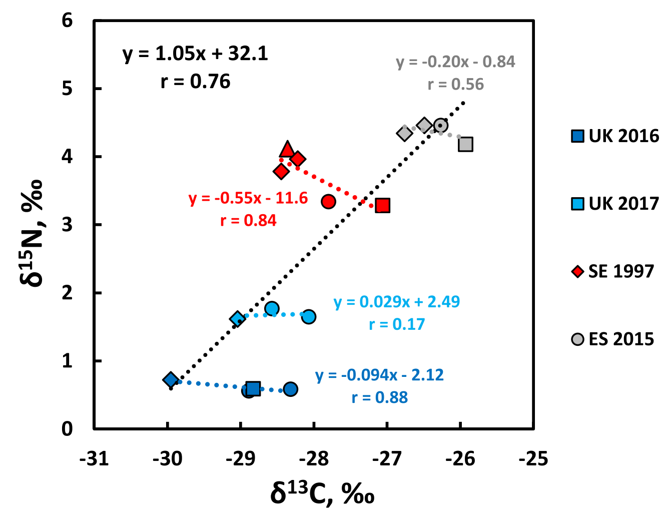

Fig. 1. Relationship between $\delta^{15}N$ and $\delta^{13}C$ in the four experiments for which both variables were available. The black line represents the relationship based on all experimental treatments, while the coloured lines are for the individual experiments. Daytime average ozone concentrations $[O_3]$ of the experimental treatments were indicated as: triangle, < 20 ppb; diamond, 20–40 ppb; circle, 40–60 ppb; square, 60–80 ppb; UK, United Kingdom; SE, Sweden; ES, Spain.

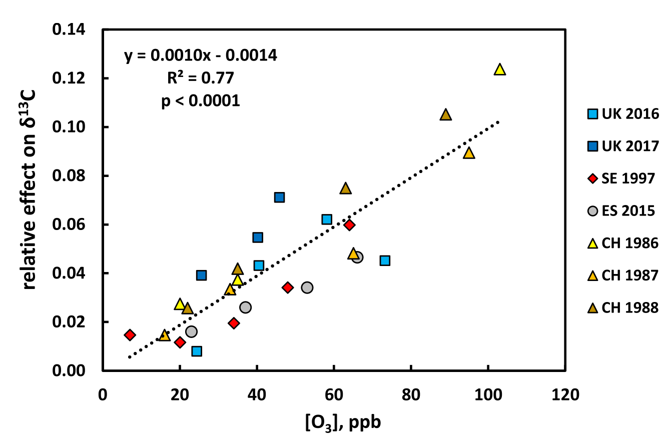

Fig. 2. Relative effects on wheat grain $\delta^{13}C$ in relation to daytime average ozone concentration $[O_3]$. The legend shows country and year of experiment: UK, United Kingdom; SE, Sweden; ES, Spain; CH, Switzerland.

demonstrated (Fig. 2). The largest effects were obtained in the high O_3 treatments of the Swiss experiments, but also the remaining experimental treatments conformed to the consistent linear relationship.

The absolute values of $\delta^{13}C$ (Fig. 3) also showed a strongly statistically significant positive relationship with O_3 exposure, although weaker ($R^2 = 0.37$; $p = 0.0008$) than for the relative $\delta^{13}C$ effect values (Fig. 2). This is a consequence of the substantial inter-experimental variation in the absolute level of $\delta^{13}C$, unrelated to O_3 exposure as shown in Fig. 1, which was accounted for in Fig. 2 but not in Fig. 3.

3.3. Relationships of grain yield with $\delta^{13}C$ under O_3 exposure

There was a highly significant ($R^2 = 0.72$, $p < 0.0001$) negative relationship between relative grain yield and grain $\delta^{13}C$ (Fig. 4a), where a higher relative yield was associated with a lower $\delta^{13}C$, i.e. a larger discrimination against ^{13}C. This can be compared (Fig. 4b) with the relationship between relative grain yield and $[O_3]$, which was only marginally stronger ($R^2 = 0.77$, $p < 0.0001$).

3.4. Comparison of $\delta^{13}C$ in grain and stems

For the Swedish 1997 experiment, $\delta^{13}C$ in grains and stems were compared (Fig. 5), where both fractions showed a significant positive relationship with $[O_3]$ ($R^2 = 0.84$, $p = 0.029$ and $R^2 = 0.92$, $p = 0.011$ for grain and stems, respectively). Comparing the slope coefficients for the two plant fractions indicated an almost significant difference ($p = 0.055$) with a steeper slope for grains. Fig. 5 also shows that stems are more depleted in ^{13}C compared to grains by ~1.5–3 ‰ depending on the level of O_3 exposure.

The carbon harvest index, C-HI, was negatively affected by O_3 exposure ($y = 0.0013x + 0.51$, $R^2 = 0.78$, $p = 0.047$; data not shown) and also correlated negatively with $\delta^{13}C$ (Fig. 6) in both grain ($R^2 = 0.97$, $p = 0.0022$) and, more weakly so, in stems ($R^2 = 0.83$, $p = 0.032$). Thus, a larger fraction of above-ground C allocated to grains was associated with larger discrimination $\delta^{13}C$. The three lowest O_3 treatments differed little with respect to C-HI, while the two higher O_3 treatments had considerably lower C-HI associated with higher $\delta^{13}C$.

4. Discussion

When comparing $\delta^{13}C$ and $\delta^{15}N$ of the experiments for which both variables were available, they were positively correlated. Possibly this is related to the general productivity (resulting from climate and soil) in the different experiments, but this is difficult to compare because of the varying characteristics of the experiments (soil vs. pot grown plants, exposure system). High productivity results from high rates of gas exchange and carbon assimilation, as well as high rates of N assimilation and metabolism within the plant. The discrimination of ^{15}N during N uptake is particularly important when N availability is high in relation to demand (Evans, 2001). This is likely to be associated with high growth, such as under ample fertilization, which would stimulate leaf gas-exchange, resulting in a higher stomatal conductance and a greater discrimination of ^{13}C.

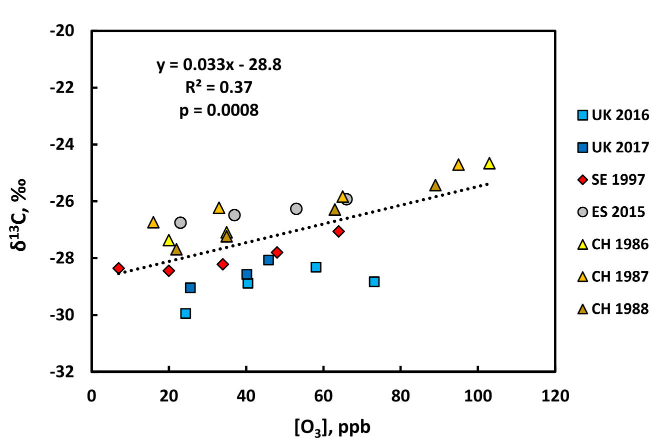

Fig. 3. Absolute $\delta^{13}C$ in wheat grains in relation to daytime average ozone concentration $[O_3]$. The legend shows country and year of experiment: UK, United Kingdom; SE, Sweden; ES, Spain; CH, Switzerland.

The O_3 treatments of the individual experiments, however, had a much smaller, in some experiments essentially absent, variation in $\delta^{15}N$ compared to $\delta^{13}C$, in line with the first hypothesis. From this we conclude that $\delta^{13}C$ of grain is a better indicator of O_3 stress than $\delta^{15}N$, for which no substantial net effect from the O_3 stress was observed. This is in line with existing data in the literature (Möcker et al., 1996; Chang Espino et al., 2021), and Brewster et al. (2024) also showed that O_3 affects N mobilisation within wheat plants, rather than causing differences in the N uptake pattern. It can likely be explained by the fact that O_3 does not have any significant effect on the isotopic composition of soil N pools in the rhizosphere, which is crucial for plant isotopic composition of the N taken up and assimilated by plants (Hu et al., 2018), while the discrimination in stomatal uptake and assimilation of C isotopes is connected to processes that are sensitive to O_3. Based on these observations and considerations we decided to restrict the rest of the analysis to O_3 effects related to $\delta^{13}C$.

Strongly significant relationships were observed of both absolute and relative $\delta^{13}C$ with $[O_3]$ for a data set containing a range of different agricultural environments and wheat cultivars, supporting the second hypothesis. Based on Eq. (2), $\delta^{13}C$ becomes less negative if g_s is reduced to a larger extent than A_n (Farquhar et al., 1989). It follows from the consistent pattern observed (Figs. 2 and 3) that the negative effect of O_3 on wheat production depends more strongly on reduced g_s than on the impairment of A_n, as suggested by Saurer et al. (1991) and discussed also by Martin et al. (1988). This should be considered in the modelling of O_3 effects on annual crops like wheat. A potential explanation is that as O_3 enters the plant leaf through the stomata, the cells surrounding the stomata are predominantly affected by O_3 as a consequence of the high reactivity of the O_3 molecule with leaf interior tissues, leading to internal O_3 concentration close to zero (Laisk et al., 1989). This pattern would promote stomatal closure, while the rest of the mesophyll remains less affected. In a screening experiment where a range of plant species were exposed to air pollutant combinations including O_3, SO_2 and NO_2 a covariation of visible leaf injury with increased $\delta^{13}C$ was mostly but not always observed (Martin et al., 1988). Ma et al. (2022) presented results pointing in the opposite direction regarding the effect of O_3 on $\delta^{13}C$ compared to our study. Their observations were based on data for leaves only and are thus not directly comparable with grain data used by us.

An important implication of the larger effect by O_3 on g_s than on A_n, as evaluated from $\delta^{13}C$, is that WUE is improved, which was found to be the case also with other types of stress, such as drought (Condon et al., 2002; 2004). This means that the accumulation of biomass in relation to the water loss through transpiration is larger and should not be understood as improved growth and yield necessarily following from higher WUE. An increase in WUE associated with suppressed growth in response to O_3 exposure has earlier been observed in radish (*Raphanus sativus*) and soybean (*Glycine max*) as inferred from the study of stable carbon isotopes (Greitner and Winner, 1988).

The negative relationship between relative grain yield and relative wheat grain $\delta^{13}C$ suggests that yield loss from O_3 is associated with the mechanisms reflected by $\delta^{13}C$. This is in accordance with the third hypothesis. It can be noted that the strength ($R^2 = 0.72$) of the relationship of relative yield with $\delta^{13}C$ is almost as high as the relationship of relative yield with $[O_3]$ ($R^2 = 0.77$), further supporting that the mechanisms of O_3 impairment of wheat grain yield are highly consistent over the seven experiments covering different sites, years and genotypes.

The higher $\delta^{13}C$ of wheat grain compared to stems shows that different plant compartments have different $\delta^{13}C$ signatures and are not representative of the plant in general. A corresponding pattern was

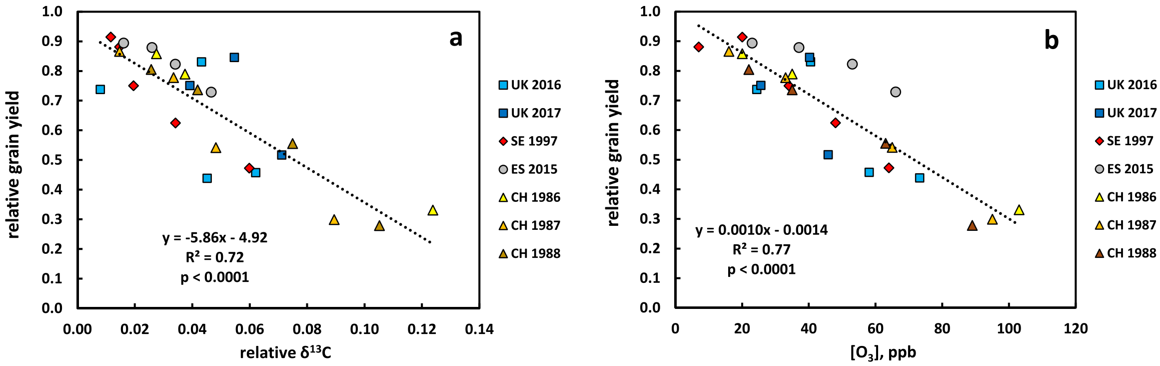

Fig. 4. Relative grain yield versus a) the relative effect of ozone on $\delta^{13}C$ in wheat grains, and b) the daytime average O_3 concentration $[O_3]$. The legend shows country and year of experiment: UK, United Kingdom; SE, Sweden; ES, Spain; CH, Switzerland.

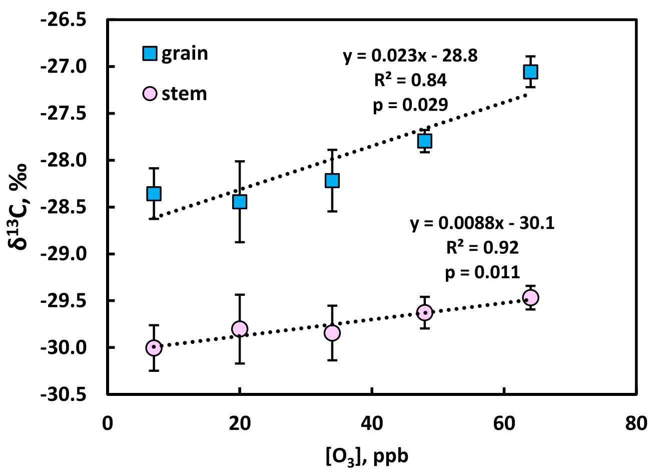

Fig. 5. $\delta^{13}C$ in grain and stems in relation to daytime average O_3 concentration $[O_3]$. Error bars show standard deviation.

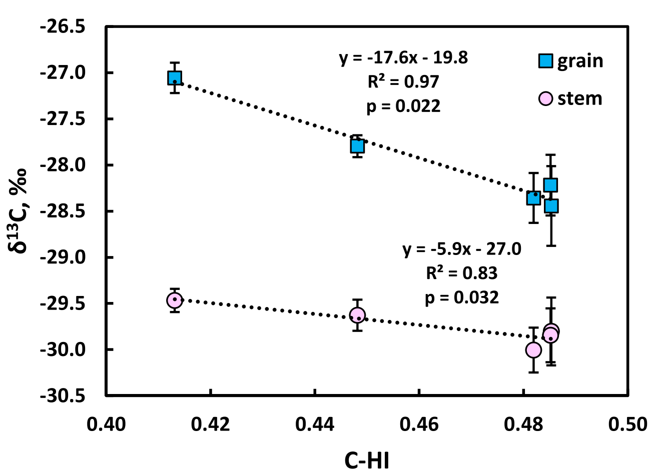

Fig. 6. $\delta^{13}C$ of grain and stems in relation to carbon harvest index (C-HI, the fraction of carbon in above-ground biomass found in grains at harvest) in five different O_3 treatments. Error bars show standard deviation.

observed by Saurer et al. (1991) when comparing $\delta^{13}C$ of grain and leaves: both were positively related to O_3 exposure, but grain more strongly so, with generally higher $\delta^{13}C$ than leaves. The difference in $\delta^{13}C$ between leaves and grain observed by Saurer et al. (1991) is of the same magnitude (approximately 1.5–3 ‰) as the difference between stems and grain found by us. It aligns with earlier observations that starch, the main component of wheat grain (Shevkandi et al., 2017), is enriched in ^{13}C vs. ^{12}C, as compared to the C assimilated in the photosynthesis (Tscherkez et al., 2011).

The stronger positive slope of the relationship of $\delta^{13}C$ with $[O_3]$ for grains compared to that for stems (Fig. 5) can possibly be explained by the fact that stems, like leaves, are formed earlier during the life cycle of wheat than grain. It has been observed that O_3 sensitivity in crops becomes larger as the plants age (Soja et al., 2000), which could contribute to the larger $\delta^{13}C$ response by wheat grain to O_3 exposure. An additional possible explanation is related to the redistribution of photosynthates stored in the stems during grain filling. Pre-anthesis reserves of carbon contribute significantly, up to ~30 % of total carbon mass in grains (Gebbing and Schnyder, 1999). The carbon in stems that is available for redistribution could be enriched in ^{13}C since ^{12}C more easily forms structural components of the plants that cannot readily be redistributed. A ^{13}C enrichment of phloem sugars has frequently been observed during transport in basipetal direction (Brüggermann et al., 2011).

The higher grain $\delta^{13}C$ at lower C-HI in the more strongly elevated O_3 treatments (Fig. 6) indicate that hampered redistribution of C from stems to grain can affect the $\delta^{13}C$ signature of wheat grain under O_3 exposure, in line with the fifth hypothesis. More data would be required to confirm this, but it points towards a conclusion that O_3 exposure effects on the carbon isotope signature of wheat grain has two main components, one depending on discrimination of ^{13}C that occurs during gas exchange and carboxylation by Rubisco, and another related to the differential redistribution of the two C isotopes within the plant from vegetative to reproductive plant parts. The redistribution of carbohydrates within the plant affecting the $\delta^{13}C$ of different plant compartments has been shown to be widespread (Badeck et al., 2005). Further studies are required to determine how important redistribution processes are for O_3 effects on $\delta^{13}C$ in wheat grain. A lower C-HI, and higher $\delta^{13}C$, at higher O_3 exposure can also be the result of premature senescence caused by O_3 affecting pre-anthesis C accumulation in stems and leaves less than post-anthesis C accumulation of grain (Grandjean and Fuhrer, 1989). Premature senescence from O_3 exposure is predominantly a post-anthesis phenomenon (Pleijel et al., 2014; Broberg et al., 2021).

Since $\delta^{13}C$ of different plant compartments of wheat differ significantly at harvest, it is relevant to ask which part of the plant is most relevant to study and if $\delta^{13}C$ should be used as an indicator of O_3 stress. The grain represents not only the agronomically significant plant fraction, but it is also the main sink, other above-ground plant fractions acting both as sources and sinks, and thus the end product of the plant life cycle, which integrates both redistribution of stored photosynthates from the earlier life stages with the direct distribution of photosynthates to the grain during grain filling (Sanchez-Bragado et al., 2014). Thus, $\delta^{13}C$ in grains integrates over the entire growth period (Condon et al., 2004), but with variable contributions of pre and post anthesis carbon assimilation (Gebbing and Schnyder, 1999). As found both by Saurer et al. (1991) and in the present study, the sensitivity in terms of the slope of the response relationship of δ^{13}C to O_3 exposure is larger for grain than for leaves and stems. This also favours grain as the plant component of largest interest when it comes to δ^{13}C as an indicator of O_3 effects. The substantial differences in δ^{13}C between vegetative and reproductive plant parts suggest that redistribution and the associated fractionation of C isotopes is of large importance for the isotopic signature. To obtain a measure of the overall δ^{13}C, representing the wheat plant and its gas exchange and CO_2 assimilation at large, requires sampling of both vegetative and non-vegetative parts of the plant and an estimation of a plant grand average δ^{13}C.

WUE can be evaluated using different approaches. While observations of δ^{13}C provide a time-integrated measure, gas exchange observations on the leaf or canopy level can inform about the situation during specific stages of the life cycle of the plants. Looking into the literature regarding different types of estimations of WUE for crops and other plants under O_3 exposure offers a somewhat complex picture. Although a consistent reduction in g_s under elevated O_3 has been observed, associated with a reduced evapotranspiration and a warmer canopy, such as in the SoyFACE experiments (Bernacchi et al., 2011), the extent to which this effect is larger or smaller than that on carbon assimilation or growth varies. Using different approaches (not based on δ^{13}C) Bou Jaoudé et al. (2008), VanLoocke et al. (2012) and Masutomi et al. (2019) suggested a decline in WUE under O_3 exposure in soybean and rice. Further, Li et al. (2012) found negative effects of O_3 on WUE in four tree species, using both leaf gas exchange observations and stable isotopes. However, a higher δ^{13}C in response to O_3 exposure, suggesting a higher WUE, has been observed in a range of studies (Greitner and Winner, 1988; Martin et al., 1988; Saurer et al., 1991; Jäggi et al., 2005).

In an OTC study of O_3 effects on wheat, (Grandjean Grimm and Fuhrer, 1992), WUE was observed based on gas exchange, both on flag leaf and (using the OTCs as cuvettes) canopy scale. There was a difference in estimated WUE at the two scales, with a consistent decline in flag leaf WUE in high O_3 concentration during the later part of the grain filling, while the canopy scale observations showed a different pattern and indicated an increase in WUE at higher O_3 exposure. The strong senescence promoting effect of O_3 on wheat leaves (Grandjean and Fuhrer, 1989) means that their assimilation and contribution to grain filling declines at a fast rate after anthesis. This means that observations of instantaneously lower WUE at high vs. low O_3 exposure during the later part of the grain filling period does not represent conditions when the O_3 exposed leaves are contributing strongly to grain filling. In addition, at lower O_3 exposure with slower leaf aging, the leaves will be in a more or less senescent condition, still contributing to grain filling, during a longer period characterized by low WUE, which could affect the grain δ^{13}C negatively. Finally, as explained above, following from the substantial differences in δ^{13}C between different parts of the plant, WUE inferred from δ^{13}C cannot be evaluated and compared with WUE observations based on gas exchange measurements without consideration of the effects of C partitioning on δ^{13}C.

The highly consistent and statistically significant positive relationship between wheat grain δ^{13}C and O_3 exposure, covering different countries, years and exposure systems, emphasizes the uniformity of the phenomenon of O_3 damage to this crop. The time-integrating character of the analysis based on δ^{13}C, and the strong link between δ^{13}C and yield effects under O_3 exposure, further strengthens this conclusion.

5. Conclusions

The main conclusions from this study were:

- δ^{13}C of wheat grain has a positive linear, statistically significant relationship with O_3 exposure.
- This can be explained by the negative O_3 impact on stomatal conductance dominates over the O_3 induced impairment of CO_2 fixation by Rubisco, resulting in an increased water use efficiency.
- The positive effect of O_3 on δ^{13}C has an additional component which depends on the fractionation of C isotopes associated with redistribution of C from stems to grain.
- Stems are more depleted in ^{13}C compared to grains, but both fractions show significant positive response to O_3 exposure, with a stronger response for grains.
- The effect by O_3 on wheat grain yield is significantly related to δ^{13}C.
- The relationships among δ^{13}C, grain yield effects and O_3 exposure over seven experiments performed in four countries is evidence that the mechanisms of O_3 effects on wheat are consistent.

CRediT authorship contribution statement

Pleijel Håkan: Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hayes Felicity:** Writing – review & editing, Visualization, Formal analysis. **Fernandez Ignacio Gonzalez:** Writing – review & editing, Funding acquisition. **Broberg Malin C.:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation. **Espino Melissa Chang:** Writing – review & editing, Visualization, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The supplementary file contains the data used that have not been published earlier.

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