Peer review history for:

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The original manuscript for review is appended below.

Reviewer 1: Round 1
Date completed: 26 August 2025
Conflicts of interest: None

Recommendation: Accept / Revisions required / Resubmit for review / Resubmit elsewhere / Decline / See comments
REVIEWER: Accept
Does the manuscript fall within the scope of SAJS? Yes/No
REVIEWER: Yes
Is the manuscript written in a style suitable for a non-specialist and is it of wider interest than to specialists alone? Yes/No
REVIEWER: Yes
Does the manuscript contain sufficient novel and significant information to justify publication? Yes/No
REVIEWER: Yes
Do the Title and Abstract clearly and accurately reflect the content of the manuscript? Yes/No
REVIEWER: Yes
Is the research problem significant and concisely stated? Yes/No
REVIEWER: Yes
Are the methods described comprehensively? Yes/No
REVIEWER: Yes
Is the statistical treatment appropriate? Yes/No/Not applicable/Not qualified to judge
REVIEWER: Yes
Are the interpretations and conclusions justified by the research results? Yes/Partly/No
REVIEWER: Yes
Please rate the manuscript on overall contribution to the field Excellent/Good/Average/Below average/Poor
REVIEWER: Good

Please rate the manuscript on language, grammar and tone
Excellent/Good/Average/Below average/Poor

REVIEWER: Excellent

Is the manuscript succinct and free of repetition and redundancies?
Yes/No

REVIEWER: Yes

The number of tables in the manuscript is
Too few/Adequate/Too many/Not applicable

REVIEWER: Too many

The number of figures in the manuscript is
Too few/Adequate/Too many/Not applicable

REVIEWER: Adequate

Are the results and discussion confined to relevance to the objective(s)?
Yes/No

REVIEWER: Yes

Is the supplementary material relevant and separated appropriately from the main document?
Yes/No/Not applicable

REVIEWER: Yes

Please rate the manuscript on overall quality
Excellent/Good/Average/Below average/Poor

REVIEWER: Good

Is appropriate and adequate reference made to other work in the field?
Yes/No

REVIEWER: Yes

Is it stated that ethical approval was granted by an institutional ethics committee for studies involving human subjects and non-human vertebrates?
Yes/No/Not applicable

REVIEWER: Not applicable

If accepted, would you recommend that the article receives priority publication?
Yes/No

REVIEWER: No

Are you willing to review a revision of this manuscript?
Yes/No

REVIEWER: No

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Yes/No

REVIEWER: Yes

Comments to the Author:

The manuscript employs sound and rigorous scientific methodologies for sampling and analysis. The statistical treatment of the data is appropriate and robust, strongly supporting the conclusions drawn. This work provides valuable original data on pollutant concentrations and their impact on sugarcane cultivars in South Africa. Although the relevance of the study can potentially be highlighted more in terms of real world scenarios, anticipated future events and use of product / biomass and global best practice or cultivar recommendations. The article highlights possible future investigations - the reason and significance should also be highlighted.

I thank the authors for their well-executed study and for contributing these important findings to the scientific community in such a professional manner. It was a pleasure to review this high-quality work.

Author response to Reviewer 1: Round 1

We would like to thank Reviewer #1 for the very positive review of this paper and accepting this work for publication in SAJS. We also acknowledge general suggestions made by Reviewer #1 above, which were carefully considered and addressed/implemented in the revised manuscript.

The relevance of this study is that the experimental treatments provide two points (elevated and ambient ozone) on the dose-response curve, which are lacking for tropical crop species in Africa. However, the usefulness of the results presented in this study can be further increased by more empirical results in future studies. Furthermore, real-world scenarios generally involve more than two stressors that modify the effects of each other and, in this case, would refer to realistic O_3 exposures, as well as climate change effects and future climate scenarios. The discussion on future investigations has been slightly expanded as follows:

Conclusions paragraph 1:

> "…elevated O_3 on sugarcane in different water regimes. In addition, future investigations should focus on obtaining more data points for producing O_3 dose-response functions and critical levels for sugarcane that can be used as a policy tool to improve air quality and vegetation health in the region. The relevance of this study can also be further enhanced by combining the interaction between current O_3 levels and other climate stressors, notably drought, in the derivation of sugarcane O_3 dose-response functions. This can guide the development of agricultural practice and cultivar selection to reduce the production risk posed by rising O_3 pollution and climate change."

Reviewer 2: Round 1

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Growth and physiological responses of two sugarcane cultivars exposed to elevated surface ozone

Abstract:

Surface ozone (O_3) pollution is known to have a detrimental impact on agriculture whilst rising carbon dioxide (CO_2) concentrations are sometimes found to offer plants protection against O_3 effects. Considering the important role of sugarcane (*Saccharum* spp. hybrids) as a major food crop in South Africa and its contribution to the national economy, the tolerance of this crop to O_3 damage must be established. A pilot study using open-top chambers was conducted whereby two local commercial sugarcane cultivars (NCo376 and N31) were fumigated during the summer growth season to explore the effects of elevated O_3 as well as the interacting effects of O_3 and CO_2 on various stress and crop quality indicators. Statistical significance of differences in treatment means was analysed by hierarchical linear modelling to account for variability between chamber and pots in explaining changes across individual plants. The results revealed a significant reduction in the number of dead leaves (senescing) for the N31 cultivar exposed to elevated O_3 compared to the other treatments. There was also a decrease in chlorophyll (chl) fluorescence used to assess photosynthetic performance, for the O_3-treated NCo376 plants that was statistically significant. This pilot study showed limited impacts of O_3 fumigation on growth and physiology, with preliminary indications that sugarcane, is less sensitive to O_3 than other crops. An increase in O_3 concentrations associated with future climate change is expected, which will have implications for cultivar selection as a possible adaptation strategy to reduce susceptibility of this crop to O_3.

Significance:

- This paper adds to the existing literature on sugarcane and ozone (O_3). We pilot a study for 2 cultivars of sugarcane and explore interacting effects of O_3 and carbon dioxide (CO_2) on various stress and crop quality indicators.
- We also employ a mixed effects model to account for variability between chamber and pots, a challenge when working with plants.
- This is the first time African sugarcane has been investigated and although the findings show limited statistical impact of O_3 and CO_2, future studies can vary the conditions of this experiment to produce more data points for a dose-response function.

Keywords: sugarcane, surface ozone, growth, physiology, South Africa

Figures, tables and supplementary material inserted at the end of the document.

Introduction

High levels of ozone (O_3) air pollution occur in southern Africa, particularly during late winter and spring from August to November where the maximum O_3 concentrations can range between 40 and 60 parts per billion (ppb) and was found to reach more than 90 ppb in middle spring[1,2,3]. The "O_3 peak season" overlaps with the summer growth season of various agricultural crops that extends from October to April of the following year[4]. Elevated concentrations of O_3 are recognised as posing a threat to the health of vegetation and agricultural crops[5,6,7]. O_3 stress effects include foliar injuries[8,9], impairment of physiological functions such as reduced photosynthesis[10] and reductions in both growth and yield of crops[11,12,13], although the extent of the effects varies between species and varieties/cultivars[6].

The main entry of ozone into the leaves is through the stomata while entry through the cuticle constitutes a negligible part of the O_3 uptake pathway by plants (Castagna and Ranieri, 2009). The impact of O_3 may also be influenced by other environmental factors such as soil water content, temperature, solar radiation intensity and vapour pressure deficit (VPD), which can cause reductions in stomatal conductance and, subsequently O_3 uptake by plants[14,15]. For instance, in low humidity (high VPD) environments, plants close their stomata to reduce plant transpiration and loss of water, which results in reduced O_3 uptake from the air, whilst plants in high humidity (low VPD) environments open their stomata and unintentionally take up O_3[16]. Another environmental factor is atmospheric CO_2 concentrations, which are steadily rising and are projected to reach between 700 and 800 ppm by the year 2100[17]. Both these gases have been shown to affect plant growth and productivity but in opposing ways. Elevated CO_2 on its own results in increased growth and yields in most crop species[18], whilst elevated O_3 alone has demonstrated reduced growth and yield of important crops[19,20]. However, the combination of elevated CO_2 and O_3 could offset the harmful effects of O_3 damage. Some studies have shown that elevated CO_2 affords plants more protection against O_3 because of lower stomatal conductance, lower O_3 uptake into leaves, and increased provision of substrates for detoxification and repair processes[14]. However, other studies found that the combined effects of elevated O_3 and CO_2 on plants provided no ameliorating effects[21].

Sugarcane (*Saccharum* spp. hybrids) is an important agricultural crop in the tropical and subtropical regions of the world, especially for producing sucrose (sugar) and ethanol. In addition to sugar being a raw material for foods, sugarcane mills have learned to harness the energy stored inside the plant to produce high-grade fuels for transport, heat or power applications[22]. Due

to its importance as a source of food and renewable energy, it is important to know how this crop will respond to O_3 pollution. Being a C_4 (i.e., produces a four-carbon molecule as the first product of photosynthesis) plant, it is assumed that sugarcane will exhibit tolerance to O_3 compared to C_3 (i.e., produces a three-carbon molecule as the first product of photosynthesis) crop species that are more O_3 sensitive [23]. In C_3 plants, studies have shown that photosynthesis is affected by a lower carboxylation efficiency induced by O_3. This means the enzyme Rubisco, which fixes atmospheric CO_2 as part of the Calvin cycle in photosynthesis, is affected. On the other hand, C_4 plants have evolved a mechanism to enhance the efficiency of Rubisco by 'concentrating' CO_2 around Rubisco so that these plants can function optimally at lower stomatal conductance than C_3 plants [24]. This also implies that C_4 photosynthesis is less responsive to higher CO_2 levels than C_3 photosynthesis but evidence supporting these assumptions is still being collected. In more recent years, studies have been published on the sensitivity or tolerance of sugarcane to O_3[22,25,26], however empirical evidence from Africa as a sugarcane growing region, is still unavailable[27].

This study aimed to assess the sensitivity of South African sugarcane cultivars to elevated atmospheric O_3 concentrations, as well as the moderating effects of elevated atmospheric CO_2 concentrations on plant response to high O_3. By investigating the direct effects of O_3 and interactive effects between O_3 and CO_2 on plant growth and photosynthesis in sugarcane plants, we can increase our understanding for crop modelling purposes and compare the findings to existing literature on sugarcane and O_3. These findings could inform the importance of breeding selection of commercial cultivars that are tolerant to O_3 and assist sugarcane producers adapt to climate change threats and O_3 air pollution.

Materials and methods

Site characteristics and plant material

An open-top chamber (OTC) research facility situated at North-West University, Potchefstroom, South Africa (26°40'50"S, 27°05'48"E, altitude 1348 m a.s.l.) was used to conduct the O_3 fumigation trial with two sugarcane cultivars. These OTCs are essentially transparent plastic cylinders having a volume of 5 m^3, ventilated by a fan that can enclose plants and allow for concentrations of air pollutants to be controlled whilst maintaining natural climatic conditions close to field conditions[28]. Each of the twelve chambers accommodated eight potted plants therefore four plants of each cultivar were placed within each chamber, giving a total of 96 plants used in the study.

Two commercial sugarcane cultivars in South Africa, NCo376 and N31, which were obtained from the South African Sugarcane Research Institute (SASRI), were used in the trial. Cultivar N31 has the ability to grow rapidly and produce an extremely high yield after an 18-24 month cycle but has a fairly low sucrose content[29]. Cultivar NCo376 produces good yields (although not as high as N31) and has a higher sucrose content than N31, but its growth during severe water stress is poorer compared to N31[30]. Seedlings of N31 and NCo376 were planted into pots and placed inside the OTCs. Irrigation was carried out every morning and evening to grow the plants under well-watered conditions. Fertilizer solution containing macro- and micro-nutrients ideal for sugarcane growth was applied to all pots once every 3 weeks and dissolved by irrigation[31]. Fumigation of the plants was initiated 5 weeks after planting. The plants were exposed to elevated O_3 and elevated CO_2 concentrations for seven months.

Treatments

Four chambers served as the control chambers (no fumigants) and received only charcoal-filtered air (< 4 ppb O_3 and 400 ppm CO_2) by passing ambient air through a Purafil filter, which effectively removes the O_3 and other contaminants present in the air stream. The eight remaining OTCs were designated as the treatment chambers. Four were used for CO_2 fumigation involving 750 ppm alone ('elevated CO_2'), two for fumigation involving 80 ppb O_3 alone ('elevated O_3') and two for fumigation involving a mixture of 80 ppb O_3 and 750 ppm CO_2 ('elevated CO_2 plus O_3'). Fumigation was maintained in the designated chambers during daylight hours (08h00 – 17h00) for the duration of the growth period of seven months. The choice of 80 ppb O_3 exposure was guided by other exposure-response data available for other South African crops at this level[32,33]. The selection of the 750 ppm CO_2 level was based on the two upper representative concentration pathways (RCP 6.0 and 8.5) future scenarios used in the Intergovernmental Panel on Climate Change (IPCC) assessment report[17].

Growth measurements

The growth measurements, measured twice a month, included the number of green and dead leaves, top visible dewlap leaf length and width, stalk height and number of tillers. The top visible dewlap (TVD) leaf blade is the plant tissue often used in sugarcane studies and refers to the uppermost fully expanded leaf with a clearly visible dewlap or collar (http://edis.ifas.ufl.edu/sc076). The number of green and dead leaves was counted, starting from

the base of the stalk to the TVD leaf. The stalk height was measured from the soil surface to the TVD leaf. The number of tillers present for each plant was counted, excluding the main tiller (primary shoot or stalk).

Chlorophyll a fluorescence measurements

Chlorophyll (chl) *a* fluorescence was measured using a Handy PEA (Plant Efficiency Analyzer, Hansatech Instrument Ltd, UK) portable fluorimeter and conducted at night to ensure that the sample leaves had already been dark-adapted for at least one hour. The first step in the measurement process using the fluorimeter was to cover the measurement area of the TVD leaf blade with a small, lightweight leaf clip. A high intensity (3 500 $\mu Mol.m^{-2}.s^{-1}$), short flash (1s) of light was applied to the measurement area, which transiently closes all PSII reaction centres, reducing the overall photochemical efficiency so that fluorescence levels rise for one to two seconds[34]. Following on from this, however, the fluorescence level typically starts to fall again, over a time-scale of a few minutes[34]. Measurements of chl *a* fluorescence were taken monthly, starting at 8 weeks after planting with three replicates per plant.

The most commonly used chlorophyll fluorescence measuring parameter is F_v/F_m[35], with the dark-adapted value of F_v/F_m signifying the maximum potential quantum efficiency of PSII in plants if all capable reaction centres were open[36]. F_v/F_m is a normalized ratio created by dividing variable fluorescence by maximum fluorescence:

$$F_v/F_m = {(F_m - F_o)}/{F_m} = {(F_{300} - F_{0.01})}/{F_{300}}$$

where

F_v/F_m is the maximum quantum yield of PSII photochemistry measured in the dark-adapted state;

F_o is the initial or minimum fluorescence intensity at 0.01 ms when light is first applied;

F_m is the maximum or peak fluorescence intensity at 300 ms; and

F_v is the variable fluorescence, the difference between maximum fluorescence and minimum fluorescence

F_v/F_m measures plant photosynthetic activity, with reduced values possibly indicating stress, photoinhibition and photosynthesis downregulation[36]. An F_v/F_m value in the range of 0.79 to 0.83 is the approximate optimal value for many plant species, with lowered values indicating plant stress, particularly the occurrence of photoinhibition[36].

Stomatal conductance measurements

To assess O_3 uptake, stomatal conductance was measured once a month using a hand-held porometer (Model AP4, Delta-T Devices, Cambridge, UK). Three measurements were taken per plant along the length of the TVD leaf. The measurements were taken between 12:00 and 2:00 pm.

Chlorophyll content measurements

Chlorophyll content (measured in units of mg/m^2) was measured once a month, starting at about 17 weeks after planting. Measurements were made using a hand-held meter (Model CCM 300, Opti-Sciences, USA) by placing the leaf clip on the TVD leaf. Three measurements were taken per potted plant.

Statistical analyses

All measured data were downloaded using the data logger software of each instrument and saved as a text/csv file on a laptop computer. The data were sorted and processed in Microsoft Excel. All measurement results were subjected to statistical analysis methods to determine the statistical significance of the differences between treatment means. Hierarchical linear mixed models (HLMs) are a class of statistical models that allow for the analysis of nested data. These models are useful when dealing with data where observations are not independent of each other. These models account for variability at different levels, from individual measurements to overall groupings[37,38]. In addition to these advantages, HLMs were selected since the estimation of individual change over time can be modelled and fewer assumptions need to hold for the procedure to be valid[39].

In this paper, hierarchical linear modelling was conducted to analyse several variables in relation to the fixed effects of treatment and time, as well as their interaction. The growth and physiological variables were separately used as dependent variables in the HLMs. Two random intercepts (random effects) were included in each model, i.e., one at the chamber level and another at the nested pot level. The average of each treatment group for each dependent variable is provided for selected time points. The *p*-values for the predictors ('treatment' and 'time') are denoted as non-significant (ns) when $p > 0.1$, with increasing numbers of stars indicating significance levels at $p \leq 0.1$, $p \leq 0.05$, and $p \leq 0.01$, respectively. These *p*-values test whether the predictors, 'treatment', 'time', or their interaction (treatment x time) has a statistically significant effect on the

dependent variable. For the random effects, the estimate of the proportion of variance in the outcome variable accounted for at each level of the model is provided. This gives a measure of the importance of each random effect ('chamber' and 'pot') by dividing the effect's variance estimate by the total variance estimate. A larger percentage indicates a more important random effect.

Results

Meteorological conditions

Near-site meteorological data for the period of the trial were obtained from the Welgegund atmospheric research station (http://www.welgegund.org/), which is located at 26.57° S and 26.94° E with an elevation of 1480 m a.s.l. The highest temperatures and relative humidities occurred during summer (December-January-February), with global radiation being the highest during spring (September-October-November) and summer (Table 1). Rainfall occurred predominantly from November to March. A comparison of ambient temperature and relative humidity levels with measurements taken inside the OTCs indicated that the temperature and relative humidity were higher (by 2-5°C and 10-20%, respectively) inside the chambers compared to the outside.

Growth parameters

The data for all plant growth parameters measured can be found in the supplementary material (Supplementary figure 3) and summarised in Table 2. Overall, green leaves, dead leaves, TVD leaf length and stalk height were slightly higher in the elevated CO_2 (i.e. elevated CO_2 and elevated O_3 plus CO_2) treatments than in the elevated O_3 treatment (Supplementary figure 3 and Table 2). However Table 2 shows that whilst the predictor variable 'time' was statistically significant for all models, 'treatment' was only significant in predicting the number of dead leaves on the N31 cultivar. The results for dead leaves presented in Fig. 1 show that the number of dead leaves was significantly higher for CO_2-treated plants than for the control plants, which can be interpreted as natural ageing (senescence) of leaves. On the other hand, the O_3-treated plants have fewer dead leaves than the other treatments. A lower number of dead leaves on the treated plants compared to the control implies that the treatments do not result in earlier ageing of leaves (accelerated leaf senescence). The treatment x time interaction was significant for green leaves, dead leaves, and leaf length for both varieties. It was also observed that the 'chamber' effect accounts for 30% or more of the variation in predicting leaf length, leaf width, and stalk height

variables for the N31 variety and 42% of the variation in stalk height for the NCo376 variety. The 'pot level' accounts for substantial variation across all growth variables, with 'tillers' accounting for 59% (NCo376) and 56% (N31), respectively. Interestingly, for NCo376 the number of tillers was the highest in the elevated O_3 treatment than in the other treatments, whereas the reverse applied for N31 over time (Supplementary figure 3), which could relate to the sensitivity of the two sugarcane cultivars to O_3. However, the differences were not statistically significant.

Physiological responses

Relative maximal variable fluorescence (F_v/F_m)

Mixed model analysis to determine statistical significance showed that the chambers did not contribute any variation to the relative maximal variable fluorescence (F_v/F_m) for NCo376 and N31. Consequently, disregarding the influence of the chambers reduces the statistical analysis to a two-way repeated measures ANOVA, which was performed to assess if there is a difference in the treatment effect (Table 3). No significant differences were found between the treatments for N31 but the differences between the treatments for NCo376 were statistically significant. A decrease in F_v/F_m with elevated O_3 over time and increase in F_v/F_m values with elevated CO_2 over time was observed for NCo376. There was a statistically significant effect of the factor 'time' for both NCo376 and N31 and a significant (but lower) treatment x time interaction, suggesting that 'time' was a better predictor of changes in photosynthetic activity than 'treatment' in this pilot trial (Table 3).

Stomatal conductance

The evolution of stomatal conductance of sugarcane through the growing season shows a decreasing trend (Supplementary figure 5) which suggests the stomata become more closed over the life cycle of the plants. In addition, NCo376 was found to have a higher stomatal conductance than N31 at each stage of development. For N31, O_3 exposure decreased stomatal conductance to levels lower than the control and CO_2 treatment, particularly in the advanced phenological stages (Supplementary figure 5). However the differences between the treatments were not statistically significant (Table 4). Neither the 'chamber' nor 'pot' levels accounted for any noticeable variation in predicting the stomatal conductance.

Chlorophyll content

It can be observed from the controls that, in general, NCo376 has a higher chlorophyll content value than N31 (Supplementary figure 6). Chlorophyll content tended to drop significantly in NCo376 close to harvest, although the treatment difference was not statistically significant (Table 5). 'Chamber' effects (e.g. reduced solar radiation, higher temperature or more watering) accounted for 12% of the variation whereas 'pot in chamber' contributed 15% of the variation in predicting the chlorophyll content variable, for NCo376 and N31 respectively.

Discussion

Hierarchical linear mixed models (HLM) were used in this study to statistically evaluate the influence of various parameters, i.e. pots, chambers, treatments and time effects, and to determine whether there is a statistically significant difference between 'treatment', 'time', as well as the interaction of 'treatment' over 'time'. The analysis indicates that the factor that most influences the growth and physiological response of each variety was 'time', while 'treatment' is only significant in predicting the dead leaves in the N31 cultivar. The 'chamber' effect influences the variation in leaf length, leaf width and stalk height in the N31 variety, while also affecting stalk height in the NCo376 variety. The 'pot' level also explains some amount of variation in the growth variables, particularly in 'tillers' for both cultivars. However, neither the 'chamber' nor 'pot' level contribute significantly to variations in stomatal conductance and chlorophyll content. As given by the residuals in the statistical analysis (statistical variation that could not be accounted for), over 80% of the variation was unexplained in the stomatal conductance and chlorophyl content data. For chlorophyll a fluorescence, the 'treatment' effect was significant for NCo376, with increased O_3 exposure decreasing the F_v/F_m ratio whilst elevated CO_2 increased F_v/F_m. A reduction in the F_v/F_m values indicate damage to the PSII II photosynthetic machinery.

The mixed model in terms of stalk height and tillers fits very well (about 70-80% of the variation in stalk height and tillers are explained by 'chamber' and 'pot' within the chambers). These variables could potentially be used as predictors in mixed models and are important factors related to sugarcane yield. For the sugarcane industry, a 15% reduction in stalk height could translate to a significant amount in terms of yield and profitability loss of the crop. In terms of tillers, multiple studies have shown that elevated CO_2 levels stimulates tillering in crops e.g. [40,41,42], which in turn provides the crop with a suitable number of stalks and forms the foundation of a good crop[43]. The specific time at which the tillers are produced is also of importance as observed in rice plants with more tillers at the early stage usually indicating they are on a healthy developmental path toward higher yield[40].

Although the HLM approach has benefits, there are also some disadvantages especially when dealing with small sample sizes. With a limited number of chambers, a choice must be made either to reproduce conditions in multiple chambers or produce more data points by varying conditions in each chamber. In this case, reproducing the conditions in multiple chambers resulted in too few data points to produce a dose-response function. If non-linear responses are expected gradient experimental designs are especially important to consider[44]. Another limitation to this study is that biomass yield and cane quality measurements of the O_3-treated sugarcane cultivars were not undertaken at the end-of-season although various articles highlight crop yield and quality affected by O_3 exposure. Yield loss due to surface O_3 is a major concern for developing countries like South Africa and holds direct relevance for future local sugarcane production.

We piloted an in-field measurement approach on local sugarcane cultivars and based on what we learned, can make recommendations to optimise the research protocol in a larger study as follows: (i) reduce the number of pots in each chamber to ensure they all receive solar radiation, (ii) increase the number of chambers per treatment to make the results more robust, (iii) improve the irrigation system to remove water stress as a factor and (iv) measure additional yield and quality (e.g., sugar content) variables to assess productivity.

Conclusions

This pilot trial set out to investigate if O_3 air pollution has adverse effects on selected growth and physiological parameters in sugarcane. At the same time, the direct benefits of elevated CO_2 concentration was investigated to determine if elevated CO_2 levels could offset the deleterious effects of O_3 damage. Preliminary indications are that the two cultivars of sugarcane are less sensitive to O_3 than other crops such as rice, wheat and potato. When the data was analysed statistically, O_3 had limited effects on growth and physiology parameters in sugarcane, although a significant decrease in dead leaves and a small decrease in chlorophyll fluorescence were detected in both cultivars. Elevated CO_2 concentrations did not significantly ameliorate the negative effects of O_3 for sugarcane. Notwithstanding, with the threat of other aspects of climate change such as multi-year droughts in semi-arid regions like South Africa, future studies should evaluate the effect of elevated O_3 on sugarcane in different water regimes.

A novel contribution in this paper is the motivation for mixed models to be used in dose-response chamber studies as a reliable statistical tool. The traditional repeated-measures ANOVA does not

take into account the effect of the chambers so this piece of information in the data is lost. HLM is recommended as a more suitable method to analyse repeated measures data as it accounts for variability between chamber and pots, a challenge when working with plants. This study used HLM model analysis to account for variance introduced by these unobserved random effects, in addition to the fixed effects of treatment, time and interaction between treatment and time.

Declarations

This work appears in a Thesis.

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TABLE 1: Monthly averages of the hourly ambient temperature, global radiation, relative humidity and monthly accumulated precipitation measured during the period of the OTC trial. The monthly averages are shown with the minimum and maximum values given in parenthesis.

Month	Temperature (°C)	Global Radiation (W/m^2)	Relative Humidity (%)	Accumulated Precipitation (mm)
Oct-14	20 (4−32)	291 (0−1086)	40 (6−99)	6
Nov-14	19 (7−30)	252 (0−1165)	60 (13−100)	25
Dec-14	21 (13−32)	270 (0−1207)	62 (11−100)	16
Jan-15	21 (12−33)	298 (0−1219)	63 (11−100)	55
Feb-15	22 (11−34)	303 (0−1143)	52 (9−99)	10
Mar-15	19 (10−30)	231 (0−1061)	64 (10−100)	21
Apr-15	17 (4−26)	212 (0−924)	58 (9−100)	8
May-15	17 (5−28)	192 (0−831)	35 (7−99)	0

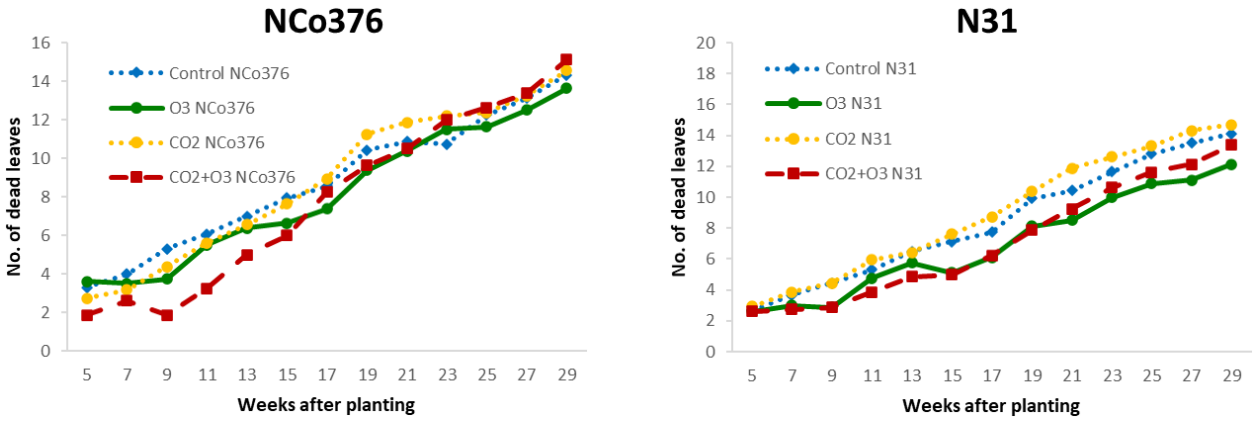


Figure 1: Evolution of the number of dead leaves through the growing season in two cultivars of sugarcane (NCo376 and N31) under the different treatments: exposure to charcoal-filtered air (control), elevated O_3 (80 ppb), elevated CO_2 (750 ppm) and elevated O_3 plus CO_2 (80 ppb and 750 ppm).

TABLE 2: Hierarchical linear modelling (HLM) on plant growth variables under the different treatments at selected weeks 5, 17 and 29 to determine if differences are statistically significant: ns indicates p>0.1 whilst *, ** and *** indicate significance at $p \leq 0.1$, $p \leq 0.05$, and $p \leq 0.01$, respectively.

NCo376

	Green leaves (number per stalk)			Dead leaves (number per stalk)			Leaf length (cm)			Leave width (cm)			Stalk height (cm)			Tillers (number per pot)		
Week	W5	W17	W29	W5	W17	W29	W5	W17	W29	W5	W17	W29	W5	W17	W29	W5	W17	W29
Control	4	5	5	3	9	14	67.4	100.9	95.5	1.8	2.2	2.5	18.7	50.0	78.2	5	13	11
O_3	3	4	4	4	7	13	61.3	99.6	96.7	1.5	2.0	2.4	18.9	50.0	74.6	5	13	11
CO_2	5	5	4	3	9	15	68.9	102.4	102.2	1.7	2.1	2.3	17.7	52.6	85.9	4	13	11
O_3+CO_2	4	4	4	2	8	15	52.1	111.1	107.8	1.4	2.0	2.2	16.7	47.5	75.6	3	12	9
Fixed effects																		
Treatment		ns			ns			ns			ns			ns			ns	
Time		***			***			***			***			***			***	
Treatment*Time		***			***			***			ns			ns			ns	
Random effects																		
Chamber		2%			10%			27%			14%			42%			8%	
Pot*Chamber		7%			33%			23%			24%			31%			59%	
Residual		91%			57%			49%			62%			25%			33%	

N31

	Green leaves (number per stalk)			Dead leaves (number per stalk)			Leaf length (cm)			Leave width (cm)			Stalk height (cm)			Tillers (number per pot)		
Week	W5	W17	W29	W5	W17	W29	W5	W17	W29	W5	W17	W29	W5	W17	W29	W5	W17	W29
Control	4	4	4	3	8	14	92.8	112.1	112.4	2.3	2.4	2.5	23.9	62.3	91.3	5	12	10
O_3	3	5	4	3	6	12	66.8	112.8	111.8	1.6	2.4	2.6	19.9	53.6	80.1	4	10	9
CO_2	4	4	4	3	9	15	78.6	112.8	114.4	2.0	2.5	2.8	20.8	57.0	85.2	4	12	10
O_3+CO_2	4	5	5	3	6	13	67.3	110.4	111.6	1.8	2.3	2.8	21.5	53.8	75.6	4	13	9
Fixed effects																		

Treatment	ns	**	ns	ns	ns	ns
Time	***	***	***	***	***	***
Treatment*Time	***	***	***	***	ns	ns
Random effects						
Chamber	5%	11%	40%	30%	67%	12%
Pot*Chamber	11%	26%	18%	15%	13%	56%
Residual	84%	62%	42%	55%	20%	33%

TABLE 3: Hierarchical linear modelling (HLM) on plant physiological parameters under the different treatments at selected weeks to determine if differences are statistically significant: ns indicates p>0.1 whilst *, ** and *** indicate significance at $p \leq 0.1$, $p \leq 0.05$, and $p \leq 0.01$, respectively.

NCo376

	Stomatal conductance (mmol m^{-2} s^{-1})			Chlorophyll content (mg/m^2)			Chlorophyll fluorescence (Fv/Fm)		
Week	W13	W21	W32	W17	W21	W30	W8	W16	W28
Control	489.31	190.66	136.02	19.03	14.13	13.33	0.785	0.780	0.784
O_3	371.75	348.00	175.95	22.75	12.53	10.21	0.797	0.793	0.778
CO_2	577.88	280.16	209.10	18.86	14.48	13.14	0.787	0.788	0.798
O_3+CO_2	687.63	142.29	107.80	16.00	13.25	13.25	0.800	0.774	0.783
Fixed effects							ANOVA treatment effect		
Treatment		ns			ns			**	
Time		***			***			***	
Treatment*Time		ns			ns			**	
Random effects									
Chamber		8%			12%				
Pot*Chamber		3%			0				
Residual		89%			87%				

N31

	Stomatal conductance	Chlorophyll content (mg/m^2)	Chlorophyll fluorescence (Fv/Fm)

Week	W13	W21	W32	W17	W21	W30	W8	W16	W28
Control	247.78	138.43	117.85	12.42	8.89	8.28	0.772	0.770	0.752
O_3	247.06	156.58	102.88	14.92	8.53	9.45	0.775	0.791	0.755
CO_2	163.50	98.87	121.23	11.12	10.57	8.08	0.774	0.770	0.773
O_3+CO_2	186.63	110.88	118.78	12.35	11.11	9.57	0.779	0.772	0.751
Fixed effects							ANOVA treatment effect		
Treatment		ns			ns			ns	
Time		***			***			***	
Treatment*Time		ns			ns			***	
Random effects									
Chamber		6%			5%				
Pot*Chamber		3%			15%				
Residual		91%			81%				

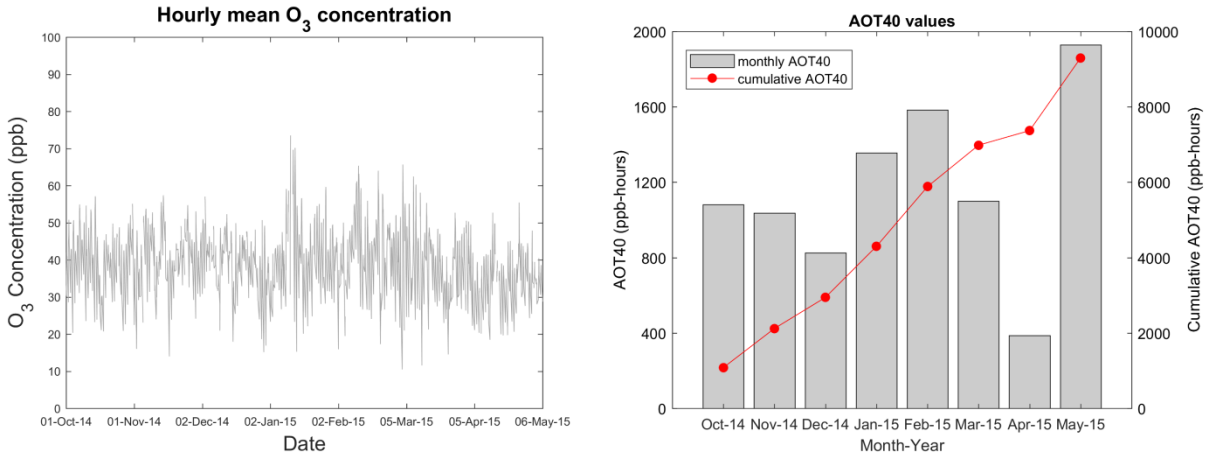
490

Supplementary material

Calculated AOT40 values for conditions in the field

To estimate the potential risk of damage by O_3 on vegetation, the most commonly known indicators applied are the seasonal cumulative exposure over a threshold such as 60 ppb or 40 ppb (SUM06 and AOT40, respectively)[1,2,3]. In order to obtain an approximate AOT40 for the ambient surroundings of the OTC facility, monthly AOT values were calculated using continuous *in situ* O_3 data from the Welgegund atmospheric research station. This site was chosen since it is approximately 30 km away from Potchefstroom and provides continuous monitoring data for a wide suite of meteorological parameters. The station is a regionally representative background site for O_3, i.e., with few local anthropogenic sources of air pollution. Monthly averages for temperature and cumulative totals for precipitation were calculated.

O_3 concentrations measured at the Welgegund station, presented in Supplementary figure 1(a), are taken at a sampling height of about 4-5 m. The AOT40 was calculated during the daylight period (08:00−17:00) for the sugarcane growing season, i.e. October to May , which differs from the AOT40 calculation method in Europe that uses only three months (May to July) and the daylight period might also be shorter[14]. Although AOT40 was calculated differently in this study, Supplementary figure 1 (b) shows that the European AOT40 critical level of 3000 ppb h for crops is reached in the first three months at the South African background site. This indicates that if the AOT40 set by the United Nations Economic Commission for Europe[3] for crop damage was applied in southern Africa, there would likely be considerable exceedances of the 3000 ppb h critical level, particularly as inter-annual variability in weather conditions also influence ambient O_3 levels. Higher concentration thresholds have been suggested for defining critical levels in southern Africa. However, European OTC experiments demonstrated that the 40 ppb threshold provided a much better linear fit to the exposure-response data than the use of higher thresholds[15]. It remains to be investigated through experiments if linear exposure-response relationships can be established at higher critical levels for locally adapted crops. As a comparison, the AOT40 inside the chamber (considering the start and end dates of fumigation) was calculated to be ~60 000 ppb h whilst the AOT40 in ambient air was calculated to be ~6 000 ppb h for the same period.



(a) (b)

Supplementary figure 1: (a) Average hourly O_3 concentration and (b) AOT40 (including cumulative values) at the regional background site Welgegund during the period 1 October 2014 to 31 May 2015. Only measurements for daylight hours (8:00 to 17:00) were used.

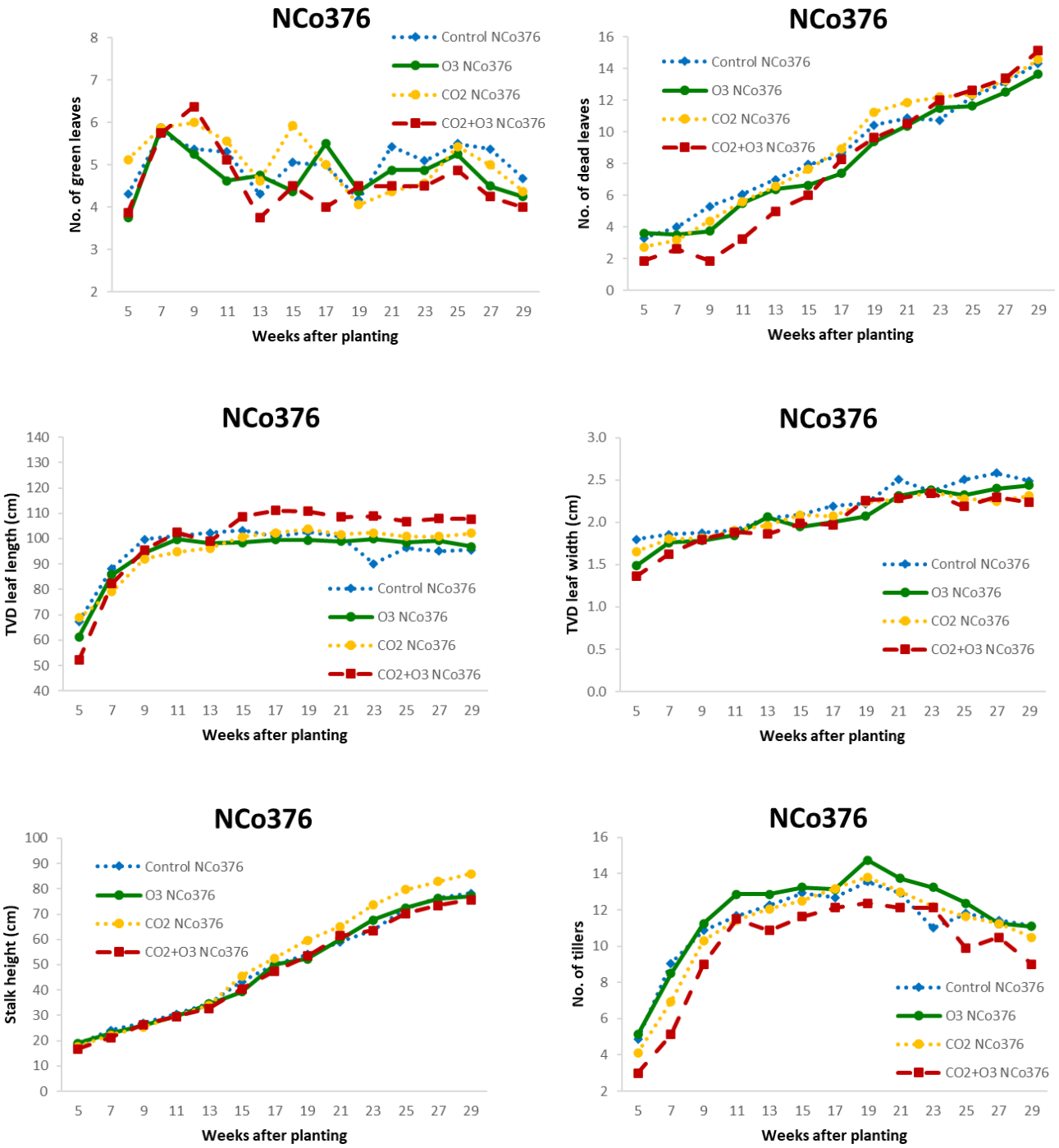
Visible foliar ozone-induced injuries

Early in the growth season, visual inspection on plants showed yellow patches and reddish brown lesions on the leave surface, which is typical of ozone-induced injuries [55]. This could be signs of chlorosis, necrosis and accelerated senescence [9]. Although visible symptoms of injury were less noticeable later in the growth season, a complete O_3 injury assessment of the plants was not done.

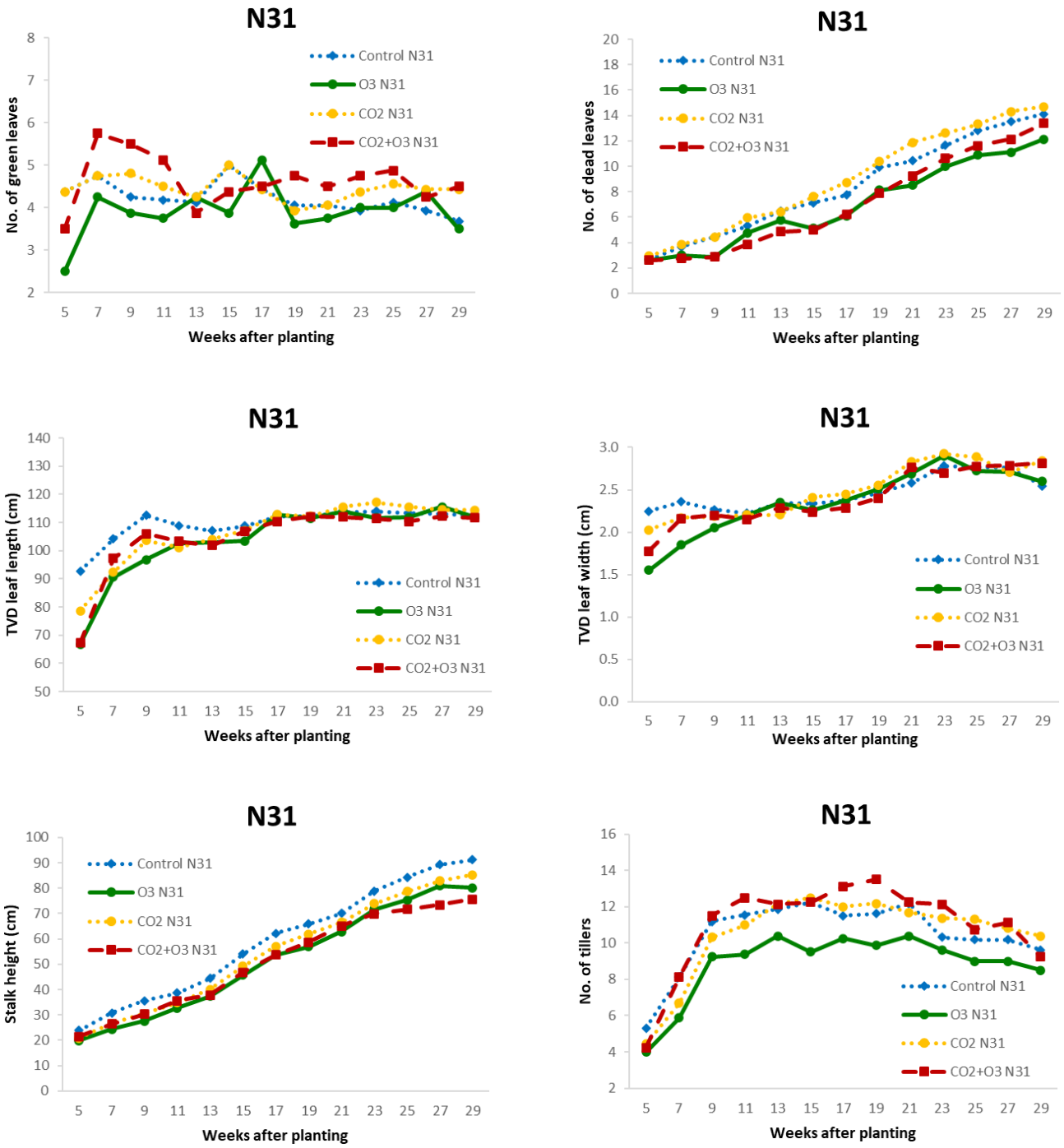
Supplementary figure 2: Ozone injury symptoms (yellowing and reddish spotting)

Growth parameters

Sugarcane yield is influenced by various plant growth responses as well as physiological traits. Indicators of plant growth over time and under the different treatments are presented in Supplementary figure 3 (a) and (b) for each variety. The parameters measured included the numbers of green and dead leaves, TVD leaf length, TVD leaf width, stalk height and number of tillers.



(a) NCo376 cultivar



(b) N31 cultivar

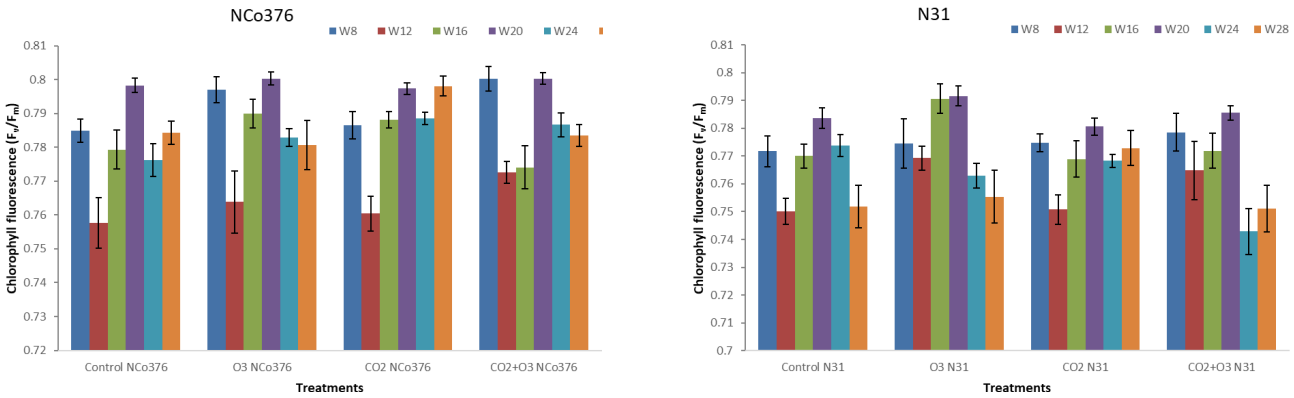
Supplementary figure 3: Growth variables in two cultivars of sugarcane, (a) NCo376 and (b) N31, under the different treatments in open-top chambers: exposure to charcoal-filtered air (control), elevated O_3 (80 ppb), elevated CO_2 (750 ppm) and elevated O_3 plus CO_2 (80 ppb and 750 ppm).

Chlorophyll fluorescence

Chlorophyll (Chl) *a* fluorescence is used to monitor photosystem II (PSII) activity and the processes that affect it [45]. The sensitivity of PSII activity to various biotic and abiotic factors can be beneficial in studying the effects of multiple stressors on the physiological state of plants [46]. The fast phase

chlorophyll kinetics can provide insight into the impact of elevated O_3 and/or CO_2 exposure on photosynthetic performance and how plants respond to environmental change [47].

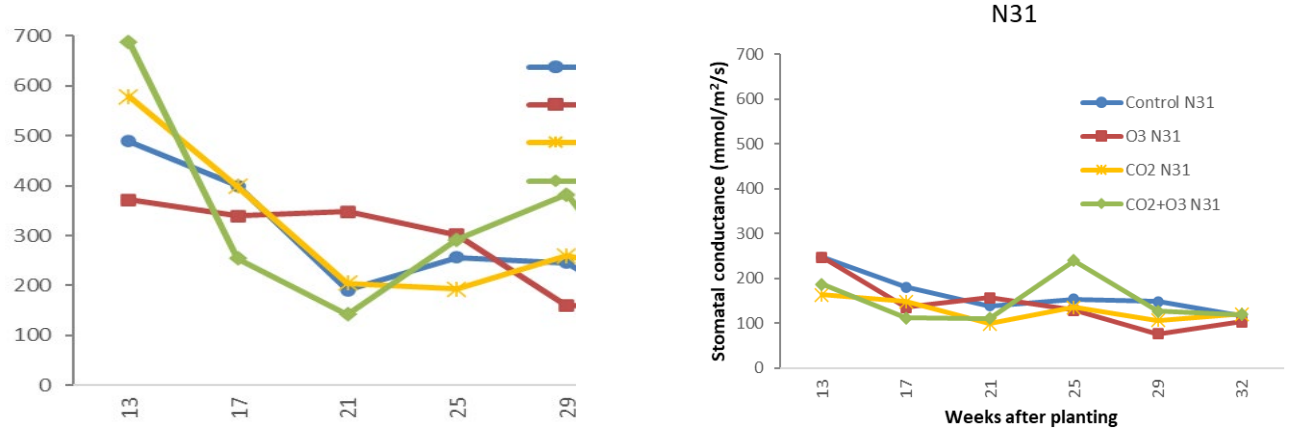
The F_v/F_m parameter is an important indicator describing the efficiency of photosystem II (PSII). The F_v/F_m values for the four treatments were calculated and are plotted in Supplementary figure 4. The results demonstrate an overall decrease in F_v/F_m for elevated O_3-treated plants over time and generally higher F_v/F_m values for elevated CO_2-treated plants compared to elevated O_3-treated plants.



Supplementary figure 4: Chlorophyll fluorescence (expressed as Fv/Fm) during the growing season for NCo376 and N31 leaves after exposure to charcoal-filtered air (control), elevated CO_2 (750 ppm), elevated O_3 (80 ppb) and elevated O_3 plus CO_2 (80 ppb and 750 ppm) in open-top chambers. Each bar represents the mean values±SE.

Stomatal conductance

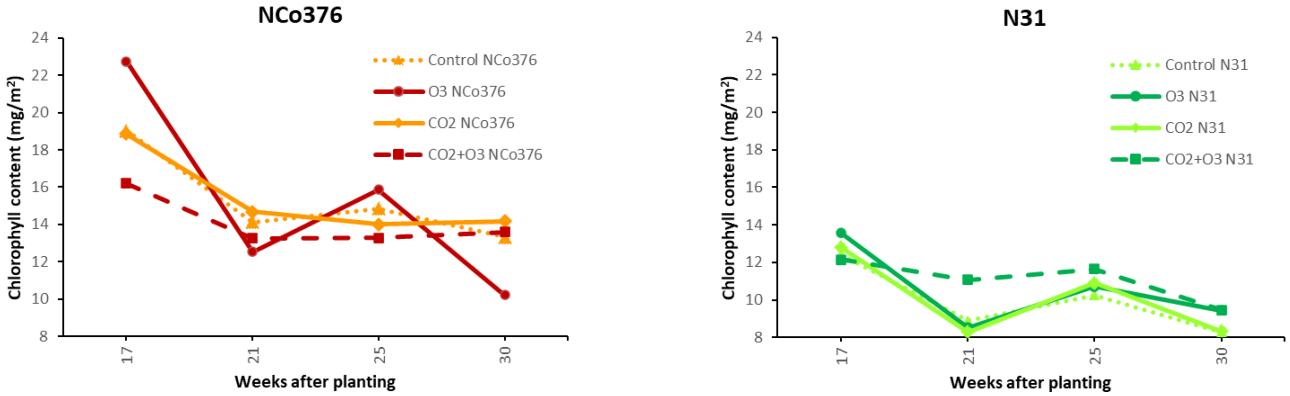
Stomatal closure by plants in response to O_3 stress can be an avoidance mechanism to prevent the uptake of O_3 by the leaves. However, by closing their stomata (pores for exchanging CO_2 and water vapour) to limit uptake of O_3 will also limit CO_2 uptake, which leads to decreased photosynthetic activity [50]. The evolution of stomatal conductance through the growing season is presented in Supplementary figure 5.



Supplementary figure 5: Mean values of stomatal conductance of NCo376 and N31 leaves after exposure to charcoal-filtered air (control), elevated O_3 (80 ppb), elevated CO_2 (750 ppm) and elevated O_3 + CO_2 (80 ppb and 750 ppm) in open-top chambers.

Chlorophyll content

Leaf chlorophyll content presented in Supplementary figure 6 provides valuable information about the physiological status of plants and is related to photosynthesis. The primary function of chlorophyll molecules is radiation absorption, which includes energy essential for photosynthesis. Reduced chlorophyll content has been linked to a decline in photosynthesis and growth in forest trees[39].



Supplementary figure 6: Mean values of chlorophyll content (in mg/m^2) of NCo376 and N31 leaves after exposure to charcoal-filtered air (control), elevated O_3 (80 ppb), elevated CO_2 (750 ppm) and elevated O_3 + CO_2 (80 ppb and 750 ppm) in open-top chambers.

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