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

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Surface ozone (O_3) pollution is known to have a detrimental effect 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 with the other treatments. There was also a statistically significant decrease in chlorophyll fluorescence (used to assess photosynthetic performance) in the O_3-treated NCo376 plants. This pilot study shows limited effects 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 article adds to the existing literature on sugarcane and ozone (O_3). We present a pilot study for two cultivars of sugarcane and explore interacting effects of O_3 and carbon dioxide (CO_2) on various stress and crop quality indicators.
- We employed 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 effect 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.

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 have been found to reach more than 90 ppb in mid-spring.[1-3] 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.[5-7] 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[11-13], 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 whilst entry through the cuticle constitutes a negligible part of the O_3 uptake pathway by plants.[14] The effect of O_3 may also be influenced by other environmental factors such as soil water content, temperature, solar radiation intensity and vapour pressure deficit, which can cause reductions in stomatal conductance and, subsequently, O_3 uptake by plants.[15,16] For instance, in low-humidity (high vapour pressure deficit) environments, plants close their stomata to reduce plant transpiration and loss of water, which results in reduced O_3 uptake from the air, whereas plants in high-humidity (low vapour pressure deficit) environments open their stomata and unintentionally take up O_3.[17] Another environmental factor is atmospheric CO_2 concentrations, which are steadily rising and are projected to reach between 700 and 800 parts per million (ppm) by the year 2100.[18] 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[19], whereas elevated O_3 alone has demonstrated reduced growth and yield of important crops[20,21]. 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.[15] However, other studies found that the combined effects of elevated O_3 and CO_2 on plants provided no ameliorating effects.[22]

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.[23] 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 with

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C_3 (i.e. produces a three-carbon molecule as the first product of photosynthesis) crop species that are more O_3 sensitive.[24] 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.[25] 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[23,26,27]; however, empirical evidence from Africa as a sugarcane growing region is still unavailable[28].

We 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 with 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 above sea level) 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.[29] Each of the 12 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- to 24-month cycle but has a fairly low sucrose content.[30] 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 with N31.[31] 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. Fertiliser solution containing macro- and micronutrients ideal for sugarcane growth was applied to all pots once every 3 weeks and dissolved by irrigation.[32] Fumigation of the plants was initiated 5 weeks after planting. The plants were exposed to elevated O_3 and elevated CO_2 concentrations for 7 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 (08:00–17:00) for the duration of the growth period of 7 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.[33,34] 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 assessment report.[18]

Growth measurements

The growth measurements, taken twice a month, included the number of green and dead leaves, top visible dewlap (TVD) leaf length and width, stalk height and number of tillers. The 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 (as can be seen in McCray et al.[35]). 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 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 1 h. 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 (3500 µMol/m^2/s), short flash (1 s) 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 1–2 s.[36] Following on from this, however, the fluorescence level typically starts to fall again, over a time scale of a few minutes.[36] Measurements of chl *a* fluorescence were taken monthly, starting at 8 weeks after planting with three replicates per plant.

The most commonly used chl fluorescence measuring parameter is F_v/F_m[37], 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.[38]

The normalised ratio F_v/F_m is created by dividing variable fluorescence by maximum fluorescence:

$$F_v/F_m = \frac{(F_m - F_o)}{F_m} = \frac{(F_{300} - F_{0.01})}{F_{300}}, \quad \text{Equation 1}$$

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.

The ratio F_v/F_m measures plant photosynthetic activity, with reduced values possibly indicating stress, photoinhibition and photosynthesis downregulation.[38] An F_v/F_m value in the range of 0.79–0.83 is the approximate optimal value for many plant species, with lowered values indicating plant stress, particularly the occurrence of photoinhibition.[38]

Stomatal conductance measurements

To assess O_3 uptake, stomatal conductance was measured once a month using a handheld 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 14:00.

Chlorophyll content measurements

Chl 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 handheld 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.[39,40] In addition to these advantages, HLMs were selected as the estimation of individual change over time can be modelled and fewer assumptions need to hold for the procedure to be valid.[41]

In this study, HLM 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 when $p > 0.1$, with increasing numbers of asterisks 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 × 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

Ambient 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 above sea level. The highest temperatures and relative humidities occurred during summer (December, January and February), with global radiation being the highest during spring (September, October and 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 with the outside. Additional data obtained from the station were ambient O_3 concentrations that were used to calculate AOT40 values (Supplementary figure 1).

Growth parameters

The data for all plant growth parameters measured can be found in the supplementary material (Supplementary figure 2) and are 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 2 and Table 2). However, the data in Table 2 indicate 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 Figure 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 with the control implies that the treatments do not result in earlier ageing of leaves (accelerated leaf senescence). The treatment × time interaction was significant for green leaves, dead leaves and leaf length for both varieties. It was also observed

Table 1: Monthly averages of the hourly ambient temperature, global radiation, relative humidity and monthly accumulated precipitation measured during the period of the open-top chamber trial. The monthly averages are shown with the minimum and maximum values given in parentheses.

Month	Temperature (°C)	Global radiation (W/m²)	Relative humidity (%)	Accumulated precipitation (mm)
October 2014	20 (4–32)	291 (0–1086)	40 (6–99)	6
November 2014	19 (7–30)	252 (0–1165)	60 (13–100)	25
December 2014	21 (13–32)	270 (0–1207)	62 (11–100)	16
January 2015	21 (12–33)	298 (0–1219)	63 (11–100)	55
February 2015	22 (11–34)	303 (0–1143)	52 (9–99)	10
March 2015	19 (10–30)	231 (0–1061)	64 (10–100)	21
April 2015	17 (4–26)	212 (0–924)	58 (9–100)	8
May 2015	17 (5–28)	192 (0–831)	35 (7–99)	0

Table 2: Hierarchical linear mixed modelling on plant growth variables under the different treatments at selected weeks 5, 17 and 29 to determine whether 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%	

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 situation applied for N31 over time (Supplementary figure 2), 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

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 analysis of variance, which was performed to assess whether 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

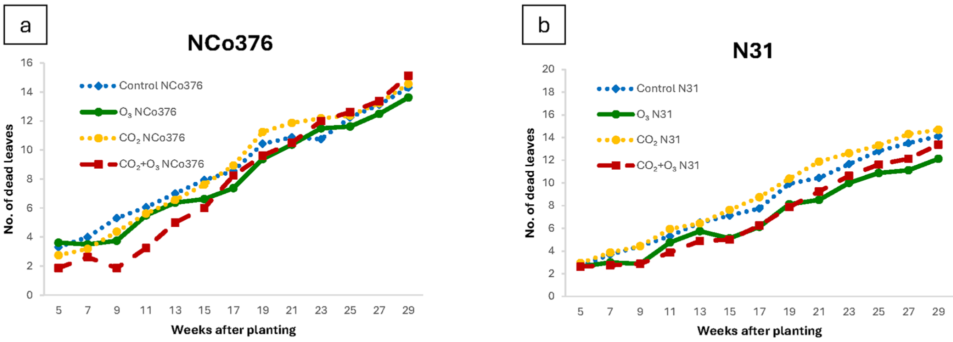

Figure 1: Evolution of the number of dead leaves through the growing season in two cultivars of sugarcane, (a) NCo376 and (b) N31, under the different treatments: exposure to charcoal-filtered air (control), elevated O_3 (80 ppb), elevated CO_2 (750 ppm) and elevated O_3 plus CO_2 (80 ppb and 750 ppm).

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 × time interaction, suggesting that 'time' was a better predictor of changes in photosynthetic activity than 'treatment' in this pilot trial (Table 3 and Supplementary figure 3).

Stomatal conductance

The evolution of stomatal conductance of sugarcane through the growing season shows a decreasing trend (Supplementary figure 4), 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 4). However, the differences between the treatments were not statistically significant (Table 3). 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 chl content value than N31 (Supplementary figure 5). Chl content tended to drop significantly in NCo376 close to harvest, although the treatment difference was not statistically significant (Table 3). '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 chl content variable, for NCo376 and N31, respectively.

Discussion

HLMs 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' and 'time', as well as the interaction of 'treatment' over 'time'. The analysis indicates that the factor that most influenced the growth and physiological response of each variety was 'time', whilst 'treatment' was only significant in predicting the dead leaves in the N31 cultivar. The 'chamber' effect influenced the variation in leaf length, leaf width and stalk height in the N31 variety, whilst 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 contributed significantly to variations in stomatal conductance and chl 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 chl content data. For chl *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[42-44], which, in turn, provides the crop with a suitable number of stalks and forms the foundation of a good crop.[45] 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 towards higher yield.[42]

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.[46] 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 such as 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: (1) reduce the number of pots in each chamber to ensure they all receive solar radiation; (2) increase the number of chambers per treatment to make the results more robust; (3) improve the irrigation system to remove water stress as a factor and (4) measure additional yield and quality (e.g. sugar content) variables to assess productivity.

Conclusions

In this pilot trial, we set out to investigate whether 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 whether 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 were 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 chl

Table 3: Hierarchical linear mixed modelling on plant physiological parameters under the different treatments at selected weeks to determine whether 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/m²/s)			**Chlorophyll content (mg/m²)**			**Chlorophyll fluorescence (F_v/F_m)**		
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/m²)**			**Chlorophyll fluorescence (F_v/F_m)**		
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%					

ANOVA; analysis of variance

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 such as South Africa, future studies should evaluate the effect of 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.

A novel contribution in this paper was the motivation for mixed models to be used in dose-response chamber studies as a reliable statistical tool. The traditional repeated-measures analysis of variance 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 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.

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Data availability

The data supporting the results of this study are available upon request to the corresponding author.

Declarations

We have no competing interests to declare. We have no AI or LLM use to declare. This paper was part of the doctoral thesis of T.L.L. (https://repository.nwu.ac.za/items/a430bd9b-ef76-4370-a2d3-18f52b26836d) but has since been significantly revised for publication in this journal.

Authors' contributions

T.L.L.: Conceptualisation, methodology, investigation, formal analysis, validation, visualisation, data curation, writing – original draft, writing – review and editing. P.G.v.Z.: Supervision, writing – review and editing. S.C.L.: Formal analysis, writing – review and editing. J.P.B.: Supervision, writing – review and editing. J.M.B.: Conceptualisation, methodology, writing – review and editing. P.D.R.v.H.: Conceptualisation, methodology, writing – review and editing. C.Y.W.: Writing – review and editing. All authors read and approved the final manuscript.

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