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Examining ozone susceptibility in the genus *Musa* (bananas)

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ABSTRACT

Tropospheric ozone (O_3) is a global air pollutant that adversely affects plant growth. Whereas the impacts of O_3 have previously been examined for some tropical commodity crops, no information is available for the pantropical crop, banana (*Musa* spp.). To address this, we exposed Australia's major banana cultivar, Williams, to a range of [O_3] in open top chambers. In addition, we examined 46 diverse *Musa* lines growing in a common garden for variation in three traits that are hypothesised to shape responses to O_3: (1) leaf mass per area; (2) intrinsic water use efficiency; and (3) total antioxidant capacity. We show that O_3 exposure had a significant effect on the biomass of cv. Williams, with significant reductions in both pseudostem and sucker biomass with increasing [O_3]. This was accompanied by a significant increase in total antioxidant capacity and phenolic concentrations in older, but not younger, leaves, indicating the importance of cumulative O_3 exposure. Using the observed trait diversity, we projected O_3 tolerance among the 46 *Musa* lines growing in the common garden. Of these, cv. Williams ranked as one of the most O_3-tolerant cultivars. This suggests that other genetic lines could be even more susceptible, with implications for banana production and food security throughout the tropics.

Keywords: banana, biomass, food security, leaf mass per area, ozone, total antioxidant capacity, total phenolic content, water use efficiency.

Introduction

Tropospheric ozone (O_3) is the third most important anthropogenic greenhouse gas and an air pollutant causing adverse impacts on plants and ecosystem services (Ainsworth *et al.* 2012, 2020). Ozone in the atmosphere is formed by photochemical oxidation reactions of carbon monoxide (CO), volatile organic compounds (VOCs), methane (CH_4) and nitrogen oxides (NO_x). Over much of the Earth's land surface, O_3 concentrations have more than doubled since the start of the industrial revolution (Monks *et al.* 2015; Ainsworth 2017). The tropics are strongly impacted by high and rapidly growing anthropogenic emissions of O_3 precursors (Kunhikrishnan *et al.* 2004). An increase in surface O_3 concentration has been noted by satellite monitoring across many tropical regions (Leventidou *et al.* 2018), with modelling studies predicting that surface O_3 concentrations will increase yet further in coming decades (Wild *et al.* 2012). Although the impact of O_3 on crops has been found to be variable across the globe (Emberson *et al.* 2009), studies estimate the current global O_3 yield gaps for rice and soybean to be 4.4% and 12.4%, respectively (Mills *et al.* 2018*a*). The highest O_3-induced yield loss in rice (*Oryza sativa*) is found in parts of India, Bangladesh, China and Indonesia, and losses of yield in soybean (*Glycine max*) are highest across the Americas (Mills *et al.* 2018*a*). It is clear that O_3 is, and will continue to be, a significant problem across much of the tropics (Van Dingenen *et al.* 2009; Moura *et al.* 2018*a*; Sharps *et al.* 2021). It has been suggested that projected increases in [O_3] across south Asia will decrease wheat yields by as much as 40% by 2050 (Tai *et al.* 2014), with important implications for global food security (Feng *et al.* 2022).

While the susceptibility to O_3 of tropical commodity crops such as rice (Sarkar *et al.* 2015), maize (*Zea mays*) (Singh *et al.* 2014), soybean (Singh *et al.* 2010) and sugarcane (*Saccharum* spp.) (Moura *et al.* 2018*b*; Cheesman *et al.* 2023) has been determined and is now beginning to be incorporated into the selection of commercial lines (Ainsworth 2017; Mills *et al.* 2018*a*), no information exists on the susceptibility of other tropical staple crops such as bananas (*Musa* spp.). The genus *Musa* represents a genetically diverse and complex assemblage of both seeded and parthenocarpic plant types, with four distinct sections: (1) Callimusa; (2) Australimusa; (3) Eumusa; and (4) Rhodochlamys (Daniells *et al.* 2001; Daniells and Janssens 2020). The majority of cultivated bananas have been derived from hybridisation within the Eumusa group, and in particular, between *Musa acuminata* (A genome) and *Musa balbisiana* (B genome), leading to a great diversity of hybrid types and ploidy levels grown around the world at both subsistence and commercial scales (Daniells and Bryde 2001). However, despite a diverse genetic potential, Cavendish cultivars, a triploid A genome subgroup (i.e. AAA), represent ~47% of all global commercial production (FAO 2022) and are the most commonly sold variety in the global north. Grown in more than 100 countries throughout the tropics and sub-tropics, the gross production value of all bananas and plantains (cooking bananas) is thought to represent US$51.5 billion annually (FAO 2022). However, precise figures on total global banana production are difficult to obtain as banana cultivation is often conducted by smallholder farmers and traded in the informal sector (FAO 2022). The global production of bananas grew at a compound annual rate of 3.2% between 2000 and 2017, reaching a record of 114 million tonnes in 2017, up from about 67 million tonnes in 2000 (FAO 2022).

The pathway for O_3 damage to plants follows O_3 uptake from the atmosphere through stomata (Ainsworth *et al.* 2012); once internal to the leaf, O_3 reacts rapidly with biomolecules forming reactive oxygen species (ROS); e.g. hydrogen peroxide (H_2O_2), superoxide ($O_2{}^-$) and hydroxyl (OH^-) radicals, which can subsequently cause cellular damage leading to metabolic dysfunction and possible cell death (Ainsworth *et al.* 2012; Emberson *et al.* 2018). Internal ROS are countered by a diverse range of antioxidants including ascorbic acid, glutathione and phenolic compounds such as flavonols (Dusart *et al.* 2019). Thus, leaf-level antioxidant capacity is important to explain the O_3 susceptibility of plant species due to its detoxification ability (Feng *et al.* 2010; Inada *et al.* 2012; Yendrek *et al.* 2015; Li *et al.* 2016). Leaves with larger contents of antioxidants exhibit greater O_3 tolerance due to a greater capacity for detoxification (Feng *et al.* 2010; Inada *et al.* 2012; Yendrek *et al.* 2015). It should also be noted that biological 'antioxidant capacity' can include enzymatic processes such as superoxide dismutase, which can either directly scavenge ROS or act during the regeneration of antioxidant compounds (Gillespie *et al.* 2007; Dusart *et al.* 2019). However, in this study we consider only the standing pool of antioxidant compounds at the time of leaf harvest.

Leaf-level antioxidant capacity has been shown to be a fundamental trait determining tolerance of *Musa* spp. to abiotic stressors such as cold temperatures and drought, which may both indirectly form ROS. For example, a comparison of the cold-tolerant plantain cv. Dajiao (*Musa paradisiaca*.; ABB group cv. Dajiao) and the cold-sensitive banana cv. Brazil (*Musa acuminata*; AAA group cv. Brazil) using proteomics suggests that an increase in antioxidant capacity confers increased cold tolerance to plantains (Yang *et al.* 2012). Antioxidant capacity, and thereby the ability to neutralise ROS has also been linked to drought tolerance in *Musa* spp., and is a suggested target for selective breeding for drought tolerance (Nansamba *et al.* 2020). It is also worth noting that transgenic banana plants have been conferred abiotic stress tolerance by an over expression of inducible cytosolic ascorbate peroxidase gene (AhcAPX) annulling damage caused by ROS (Shekhar *et al.* 2019). It is therefore reasonable to assume that accession-level differences in antioxidant capacity may underpin observed differences in response to ROS, regardless of whether they formed directly by O_3 or indirectly in response to other abiotic stressors.

Leaf internal morphology also impacts the interaction of O_3 and ROS with critical cellular processes, determining plant O_3 susceptibility. Specifically, leaves with high leaf mass per unit area (LMA) (e.g. thicker leaves and/or those with higher leaf density) have a lower O_3 load per unit leaf mass and appear more tolerant to O_3 (Bussotti 2008; Li *et al.* 2016). Indeed, O_3 susceptibility across sub-tropical tree species is negatively related to increasing LMA (Zhang *et al.* 2012; Li *et al.* 2016; Dai *et al.* 2017), while an analysis across clones of silver birch has shown that accessions with thinner leaves are more sensitive to O_3 (Pääkkönen *et al.* 1997; Karlsson *et al.* 2007).

Plant water use efficiency (WUE) is an important trait that links carbon and water cycles of terrestrial ecosystems (Beer *et al.* 2009). The intrinsic water use efficiency (iWUE) describes the ratio of photosynthetic rate to stomatal conductance (Cernusak *et al.* 2021). We therefore expect higher iWUE to correspond to less O_3 uptake through stomata per unit of CO_2 fixed by photosynthesis, thereby resulting in less O_3 exposure risk for a given amount of photosynthesis (Kolb and Matyssek 2001; Hoshika *et al.* 2018). Indeed, an experimental study of soybean genotypes showed that O_3-tolerant cv. Fiskeby III had a higher WUE than O_3-susceptible cv. Mandarin (Ottawa) (Bailey *et al.* 2019). For crops, leaf-level WUE is already used as an important parameter in crop improvement (Masutomi *et al.* 2019), with iWUE determined in a time-integrated way by measuring the stable carbon isotope composition of leaf dry matter, following the formulation developed by Farquhar and colleagues (Farquhar *et al.* 1982, 1989; Farquhar and Richards 1984).

Despite the importance of banana as a staple food crop in the tropics, there is to date, no work that we are aware of examining the potential impacts of tropospheric O_3 in *Musa* spp., with the only published work on O_3 and banana being focused on the use of O_3 either as a fumigant during packing or in solution as a post-harvest wash (Alencar *et al.* 2013). In the face of predicted rises in O_3 concentration across the tropics (Brown *et al.* 2022). It is therefore vital to identify the potential O_3 risks on banana production and at the same time explore the potential held within the genetic diversity of the *Musa* genus to mitigate such risks. Hence, in this work we aimed to:

1. carry out the first known characterisation of O_3 susceptibility in the highly important pantropical herbaceous crop – bananas (*Musa* spp.) using Australia's major cultivar, Williams (AAA group, Cavendish subgroup); and
2. test the range of physiological and biochemical traits thought to influence response to O_3 across the *Musa* genus under common garden conditions. This allowed us to place our observed impact of O_3 in cv. Williams Cavendish within the context of putative susceptibility to O_3 across the *Musa* genus more broadly.

In this study, we tested two hypotheses: (1) O_3 exposure would lead to decreases in biomass and increases in the leaf physiological and biochemical traits LMA, iWUE and antioxidant capacity in cv. Williams; (2) among a large collection of *Musa* lines, there would be some with high LMA, iWUE and antioxidant capacity, and that lines with high levels of these putative O_3 tolerance traits would be associated with higher ploidy levels and/or a higher proportion of B genome.

Materials and methods

The O_3 susceptibility of banana (*Musa* spp.) cv. Williams (https://www.promusa.org/Williams) was tested in open top chambers (OTC) built at the UK University of Exeter's TropOz Research facility located at James Cook University's Environmental Research Complex (ERC) on the Nguma-bada campus in Far North Queensland, Australia (www.tropoz.org). In addition, examination across the genus *Musa* of biophysical variation in functional traits known to determine O_3 susceptibility was conducted by sampling a living germplasm collection held at the South Johnstone Research Facility of the Queensland Department of Agriculture and Fisheries.

Open top chamber (OTC) and O_3 treatment

The experiment was conducted in nine independently controlled and monitored OTCs. The chambers (internal volume 22.2 m^3) were continuously ventilated at $\sim 2\ m^3\ s^{-1}$ using separate inline square centrifugal fans (ICQ560-VEE, Pacific Ventilation, Melbourne, Vic., Australia). External air to each chamber was drawn through two 592 mm × 592 mm High-Capacity Honeycomb Grid Carbon Filters (AF4VC14412, AireFlow-VC, Airepure Australia, Mulgrave, Vic., Australia) each fitted with a BR4 G4 pre-filter (80022020, Airepure Australia). Both fans and filters were housed within individual custom-made air handling units with air directed into the OTC's through a galvanised steel HVAC conduit. Into the charcoal filtered air stream of each chamber, pure O_3 generated on site (AOT-60G, Aquapure Ozone technology, Shenzhen, China) was delivered using a ¼" PTFE tubing manifold with the delivery regulated by both a PTFE 24V NC solenoid, pressure regulator and a PFA needle valve (MV-13-120, Parker Hannifin, Tucson, AZ, USA). This allowed for regulated delivery of pre-determined doses of O_3 into each chamber's air stream. The solenoid control systems were run by a networked Raspberry Pi-4 computer allowing for individual control over O_3 supply to specific chambers at specific times, with the aim of achieving a range of ambient O_3 concentrations between 0 and 120 ppb above ambient in 15-ppb increments across the nine chambers.

Ozone concentrations in each chamber were monitored sequentially using an ultraviolet (UV) absorption O_3 analyser (Model 205, 2B Technologies, Boulder, CO, USA) in air brought to a centralised service hub via a vacuum pump (Labport 840FT.18, KNF, Moreland West, Vic., Australia). A series of 24 V solenoids on a custom PTFE manifold, regulated by a networked Raspberry Pi-4 computer allowed for sampling from each chamber (via ¼" PTFE lines) in series. Assuming a 2-min sampling period per chamber and all nine chambers operational (plus zero check), this allowed for approximately three O_3 concentrations readings per hour per chamber. Environmental variables such as air temperature (T), air relative humidity (RH), shortwave radiation and photosynthetically active radiation (PAR) were monitored using a single meteorological monitoring station (Campbell Scientific, Logan, UT, USA) established in the central OTC.

Plant material

Tissue culture plantlets of cv. Williams were initially grown in a shade house, for 2 months and then transplanted into 60-L pots filled with locally sourced 'garden mix' top soil. After 1 month acclimation to sun, we selected 27 homogeneous plants approximately 20 cm in height and transferred them to OTCs for O_3 treatment, with three replicates grown in each chamber. The plants were grown under O_3 fumigation for 109 days from 23 October 2020 to 9 February 2021 with O_3 fumigation for 9 h per day (from 08:00 hours to 17:00 hours). All plants were watered daily using an automated drip irrigation system to ensure pots were kept close to field capacity, fertilised using a controlled release fertiliser (Scotts Landscape Formula All Purpose Osmocote, Scotts Miracle-Gro, OH, USA) and rotated every week to avoid the positional effect within chamber (Potvin and Tardif 1988).

At the end of the O_3 fumigation period and when the plants were on average 97 cm in height, two leaves were collected from every plant, specifically the third most recently expanded and therefore newly mature leaf (new leaf) and the eighth-most recently expanded (old leaf), both new and old leaves having fully developed under O_3 fumigation. From each leaf two mid-lamina leaf sections $\sim 300\ cm^2$ from both sides of the midrib were taken and measured for total fresh weight. After weighing, and scanning to determine area, one section was wrapped in tinfoil, snap-frozen in liquid N_2 and stored at −20°C before freeze-drying for biochemical analyses (see below); the other section was dried at 70°C for determination of LMA.

At the end of the experiment, leaves, pseudostem, corm and small suckers were harvested separately and dried in an oven at 70°C until constant weight for biomass determination.

Ozone dose response

As well as determination of the concentration-based O_3 metric, AOT_{40} (accumulated O_3 concentrations over a threshold of 40 ppb), we estimated O_3 flux into leaves using the Deposition of O_3 for Stomatal Exchange (DO_3SE) model based on an empirical Jarvis model of stomatal conductance (g_s) (Jarvis 1976; Emberson *et al.* 2000). To parameterise the model, stomatal conductance to water vapour (g_s) was measured using a SC-1 Leaf Porometer (Meter Group, Pullman, WA, USA) on the abaxial and adaxial surfaces of the youngest fully expanded mature leaf on each plant. Point measurements of g_s were conducted over a range of conditions with a total of 408 measurements of g_s used to parameterise the model. The air temperature, PAR and relative humidity varied from 26.8 to 39.5°C, 0 to 2515 $\mu mol\ m^{-2}\ s^{-1}$ and 46.5 to 94.6%, respectively, during the g_s measurements. The O_3 flux-based metric POD (Phytotoxic O_3 Dose, the accumulated stomatal O_3 flux above a threshold of 1 or 6 nmol $O_3\ m^{-2}$ projected leaf area (PLA) s^{-1}) was calculated according to CLRTAP (2017).

Musa diversity sampling

Banana cultivars were sampled from the living collections held at the South Johnstone Research Facility under common garden conditions and ambient $[O_3]$. At this site, a diverse range of ~200 banana types and hybrids are grown under standard fertilisation and watering regimes. On a single day (15 December 2020), we sampled 46 genetic lines chosen to represent a good cross-section of the diversity held in the collection. During sampling, three fully expanded new leaves were collected from each genetic line, with a mid-lamina leaf section ~400 cm^2 from each being used for determination of leaf morphological traits (e.g. lamina leaf mass per unit area, LMA) and biochemical analysis (see below).

Leaf morphological traits

For all lamina samples collected from the OTC experiment and diversity sampling, LMA was calculated using the leaf dry mass (DM) and the leaf area (LA) determined by image analyser software (ImageJ, National Institutes of Health (NIH), Bethesda, MD, USA). LMA was calculated as LMA = DM/LA in units of $g\ m^{-2}$. Freeze-dried leaf samples were subsequently ground into fine powder (Rocklabs Bench Top Ring Mill) and stored in airtight vials until determination of leaf biochemistry and stable isotope concentrations.

Biochemical analyses

Powdered leaf samples (~30 mg) were extracted in cold 50% acetone (Ritmejerytė *et al.* 2019). Total antioxidant capacity (TAC) was determined in the leaf extract by the ferric reducing antioxidant power (FRAP) assay. The assay was carried out according to Benzie and Strain (1996) with some modifications. Fresh FRAP solution was made by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution (in 40 mM HCl) and 20 mM $FeCl_3.6H_2O$ solution. The volume ratio of the three reagents was 10:1:1, respectively. The solution was kept warm at 37°C in a water bath until used. Leaf extracts (10 μL) were allowed to react with the FRAP solution (190 μL) for 30 min in the dark. The absorbance of the reaction mixture was measured at 593 nm and TAC was expressed as ascorbic acid equivalents (mg AAE g^{-1} dry weight). Ascorbic acid was used as the standard and a calibration curve in the range of 0–250 $\mu g\ mL^{-1}$ was prepared for this assay.

Total phenolic content (TPC) was measured in the same leaf extract by the Folin–Ciocalteau method (Singleton and Rossi 1965; Cork and Krockenberger 1991) with some modifications (Ritmejerytė *et al.* 2019). Briefly, a 20-μL aliquot of the leaf extract and 380 μL distilled H_2O were mixed with 25 μL Folin–Ciocalteu reagent. After 3 min, 75 μL 20% Na_2CO_3 (w/v) was added to the reaction mixture and incubated in the assay tubes at room temperature for 20 min. The absorbance of the mixture was then measured at 765 nm with a microplate reader (FLUOstar OPTIMA, BMG Labtech Pty. Ltd, Vic., Australia). Gallic acid was used as a standard and TPC was expressed as Gallic acid equivalents (mg GAE g^{-1} dry weight).

Stable isotope measurements

The carbon stable isotope ratio ($\delta^{13}C$, ‰) and weight percent (%C) were determined using a Costech Elemental Analyser fitted with a zero-blank auto-sampler coupled via a ConFloIV to a ThermoFinnigan DeltaVPLUS using Continuous-Flow Isotope Ratio Mass Spectrometry (EA-IRMS) at James Cook University's Advanced Analytical Centre. Stable isotope results are reported as per mil (‰) deviations from the VPDB reference. Precisions (s.d.) on internal standards were better than 0.1‰ for $\delta^{13}C$. The iWUE was calculated from $\delta^{13}C$ according to the equation of Farquhar *et al.* (1989):

$$\mathrm{iWUE} = \frac{C_a}{1.6}\left(\frac{b - \Delta^{13}C}{b - a}\right) \quad (1)$$

where $\Delta^{13}C$ is the carbon isotope discrimination by the plants (‰), and

$$\Delta^{13}C = \left(\frac{\delta^{13}C_a - \delta^{13}C}{1 + \delta^{13}C/1000}\right) \quad (2)$$

where C_a (400 ppm) is the mean ambient CO_2 concentration; the parameters a (4.4‰) and b (27‰) are the diffusional fractionation for CO_2 in air and isotopic discrimination of the RuBP carboxylase enzyme against $^{13}CO_2$, respectively; $\delta^{13}C_a$ is the carbon isotope ratio of atmospheric CO_2 (−8‰ under ambient air conditions).

Statistical analyses

Rather than adopt a fixed treatment level approach, we chose to fumigate the nine OTC's each with a different [O_3], such that we established a gradient to determine the full range of impacts. Such an approach has been advocated as better than a fixed level approach in global change studies of continuous environmental drivers, because it can also identify non-linear responses, should they occur (Kreyling *et al.* 2018). As specifically pointed out by Hurlbert (2004), this approach does not lead to problems of pseudoreplication and represents a valid method of testing for significant treatment effects. With each OTC representing a single point on the gradient of O_3 exposure, a linear regression of averaged data at the chamber level (n = 3 plants) was used to investigate the relationship between the O_3 exposure metric (e.g. AOT_{40}, POD_1 and POD_6) and both plant biomass and leaf functional traits (LMA, iWUE, TAC and TPC).

Across the diverse *Musa* lines, leaf traits (LMA, TAC and iWUE) were checked with Shapiro–Wilk and Levene's tests for normality and homogeneity of variance. To compare the means of the leaf trait data across different ploidy levels, data were then subjected to analysis of variance (ANOVA) coupled with Tukey's *post hoc* test (i.e. LMA and iWUE) or the non-parametric Kruskal–Wallis test (i.e. TAC) if the assumption of normality was not met. Pearson's correlation coefficient and Spearman's rank were conducted to identify correlations between the leaf traits. Lastly, a principal component analysis (PCA) was conducted to evaluate the leaf trait relationships among 46 genetic lines in PCA space. To do this analysis, the z-scores of LMA, TAC and iWUE traits were calculated using the scale function in the R-package scales (Wickham and Seidel 2022) and then summed. The genetic lines were ordered according to the sum of z-scores for the PCA plot. A permutational multivariate analysis of variance was performed to identify whether different ploidy levels were different in the PCA space. All statistical analyses were performed using the software R ver. 3.6.2 (R Core Team 2019) utilising base R and the packages *dplyr* (Wickham *et al.* 2023), *vegan* (Oksanen *et al.* 2020), *ggbiplot* (Vu 2011), *pairwiseAdonis* (Martinez Arbizu 2020).

Results

Open top chamber O_3 experiment

Banana cv. Williams was exposed to nine different levels of O_3 exposure in OTCs. Over the experimental period, the average values of daytime (i.e. between 08:00 hours and 17:00 hours) O_3 concentration ranged between 14.6 ± 4.5 and 91.5 ± 39.3 ppb, corresponding to an AOT_{40} of between 0 and 55 ppm h^{-1} (see Supplementary Table S1). During the experimental period, the average daytime air temperature, PAR and relative humidity were 32 ± 2.7°C, 1154.1 ± 693 μmol m^{-2} s^{-1} and 65 ± 13.4%, respectively. For determination of POD_y parametrisation of g_s in the DO_3SE model is in Table 1, with calculated POD_1 ranging from between 4.4 and 43.5 mmol m^{-2}, and POD_6 ranging between 0 and 27.4 mmol m^{-2} (Table S1).

Ozone dose response relationships for cv. Williams

Aboveground biomass of cv. Williams was significantly impacted by exposure to O_3, whether O_3 exposure was expressed as either a concentration (i.e. AOT_{40}) or flux (i.e. POD_1 and POD_6) metrics (Fig. 1, Table S2). Specifically, there was a significant (P < 0.05) and a highly significant (P < 0.01) decline in pseudostem and corm, and small suckers biomass, respectively. Similar patterns were seen irrespective of whether AOT_{40}, POD_1 or POD_6 was used (Table S2). However, in all cases, there was no significant change in leaf biomass with increasing O_3 exposure (Table S2). As significant trends observed with POD_1 provided the highest Adj-R^2 values, we conducted further analyses using this metric of O_3 exposure.

Leaf functional traits (TAC, TPC, LMA and iWUE) were tested for O_3 dose response in both old and new leaves. The old-leaf TAC and TPC both showed highly significant relationships with POD_1 (Adj-R^2 = 0.68, P < 0.01, Fig. 2*a* and Adj-R^2 = 0.78, P < 0.001, Fig. 2*b*); the relationships were positive in both cases. However, the new-leaf TAC and TPC both were not significantly related to the POD_1 (Adj-R^2 = −0.042, P = 0.44 and Adj-R^2 = −0.11, P = 0.67). The mean values of new-leaf TAC were between 27.0 and 33.8 mg AAE g^{-1} dry weight and new-leaf TPC were between 23.7 and 27.4 mg GAE g^{-1} dry weight. Similarly, mean values for

Table 1. Stomatal conductance model parameterisation of banana cv. Williams.

Parameter		Unit	cv. Williams
g_{max}		mmol O_3 m^{-2} s^{-1}	530
L_m		m	0.46
f_{min}		fraction	0.13
f_{PAR}		unitless	0.004
f_{VPD}	VPD_{min}	kPa	4.7
	VPD_{max}	kPa	2.35
f_{temp}	T_{min}	°C	26
	T_{opt}	°C	35
	T_{max}	°C	43

g_{max}, maximum conductance; f_{min}, fraction of g_{max} at minimum stomatal conductance (g_{min}); L_m, effective leaf blade width; f_{temp}, f_{light}, f_{VPD} are the functions of g_s response to air temperature (T, °C), photosynthetically active radiation at the leaf surface (PAR, μmol m^{-2} s^{-1}) and vapour pressure deficit (VPD, kPa), respectively.

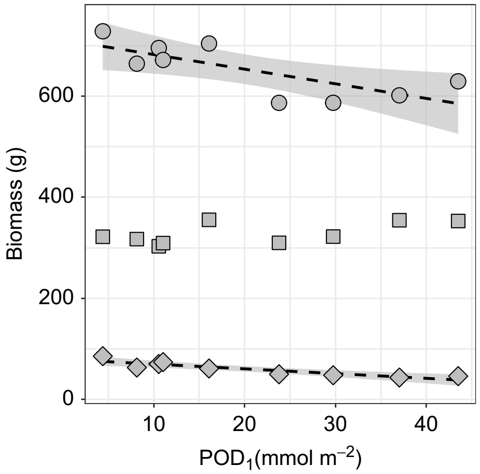

Fig. 1. Variation in aboveground biomass of banana cv. Williams (AAA group, Cavendish subgroup) grown under various O_3 concentrations. Biomass broken down into leaf biomass (square), pseudostem and corm biomass (circle), and small suckers (diamond) biomass with O_3 metric POD$_1$. Both pseudostem and corm, and small sucker biomass show significant negative declines (Adj-$R^2 = 0.50$, $P < 0.05$ and Adj-$R^2 = 0.77$, $P < 0.01$) with increasing O_3 flux metric POD$_1$.

old-leaf TAC were between 23.8 and 33.4 mg AAE g^{-1} dry weight and old-leaf TPC were 25.9 and 31.3 mg GAE g^{-1} dry weight. Thus, while relationships between TAC and TPC with POD$_1$ were significant in older leaves, there were no significant relationships with POD$_1$ in new leaves.

For physiological traits, mean values of new-leaf LMA ranged between 76.8 and 83.6 g m^{-2}, and old-leaf LMA ranged between 75.3 and 81.8 g m^{-2}. However, there was no significant trend in LMA values with the POD$_1$ for either new (Adj-R^2 = –0.14, P = 0.93) or old leaves (Adj-R^2 = 0.11, P = 0.21). In the case of iWUE, the mean values of new-leaf iWUE varied from 42.0 to 55.3 μmol mol^{-1} and old-leaf iWUE varied from 42.3 to 50.2 μmol mol^{-1}. They also showed no significant trend with POD$_1$ for either new (Adj-R^2 = 0.10, P = 0.21) or old leaves (Adj-R^2 = 0.05, P = 0.28). Thus, there were no changes in LMA and iWUE across the O_3 exposure gradient.

Leaf functional traits evaluation of diverse *Musa* genetic lines

Different correlation tests were performed to assess the relationships among leaf functional traits LMA, TAC and iWUE of 46 *Musa* lines. The Pearson's test showed a moderate positive relationship between LMA and iWUE ($r = 0.36$, $P = 0.02$). For non-parametric data, the Spearman's rank correlation rho (ρ) values showed a moderate correlation between the pairs LMA and TAC (ρ = 0.30, P = 0.04) and TAC and iWUE ($\rho = 0.40$, $P = 0.006$).

The one-way ANOVA and Kruskal–Wallis test were performed to evaluate if the three leaf traits (LMA, TAC and iWUE) were different for the three different ploidy levels: diploid, triploid and tetraploid. The ANOVA test showed that LMA was statistically significantly different among the ploidy levels ($F_{(2,\ 43)}$ = 4.70, P = 0.01), with mean value of LMA being 15.8% lower and significantly different (Tukey's *post hoc*, P-value 0.015) in diploid lines as compared to triploid lines, while tetraploid lines were not significantly different. For both iWUE ($F_{(2,\ 43)}$ = 0.99, P = 0.38) and leaf TAC ($H_{(2)}$ = 0.68, P = 0.71), there were no significant differences among the three levels of ploidy.

In just the 32 triploid genetic lines (Table 2), a one-way ANOVA was performed to determine whether the three leaf traits (LMA, TAC and iWUE) were significantly different between cultivars with a difference in their proportion of B genome. Using three levels of B genome contribution, none (e.g. AAA, 0%), low (e.g. AAB or 33%), and high

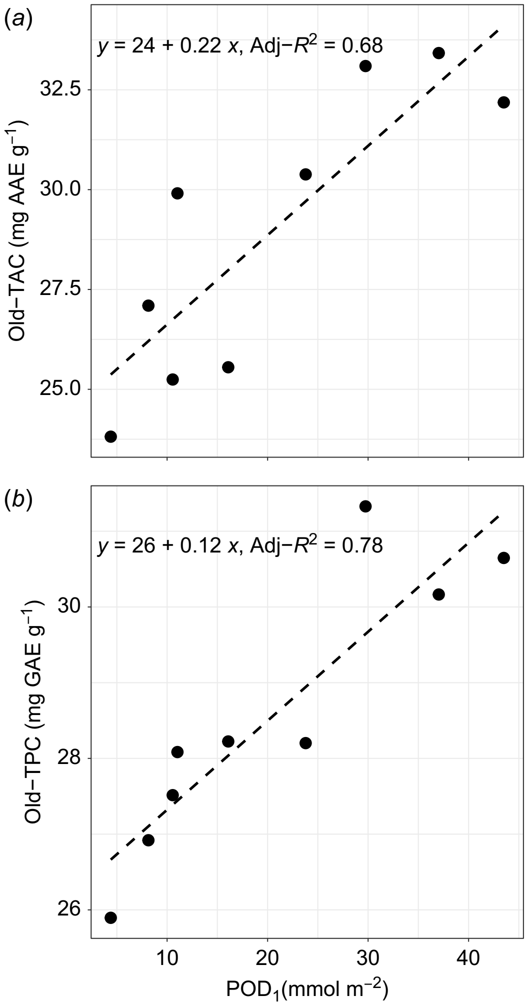

Fig. 2. Leaf lamina (*a*) total antioxidant capacity (TAC) and (*b*) total phenolic content (TPC) in the eighth (old) leaf of *Musa* spp. cv. Williams grown under various O_3 concentrations. Values represent chamber average of three plants. Both TAC and TPC show a highly significant ($P < 0.01$) and positive relationship with the O_3 flux metric, POD$_1$.

Table 2. Leaf functional traits of 46 *Musa* lines ranked according to the sum of z-scores of leaf mass per area (LMA), total antioxidant capacity (TAC) and intrinsic water use efficiency (iWUE) calculated from $\delta^{13}C$ values.

ID	Accession	Ploidy	Genome[A]	Proportion of 'B' genome	LMA (g m^{-2})	TAC (AAE g^{-1})	iWUE (µmol mol^{-1})	z-LMA	z-TAC	z-iWUE	z-Sum	PC1	PC2	PC3
1	Pisang Kelat	Trip.	AAB	0.33	65.4	27.7	97.9	−0.40	1.97	1.92	3.50	2.11	1.68	0.71
2	Williams	Trip.	AAA	0	86.6	21.8	93.3	1.34	0.68	1.44	3.46	2.02	−0.47	0.16
3	Ney Poovan	Dip.	AB	0.5	68.3	29.9	87.4	−0.16	2.46	0.85	3.14	1.78	1.85	−0.43
4	Goldfinger	Tetra.	AAAB	0.25	95.5	22.0	82.2	2.07	0.74	0.31	3.12	1.70	−0.95	−1.06
5	Niukin	Dip.	AA	0	90.9	22.5	83.3	1.68	0.84	0.43	2.95	1.63	−0.60	−0.85
6	Da Jiao	Trip.	NA	0.33	82.0	24.2	85.2	0.96	1.20	0.62	2.77	1.56	0.17	−0.54
7	Pisang Raja	Trip.	AAB	0.33	71.0	24.4	93.0	0.06	1.25	1.42	2.72	1.63	0.84	0.46
8	Hom Thong Mokho	Trip.	AAA	0	88.6	17.3	92.9	1.50	−0.29	1.41	2.62	1.57	−1.26	0.51
9	Sar 294	Trip.	TTT	0	92.0	22.2	78.6	1.78	0.78	−0.06	2.50	1.32	−0.71	−1.23
10	Asupina	Trip.	TTT	0	76.0	20.7	92.6	0.46	0.44	1.38	2.28	1.39	−0.02	0.62
11	Blue Java	Trip.	ABB	0.66	75.8	22.1	89.3	0.45	0.75	1.03	2.23	1.32	0.21	0.22
12	Dwarf Cavendish	Trip.	AAA	0	94.0	16.7	83.9	1.94	−0.43	0.48	1.99	1.12	−1.68	−0.33
13	Tigua	Trip.	AAB	0.33	81.0	18.9	86.7	0.87	0.06	0.77	1.70	1.00	−0.58	0.15
14	Dwarf Red	Trip.	AAA	0	90.1	18.1	79.7	1.62	−0.12	0.06	1.56	0.84	−1.23	−0.65
15	Kirkirnan	Dip.	AA	0	59.0	19.6	99.2	−0.93	0.21	2.05	1.33	0.97	0.81	1.87
16	*Musa acuminata* ssp. *zebrina*	Dip.	AA	0	63.5	29.6	72.8	−0.56	2.39	−0.65	1.18	0.55	2.08	−1.35
17	Ainu	Trip.	AAB	0.33	74.1	16.1	88.1	0.31	−0.57	0.92	0.66	0.46	−0.62	0.82
18	Pisang Nangka	Trip.	AAA	0	65.9	19.4	85.5	−0.36	0.17	0.65	0.47	0.33	0.38	0.58
19	Mangat	Trip.	AAB	0.33	76.1	16.1	84.2	0.47	−0.55	0.51	0.44	0.30	−0.72	0.42
20	Lady Finger	Trip.	AAB	0.33	86.0	19.5	68.8	1.28	0.19	−1.06	0.41	0.09	−0.78	−1.48
21	Monthan Short	Trip.	ABB	0.66	69.6	20.0	80.4	−0.06	0.30	0.13	0.37	0.21	0.25	−0.02
22	Sugar	Trip.	AAB	0.33	61.4	22.1	81.5	−0.73	0.76	0.24	0.27	0.17	1.06	0.16
23	Kluai Teparot	Trip.	ABB	0.66	75.6	15.0	84.3	0.43	−0.80	0.52	0.16	0.15	−0.87	0.57
24	Lakatan	Trip.	AAA	0	77.4	14.0	84.6	0.58	−1.02	0.56	0.11	0.13	−1.13	0.63
25	Igisahira Gsanzwe	Trip.	AAA	0	75.9	16.4	80.0	0.46	−0.49	0.09	0.05	0.04	−0.67	0.09
26	*Musa coccinea*	Dip.	NA	0	46.9	30.8	72.6	−1.92	2.64	−0.67	0.04	−0.06	3.22	−0.86
27	Pisang Susu	Trip.	AAA	0	67.2	15.8	84.0	−0.26	−0.62	0.50	−0.38	−0.14	−0.26	0.79
28	PA 03.22	Tetra.	AAAB	0.25	68.1	16.9	75.9	−0.18	−0.37	−0.33	−0.88	−0.51	−0.14	0.01
29	IC2	Tetra.	AAAA	0	65.5	19.7	71.4	−0.39	0.23	−0.80	−0.97	−0.61	0.44	−0.52
30	Goly Goly Pot Pot	Trip.	ABB	0.66	76.2	19.0	62.4	0.48	0.08	−1.71	−1.15	−0.83	−0.29	−1.55
31	Horn Plantain	Trip.	AAB	0.33	68.6	14.1	76.7	−0.14	−0.99	−0.26	−1.38	−0.77	−0.59	0.33
32	Pacific Plantain	Trip.	AAB	0.33	63.1	16.6	75.4	−0.59	−0.45	−0.38	−1.42	−0.81	0.10	0.20
33	Bluggoe	Trip.	ABB	0.66	64.8	17.0	70.9	−0.45	−0.36	−0.85	−1.66	−0.99	0.07	−0.26
34	Gros Michel	Trip.	AAA	0	57.1	17.1	76.5	−1.08	−0.33	−0.28	−1.69	−0.93	0.53	0.44
35	Kandrian	Trip.	ABB	0.66	60.3	17.2	73.3	−0.82	−0.31	−0.60	−1.73	−1.00	0.37	0.07
36	Pisang Gajih Merah	Trip.	ABB	0.66	56.8	12.4	84.6	−1.11	−1.36	0.56	−1.91	−0.95	−0.17	1.57
37	Vunamami	Dip.	AS	0	53.4	14.4	81.6	−1.39	−0.93	0.25	−2.07	−1.07	0.33	1.26
38	Ducasse	Trip.	ABB	0.66	60.1	16.4	70.8	−0.84	−0.49	−0.86	−2.19	−1.27	0.24	−0.03
39	Inarnibal	Dip.	AA	0	66.1	13.7	71.2	−0.34	−1.09	−0.81	−2.25	−1.30	−0.52	0.06
40	Pisang Mas	Dip.	AA	0	61.9	13.7	72.7	−0.69	−1.08	−0.67	−2.44	−1.38	−0.27	0.32

(*Continued on next page*)

Table 2. (*Continued*).

ID	Accession	Ploidy	Genome[A]	Proportion of 'B' genome	LMA ($g\ m^{-2}$)	TAC (AAE g^{-1})	iWUE ($\mu mol\ mol^{-1}$)	z-LMA	z-TAC	z-iWUE	z-Sum	PC1	PC2	PC3
41	FHIA-25	Trip.	AAB	0.33	69.2	14.3	61.6	−0.09	−0.95	−1.80	−2.84	−1.74	−0.60	−0.87
42	*Musa balbisiana*	Dip.	BB	1	60.9	16.1	63.8	−0.77	−0.55	−1.57	−2.90	−1.74	0.16	−0.57
43	Yangambi Km5	Trip.	AAA	0	59.2	14.4	67.3	−0.91	−0.93	−1.22	−3.06	−1.78	−0.01	−0.06
44	*Musa ornata*	Dip.	NA	0	56.1	14.9	66.9	−1.17	−0.81	−1.26	−3.23	−1.88	0.25	−0.03
45	Pisang Ceylan	Trip.	AAB	0.33	63.7	12.9	61.9	−0.54	−1.26	−1.76	−3.57	−2.12	−0.51	−0.49
46	Calcutta 4	Dip.	AA	0	47.8	14.1	64.5	−1.85	−0.99	−1.50	−4.34	−2.50	0.61	0.18

Ploidy, ploidy level; Dip., diploid; Trip., triploid; Tetra., tetraploid.
[A] Presumed genome if known (www.promusa.org).

(e.g. ABB or 66%), we saw no significant differences in LMA ($F_{(2,\ 29)} = 2.46$, $P = 0.1$), leaf TAC ($F_{(2,\ 29)} = 0.47$, $P = 0.63$) or iWUE ($F_{(2,\ 29)} = 1.03$, $P = 0.37$).

PCA results for diverse *Musa* lines

A PCA analysis of leaf traits data was performed for 46 *Musa* genetic lines in which the lines were ordered according to the sum of *z*-scores for LMA, TAC and iWUE traits (Table 2). The first two PCA axes (PC1 and PC2) explained a total of 81.4% of the observed variation in the leaf trait dataset (52.7% and 28.7% in PC1 and PC2, respectively). The loading plot (Fig. 3) showed that PC1 was positively related to all variables iWUE, LMA and TAC. Of the three variables, the contribution of iWUE to the PC1 was higher than LMA and TAC, as the iWUE vector was more parallel to the PC1 axis. On the other hand, LMA and TAC were the main contributors to the PC2 axis.

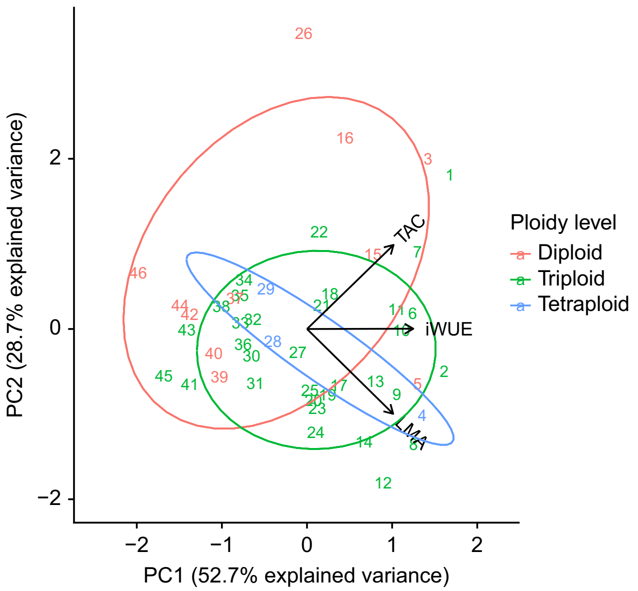

Fig. 3. Principal components analysis of leaf functional traits. The PCA biplot shows the 46 *Musa* lines as numbers in the plane formed by two principal components and the leaf functional trait variables, leaf mass per area (LMA), intrinsic water use efficiency (iWUE) and total antioxidant capacity, are shown as vectors (arrows). The *Musa* lines were grouped by ploidy levels.

A permutational multivariate analysis of variance was conducted, to test whether the distribution of ploidy groups in PCA space differed (Fig. 3). The permutation test showed that there were significant differences among the ploidy levels ($F_{(2,\ 43)} = 3.27$, $P = 0.02$). A pairwise Adonis analysis revealed a significant difference between diploid and triploid lines ($P = 0.005$, Fig. 3), but no significant differences between tetraploid and either triploid or diploid lines. Further, only for the triploid dataset, a similar PCA analysis was conducted to identify the effect of B genome. The result showed that the proportion of B genome had no significant effect on the PCA distributions ($F_{(2,\ 29)} = 1.39$, $P = 0.26$). Thus, we found evidence of an association between ploidy level and traits relevant to O_3 susceptibility but no such association with the proportion of B genome in the triploid lines.

Discussion

In this study, we investigated the effects of O_3 on above-ground biomass and leaf functional traits of the banana cv. Williams grown under different O_3 concentrations in OTCs. In addition, we examined the range of functional traits known to mitigate O_3 susceptibility in other species expressed across the *Musa* genus grown under common garden conditions. As the future predictions of O_3 concentrations across the tropics continue to rise (Mills *et al.* 2018*b*; Brown *et al.* 2022), it is important to identify the potential O_3 risks on banana production, and at the same time explore the range of potential O_3 tolerance across genetic lines as a basis for future plant breeding programs.

Responses of biomass to O_3 for cv. Williams

O_3 exposure had significant effects on the biomass of cv. Williams. The results showed a significant reduction in both pseudostem (including corm biomass) and new shoots (small suckers) biomass with increasing O_3 flux. However, there was no change in leaf biomass with the POD_1 (or indeed other metrics of O_3 exposure AOT_{40} or POD_6). In general, studies show that O_3 has negative impacts on total plant biomass (Wittig *et al.* 2009; Emberson *et al.* 2018); however, in those studies that partitioned above-ground biomass into components, similar results have been found to our findings with banana. In Holm oak (*Quercus ilex*), Gerosa *et al.* (2015) found that while stem biomass significantly reduced under high O_3, leaf biomass showed no decline. It appears that at high O_3 exposure, plants preferentially drive energy to the maintenance of leaf metabolism and/or to production of new leaves, decreasing the allocation of biomass to below-ground, structural or storage components of the plant (Moura *et al.* 2018*b*; Holder and Hayes 2022).

Responses of leaf functional traits to O_3 for cv. Williams

The study of biochemical traits across O_3 exposure showed a significant increase in old-leaf TAC and TPC with increasing O_3 flux (e.g. POD_1 and POD_6). Gao *et al.* (2016) also reported that elevated O_3 significantly increased both leaf TAC and TPC in four Chinese tree species. Similarly, an increase in TPC alone due to O_3 exposure has been regularly observed (Saleem *et al.* 2001; Yamaji *et al.* 2003). The increase in TPC can be related to their defensive role against oxidative stress through their action as ROS scavengers (Oksanen *et al.* 2013). The change in TAC is consistent with changes in TPC, as phenolic compounds are the main contributors to the antioxidant properties of plant extracts (Dudonné *et al.* 2009). However, the new-leaf TAC and TPC were found to not relate to O_3 flux. Hence, this study suggests that plant leaves respond to cumulative O_3 exposure by increasing antioxidants, rather than constitutively upregulating antioxidant content in new leaf tissue.

Our results from cv. Williams show that there was no significant trend in LMA with increasing O_3 flux. Although interspecific variation in O_3 susceptibility among tree species can, to a large extent, be explained by variation in LMA (Feng *et al.* 2018), meta-analysis has shown that LMA itself is not much altered by elevated O_3 (Poorter *et al.* 2009). A recent study on C_4 species showed that elevated O_3 had no significant effect on LMA (Li *et al.* 2022), while Shang *et al.* (2017) found that LMA significantly reduced with increasing O_3 metric in particular poplar (*Populus* spp.) clones. These differences may be related to the O_3 susceptibility of plant species themselves, with LMA responses being both cultivar- and species-specific (Dai *et al.* 2017; Feng *et al.* 2018).

Several studies have shown that elevated O_3 decreases plant iWUE (Masutomi *et al.* 2019; Xu *et al.* 2020; Li *et al.* 2021). Commonly, elevated O_3 exposure reduces plant net photosynthesis much more than it does stomatal conductance, leading to a decline in leaf iWUE, as iWUE is the ratio of photosynthesis to stomatal conductance (Hoshika *et al.* 2015; Xu *et al.* 2020, 2021). However, other studies also have indicated that iWUE is not impacted by elevated O_3 (Zhang *et al.* 2014; Sugai *et al.* 2018; Li *et al.* 2019). In cv. Williams, we did not find any significant relationship between iWUE and O_3 flux. This may be due to the change in both A and g_s proportionally by O_3 exposure (Zhang *et al.* 2014; Li *et al.* 2019), or the dominance of the $\delta^{13}C$ signal by structural carbon laid down in the young leaf during development (Vogado *et al.* 2020).

Correlations among leaf functional traits in diverse *Musa* lines

We found large variability and correlation in the values of LMA, TAC and iWUE among the 46 lines of *Musa* studied. In general, LMA is used to express the degree of a plant's adaptations and acclimation to its surroundings. Leaves with high LMA have thicker and/or denser leaves compared to those with low LMA. Accordingly, they typically have a higher nitrogen content per unit leaf surface; as a result, they have a high photosynthesis rate and a high iWUE (Bussotti 2008). These traits make plants less reactive to environmental stressors and enable them to support the detoxification process (Bussotti 2008). In this regard, the correlation among these leaf traits of 46 *Musa* lines was consistent with more general observations. Moreover, our PCA results were consistent with our correlation tests. Of the three variables, the contribution of iWUE to the PC1 was higher than LMA and TAC, indicating selection for high iWUE, already used as an important indicator for crop improvement (Masutomi *et al.* 2019), may have additional benefits for conferring O_3 tolerance. The observed positive relationship between putative determinants of O_3 tolerance provides support for an additive z-value (i.e. iWUE + LMA + TAC) to determine projected O_3 susceptibility (Table 2).

There was a significant effect of ploidy level on the leaf functional traits of the 46 *Musa* lines tested. Our analyses showed that diploid lines were significantly different from triploid lines (Fig. 3), with our results suggesting that the diploid lines could have a lower tolerance to O_3 as compared to triploid lines. General observations have shown that AA diploids are more susceptible to drought than most triploids (Ravi *et al.* 2013), leading to the suggestion that triploid accessions are generally more stress tolerant. We saw no significant differences between tetraploid and either diploid or triploid lines, however, this was probably due to low numbers of observations with only three tetraploids among 46 lines tested. It has been suggested that the presence of B genome may confer some advantage under abiotic stress, with the B genome showing greater tolerance to drought than those entirely based on the A genome (Thomas *et al.* 1998; Ravi *et al.* 2013; Delfin *et al.* 2016). However, we could not find any significant effects of B genome levels on the leaf trait variables tested. A possible reason is that the assumption of full segregation between the A and B genomes simply does not exist as demonstrated by Baurens *et al.* (2019).

Evaluation of combined results

Examining functional trait diversity present across the *Musa* genus, we projected an O_3 tolerance ranking (Table 2) among the 46 lines tested. The highest-ranked (putative O_3 tolerant) lines include accessions like cv. Goldfinger (FHIA-01), noted for their tolerance to cold, pests and disease (Orjeda *et al.* 1999; Daniells and Bryde 2001; Mintoff *et al.* 2021), and cv. Williams noted as being resistant to mechanical stress due to robust leaves (J. Daniells, pers. comm.). Therefore, our ranking of banana accessions may be consistent with generalised stress tolerance. The predicted ranking provides a context for the extrapolation of our OTC results in cv. Williams to the *Musa* genus more broadly as well as identifying accessions for future O_3 risk assessment.

The results of our *Musa* genus study with respect to LMA, iWUE, and TAC suggest that cv. Williams could be considered amongst the more O_3 tolerant cultivars of the 46 lines tested here. Considering our OTC experiment results showing the susceptibility of cv. Williams to O_3, we would expect the other lower-ranked *Musa* genetic lines to be even more susceptible to O_3. This information will be important for predicting food security in regions where O_3 concentrations are already high or will further increase in the future. For example, in Africa, banana production represents an important staple food crop. Whereas other staple food crops such as *Triticum aestivum* (wheat) and *Phaseolus vulgaris* (bean) are already known to be sensitive to O_3 (Hayes *et al.* 2020), nothing is yet known about the susceptibility of local banana varieties to O_3. Our experimental results suggest there could be a substantial risk from O_3 on banana production in Africa. Experimental work on different *Musa* genetic lines would be required to confirm our presumed variation in susceptibility, while work with larger plants would be required to extend our observation on biomass gain to impacts on fruit yield and crop production losses.

Conclusion

We have demonstrated the scale and possible prevalence of O_3 susceptibility in the important tropical food group *Musa*. Securing agricultural production and food security across tropical regions will require the development of agricultural practice including improved cultivar selection and efficient irrigation management. However, both of these practices may incur unintended consequences in the form of increased impacts of O_3 damage (Harmens *et al.* 2019). For example, the selection of high-yielding cultivars with associated increased stomatal conductance (g_s) has been postulated as the reason behind the substantial variation observed in O_3 susceptibility of wheat lines (Biswas *et al.* 2008). Similarly, the alleviation of soil water deficit via targeted irrigation may lead to higher g_s thereby increasing the flux of O_3 into crop leaves during periods (such as local biomass burning) in which O_3 concentration may be high. Here, we have established susceptibility to O_3 in the biomass production of a widely planted banana cultivar. We have also explored on an empirical basis the potential for O_3 tolerance among a diverse collection of wild species, cultivars and synthetic hybrids in the genus *Musa*. Development of cultivars with O_3 tolerance traits, in addition to those traits desirable for food production, could contribute to safeguarding tropical food supplies as O_3 pollution continues to rise.

Supplementary material

Supplementary material is available online.

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