Differential response of photosynthetic electron transport and CO₂ assimilation in sensitive (S156) and resistant (R123) *Phaseolus vulgaris* L. (bush bean) genotypes to chronic ozone exposure

Tropospheric ozone is currently regarded as one of the most important air pollutants, since it causes more damage to vegetation world-wide than all the other pollutants combined (Ashmore and Bell 1991). Due to its oxidative nature ozone causes leaf damage and a decrease in photosynthesis. Ozone tolerance varies widely between species and genotypes. The aim of this study was to identify and quantify the physiological and biochemical constraints imposed by chronic ozone exposure of two bush bean (*Phaseolus vulgaris* L.) genotypes with known difference in sensitivity, namely S156 (sensitive) and R123 (resistant), to charcoal-filtered air and 80 nmol.mol⁻¹ O₃. The study was conducted in open-top growth chambers (OTCs) over the entire growth period by measuring chlorophyll a fluorescence (JIP-test) and photosynthetic gas exchange of the test plants weekly. The status of the photosynthetic apparatus was assessed by analysis of chlorophyll a fluorescence kinetics (JIP test) and CO₂ response curves (A:C). O₃-induced physiological effects were detected in S156 long before appearance of necrotic spots on the trifoliate leaves. Photosynthesis was substantially inhibited in S156, mainly due to disengagement of the oxygen evolving complex (OEC), inhibition of intersystem electron transport and the reduction of end-electron acceptors of PSI (ferredoxin, NADP⁺), causing the concomitant decrease in the carboxylation and regeneration of ribulose-1,5-bisphosphate. Seed and pod yield closely reflected the photosynthetic response of the test plants.

Although leaves of both the genotypes were affected visually, it was S156 that displayed severe necrotic ozone injury on the trifoliate leaves. Our data contribute to and complement the existing knowledge on the processes underlying the phytotoxicity of O₃ needed for development of tolerant genotypes.

**Keywords:** *Phaseolus vulgaris*, ozone, photosynthesis, chlorophyll a fluorescence, gas exchange, photosynthetic electron transport, seed yield, open-top chambers.
Introduction

Atmospheric pollution has risen sharply since 1973 due to a drastic increase in commercial energy consumption (McCormick 1997). Some of these emissions, such as CH₄, NOₓ, CO and volatile organic carbons (VOCs) are associated with the production of the secondary air pollutant, ozone (O₃) (Monks et al. 2015). The continuous rise of tropospheric O₃ (IPCC 2007) is considered to be one of the major environmental stress factors, causing more damage to crops and forests than any other air pollutant (Ashmore and Bell 1991). The biological effects of O₃ on plants have been studied for more than 60 years (Manning et al. 1972; Heggestad 1991; Davison and Reiling 1995). Although smaller rates of deposition to non-stomatal surfaces occur, O₃ enters the plant mainly through open stomata during daytime (Fowler et al. 2009). O₃, a powerful oxidant, generates toxic free radicals within the apoplastic and cell fluids and is responsible for damage to cell metabolism (Mills et al. 2011). While elevated background levels of O₃ are often insufficient to produce visible injury, lower photosynthesis is often reported (Mckee et al. 1997). They ascribed the reduction in net photosynthesis of plants exposed to O₃ for short terms or chronically, to inter alia impairment of stomatal action, inhibition of electron transport and decreased Rubisco activity and content. Measuring the effect of chronic ozone exposure (38-120 nmol.mol⁻¹) on soybean, in an open air experiment, the final verdict of Betzelberger et al. (2012) was that because canopy radiation interception, efficiency of photosynthesis and harvest index were all negatively affected, that the attack by this pollutant is multi-faceted.

Ozone tolerance varies widely between species and genotypes. The families Fabaceae and Solanaceae, which include many crop plants, seem to be particularly sensitive (Heagle 1989). Ozone-sensitive and -tolerant cultivars have been reported for many plant species, including bush bean (Phaseolus vulgaris L.) (Guzy and Heath 1993). Using chlorophyll a fluorescence and leaf gas exchange to study the response to O₃ of the Phaseolus vulgaris cultivars Pinto (sensitive) and Groffy (resistant) in a short-term O₃-exposure experiment (80 nmol.mol⁻¹, 4h), Guidi et al. (1997) reported constraints on stomatal conductance and quantum efficiency (Fv/Fm), leading to a decrease in the maximum CO₂ assimilation (Amax). However, no clear explanation for the differential response was given. Evaluating physiological and biochemical traits that may confer O₃ tolerance in bean cultivars exposed to acute O₃-stress, Guidi et al. (2010) reported that down-regulation of PSII occurred in the tolerant cultivar resulting in protection against the generation of active oxygen species.

Farage et al. (1991) using single acute (200 nmol.mol⁻¹, 4-16h) O₃ exposures of wheat, came to the conclusion that the predominant factor and initial cause of O₃-induced reduction in light saturated CO₂ uptake, was a decrease in apparent carboxylation efficiency and that the capacity for ribulose-1,5-bisphosphate (RuBP) regeneration was less affected. In an effort to identify physiological subsystems that may mediate differences in sensitivity to ozone of three bush bean (Phaseolus vulgaris L.) genotypes with known differences in sensitivity to O₃ (S156, R123 and R331), Flowers et al. (2007) could not resolve the sequence of loss of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content or activity and changes in mesophyll conductance, Fm and Fv/Fm. They were of the opinion that constraints on all of these processes could have been the result of general destruction rather than sequential attack on individual subsystems. Flowers et al. (2007) also reported that unstressed S156 had a higher photosynthetic rate than R123, but had no significant capacity to protect Rubisco from attack at high O₃.

Chlorophyll a fluorescence induction measurements are widely used to monitor stress. However the analysis of the fluorescence curves often remained limited to the initial rise phase and the maximum (Fm) and the minimum (Fo) of the fluorescence emission.

In our study, we investigated the effect of O₃ on primary photosynthesis by quantification of the OJIP fluorescence curve by the JIP-test and by plotting and analysing fluorescence curves according to different expressions (Strasser et al. 2004; Strasser et al. 2007). This revealed information on the effect of the applied stress on all partial processes of photosynthesis namely, absorption of light energy, trapping of excitation energy, intersystem electron transport and reduction of end-electron acceptors. In parallel, the stress effect was studied by analysis of CO₂ response curves of photosynthesis (A/Ci curves) of the intact leaves, characterising O₃-induced effects on CO₂ assimilation, carboxylation efficiency, Rubisco regeneration and stomatal limitation. The test plants used were the ozone-sensitive (S156) and ozone-resistant (R123) Phaseolus vulgaris genotypes (bush bean) chronically fumigated in well-controlled OTCs over the full growth period. By parallel measurement of chlorophyll a fluorescence and photosynthetic gas exchange we intended to obtain a correlation of complementary information of indirect and direct signals on the photosynthetic response of the test plants. Recording in addition chlorophyll content and seed yield of the test plants, allowed comparison of the response of the plants on biophysical, biochemical and physiological level of photosynthesis as well as on seed yield level. The data are intended to complement the existing knowledge of O₃ effects on photosynthesis of crop plants needed for development of tolerant lines.

Materials and Methods

Experimental site and treatments

The study was conducted at the Potchefstroom campus of the North West University in open-top chambers (OTCs), each consisting of a cylindrical aluminium framework with rain cap, covered with transparent PVC sheeting. Four 5 m³ chambers, ventilated at 1.5 replacements per minute, were used in this study. Two chambers served as control
and were ventilated with charcoal filtered (CF) air. The two treatment chambers were ventilated with air enriched with O$_3$ from gas cylinders. The O$_3$ concentration in the diurnal O$_3$ levels maintained in the treatment OTCs. For technical details of the OTC system and for a record of the entire growing season (40 days). The O$_3$ concentration was measured with an ozone monitor (Model 205, 2B Technologies, Inc., Colorado, USA). See Heyneke et al. (2012) for technical details of the OTC system and for a record of diurnal O$_3$ levels maintained in the treatment OTCs.

**Plant material**

Two bush bean (Phaseolus vulgaris L.) genotypes namely ‘S156’ (O$_3$-sensitive) and ‘R123’ (O$_3$-resistant), selected from ‘Oregon 91’ for O$_3$ sensitivity (Dr. Richard Reinert, USDA, Raleigh, NC), were obtained from Dr. Kent Burkey from USDA Air Quality Research Unit, Raleigh, NC. Two seeds were planted in 16 dm$^3$ (30 cm diameter) in four pots per genotype per two treatment chambers and two control chambers respectively. The pots were filled with a mixture of sand, soil and vermiculite (1:2:1). Constant water supply to each pot was mediated by glass fibre wicks (Thoenes Dichtungstechnik GmbH, Germany) placed at different levels in the soil of the pots through which water was taken up by capillary action from water reservoirs positioned underneath each pot. This method ensured good control of the water regime of the test plants in an effort to eliminate possible effects of vapour pressure deficit (VPD) through its influence on stomatal conductance affecting access of ozone to the mesophyll of the leaves.

**Chlorophyll content index**

Chlorophyll content index (CCI) of youngest fully expanded leaves was measured with a hand-held chlorophyll content meter (CCM-200, Opti-Sciences, Inc., USA). Eight measurements were taken per trifoliate leaf of two plants of each treatment.

**Photosynthetic gas exchange**

Photosynthetic gas exchange was measured with an infrared gas analysis system (CIRAS-2, PP-Systems, Hertz, U.K.) on four randomly selected plants in each chamber of which two replicate measurements were of the sensitive S156 line, and two were of the resistant R123 line. A 2.5 cm$^2$ section of the third fully expanded leaf was clamped into a broad leaf photosynthetic leaf chamber (PLC) with light and temperature control. The leaf in the cuvette was first acclimated at a CO$_2$ concentration of 360 μmol.mol$^{-1}$ at a saturating net photosynthetic photon flux (1200 μmol m$^{-2}$ s$^{-1}$) for five minutes to ensure full activation of Rubisco (Taylor and Terry, 1984). Leaf temperature was kept at 26 °C during measurements. After gas exchange reached a steady state, the intercellular CO$_2$ concentration ($C_i$) was manipulated by varying ambient CO$_2$ concentration ($C_a$). The linear slope of the response curve was determined by lowering $C_i$ in the cuvette from 360 μmol.mol$^{-1}$ in five steps to 200, 100, 50 and 25 μmol.mol$^{-1}$. $C_a$ was again stabilised at 360 μmol.mol$^{-1}$ to ensure open stomata and to verify the stability of the photosynthetic apparatus. Lastly, $C_i$ was stepped up from 360 μmol.mol$^{-1}$ in four steps to 500, 700, 1000 and 1500 μmol.mol$^{-1}$, allowing CO$_2$ assimilation rate ($A$) vs. intercellular CO$_2$ concentration ($C_i$) response curves ($A/C_i$ curves) to be drawn (Singsaas et al., 2001). A three minute measuring period at each successive increment was sufficient to attain stable values. The supply functions [$A = \gamma (C_i - C_a)$] corresponding to the demand functions [$A = CE(C_i - \Gamma)$] were drawn by simply joining the value of $C_i = C_a$ (at 360 ppm) on the abscissa to the point giving $A_{\text{sat}}$ at this value of $C_i$ (Pammenter, 1989). The degree of stomatal limitation of photosynthesis was calculated by the equation: $l = (A_{\text{sat}} - A) / A_{\text{sat}} x 100$ (Farquhar and Sharkey 1982).

**Abbreviations are as follows:** $A$ = net photosynthesis; $A_{\text{sat}}$ = CO$_2$ assimilation rate at ambient CO$_2$ concentration; $A_i$ = CO$_2$ assimilation rate in absence of stomatal limitation ($C_i \geq 360$ μmol.mol$^{-1}$); $\gamma = \text{stomatal conductance to CO}_2$; $C_a$ = ambient CO$_2$ concentration; $C_i$ = intercellular CO$_2$ concentration; $CE$ = $\delta A/\delta C_i$ = apparent carboxylation efficiency; $\Gamma$ = CO$_2$ compensation concentration; $E$ = transpiration rate; $l$ = % stomatal limitation; PFD = photon flux density.

Interpretation of the parameters was done according to Farquhar and Sharkey (1982). We were aware of the fact that the estimated parameter values may differ for the same data set depending on the fitting methods used (Gu et al. 2010).

**Chlorophyll a fluorescence induction**

Chlorophyll a fluorescence induction transients were recorded weekly. Six measurements were taken on two leaves of four different plants in the treatment and control OTCs respectively. Measurements were recorded at night on at least one hour dark adapted leaves with a Plant Efficiency Analyser (Handy-PEA, Hansatech Instruments Ltd., Kingslynn, UK). Each induction transient was induced by red light (peak 650 nm) at 2000 μmol photons m$^{-2}$ s$^{-1}$ (sufficient excitation intensity to ensure complete closure of PSII reaction centres to obtain the true fluorescence intensity of F$_{v}$/F$_{m}$) and recorded for 1 second on a 4 mm diameter area of a dark-adapted, attached leaf sample. The recorded OJIP transients were analysed by the JIP-test according to Strasser et al. 2004. OJIP refers to the polyphasic fast fluorescence rise from minimal fluorescence intensity at step O (50 μs), through steps J (~2 ms) and I (~30 ms) to maximal fluorescence at step P (~300 ms). The kinetics of the OJIP fluorescence transient of plants have been found to be very sensitive to environmental conditions (Strasser and Strasser 1995; Krüger et al 1997).
The fast phase fluorescence rise (OJIP) occurring within less than a second after illumination of a dark-adapted plant sample, reflects the concentration of primary reduced quinone electron acceptors of PSII in its reduced state (Qr/ Qtotal) in the thylakoid membranes as affected by the kinetics of several different redox reactions taking place in the photosynthetic ET-chain. The JIP-test represents a translation of the original fluorescence data to biophysical parameters that quantify the stepwise flow of energy through PSI at the reaction centre (RC) as well as at the excited cross-section (CS) level (Strasser and Strasser 1995; Force et al. 2003; Strasser et al. 2004). The average values of these parameters were calculated using the computer program “Biolyzer” (http://www.fluoromatics.com). The parameters which all refer to time zero (start of fluorescence induction) are: (i) the specific energy fluxes (per reaction RC, of PSI) for absorption (ABS/RC), trapping (TRo/RC), electron transport (ETo/RC) and dissipation at the level of the antenna chlorophylls (DIo/RC); (ii) the flux ratios or yields, namely, the maximum quantum yield of primary photochemistry (ϕo = TRo/ABS = Fo/Fo), the efficiency with which a trapped exciton, having triggered the reduction of Qr to Qr− can move an electron further than Qr− into the electron transport chain (ψi = ETo/ET), the quantum yield of electron transport (ϕet = ET/ABS = ψi ψj) and the quantum yield of dissipation (ϕd = DIo/ABS = (1 - ϕo); (iii) the phenomenological energy fluxes (per excited cross-section, CS) for absorption (ABS/CS), trapping (TRo/CS), electron transport (ETo/CS) and dissipation (DIo/CS); (iv) the fraction of active PSI-reaction centres per total absorption (RC/ABS) and per excited cross-section (RC/CS). The initial stage of photosynthetic activity of a RC complex is regulated by four functional steps namely absorption of light energy (ABS), trapping of excitation energy (TR), conversion of excitation energy to electron transport (ET) and reduction of end electron acceptors beyond PSI (RE). A multi-parametric expression, the so called photosynthetic performance index (PItotal), reflecting the contribution of these four partial processes, was introduced by Tsimilli-Michael and Strasser (2008) and Yordanov et al. (2008):

$$P_{\text{total}} = \frac{\gamma_{\text{RC}}}{1 - \gamma_{\text{RC}}} \cdot \frac{\psi_{\text{Po}}}{1 - \psi_{\text{Po}}} \cdot \frac{\psi_{\text{Eo}}}{1 - \psi_{\text{Eo}}} \cdot \frac{\delta_{\text{Ro}}}{1 - \delta_{\text{Ro}}}$$

where $\gamma_{\text{RC}}$ stands for the fraction of chlorophyll molecules which are active reaction centers of PSI. Therefore, $\gamma_{\text{RC}}/(1 - \gamma_{\text{RC}}) = \text{RC/Chl}_{\text{Antenna}} = \text{RC/ABS}$ in the JIP-test terminology. RC/ABS is the fraction of reaction centre chlorophyll (ChlRC) per total chlorophyll (ChlRC + ChlAntenna). This expression can be deconvoluted into two JIP-test parameters and estimated from the original fluorescence signals as RC/ABS = RC/TRo • TRo/ABS = [(F2ms - F50μs)/(F2ms - F50μs)] • Fo/Fo. The factor 4 is used to express the initial rise of the relative variable fluorescence between steps O and P of the OJIP transient (VOP) per 1 ms. The expression RC/ABS shows the contribution to the Ptotal due to the RC-density relative to all chlorophylls of PSII. The contribution of the light reactions for primary photochemistry is estimated according to the JIP-test as $\varphi_{\text{Po}}/(1 - \varphi_{\text{Po}}) = \text{TRo/DIo} = k_{\text{Po}}/k_{\text{Oi}} = F_{o}/F_{o}$. The contribution of electron transport past Qr is derived as $\psi_{i}/(1 - \psi_{i}) = \text{ETo/ETo - ET} = (F_{\text{M}} - F_{\text{Tr}})/(F_{\text{M}} - F_{\text{O}})$. The contribution of the reduction of end electron acceptors (finally NADP+) is derived as $\delta_{o}/(1 - \delta_{o}) = \text{RE/ABS} = (1 - V_{\text{Tr}})/(1 - V_{\text{Tr}})$.

Extended analysis of the fluorescence transients was done by calculation of the difference in relative variable fluorescence to present so-called $\Delta V$ curves (expressed as $V = f(t)$), i.e. subtracting normalised variable fluorescence values of the controls of transients normalised between (1) Fo and Fv (Vijo = (F − Fv)/(F − Fo)) and therefore as a function of time, $\Delta V_{ij} = (V_{ij} \text{treatment} - V_{ij} \text{control})$. (2) Fj and Fp (Vjp = (Fj - Fp)/(Fj - Fj)) and therefore as a function of time, $\Delta V_{jp} = (V_{jp} \text{treatment} - V_{jp} \text{control})$ respectively, from the fluorescence values of their respective treatments (Figure 2b and 2d). The $\Delta V$ plots revealed bands hidden in the J and I steps of the fluorescence kinetics which are much richer in information than the original O-J-I-P transients. From these plots, valuable information was obtained regarding the functionality of the OEC (oxygen evolving system), accumulation of electron carriers such as Qr− and reduction of end electron acceptors of the photosynthetic ET chain (Strasser et al. 2004).

To fully interpret the effect of O3 on the kinetics of the I-P fluorescence transients of the test plants, additional normalisations were done to present the relative amplitude of the I-P phase (Figure 2c and 2e).

See Strasser et al. (2004) for a list of the formulae and a glossary of terms used by the JIP-test for the analysis of the OJIP chlorophyll a fluorescence transient.

Crop yield

The pods were harvested as soon as they reached maturity. The pods were separated from the plants and dried for 24h at 60 °C or until constant mass. Yield was determined in terms of total pods per plant, number of seeds per pod, total seed per plant and total gram of seed per plant.

Statistical analysis

Statistical analysis was implemented using the ‘Statistica’ software package for Windows version 6 (StatSoft, Inc., USA). In data sets with parametric distribution, significant differences between treatments were determined using the Students’ t-test. In the strict sense the 8 replicate pots per treatment and per genotype used, are pseudo-replicates. Given the size of the OTCs (5 m3) used and the excellent treatment and per genotype used, are pseudo-replicates. The pods were harvested as soon as they reached maturity. The pods were separated from the plants and dried for 24h at 60 °C or until constant mass. Yield was determined in terms of total pods per plant, number of seeds per pod, total seed per plant and total gram of seed per plant.
Results and Discussion

Plant development and foliar injury

After 35 days of O₃ fumigation a marked decrease in growth was evident in the S156 Phaseolus vulgaris genotype (treatment O₃S) when compared to the carbon-filtered test plants (treatment FS) (Figure 1). In contrast, little visual damage and inhibition of growth were evident compared to its control (O₃R).

Symptoms characteristic of O₃ stress were visible on the S156 genotype’s (O₃S) leaves 12 days after fumigation at 80 nmol.mol⁻¹ O₃, a major treatment effect relative to O₃ during the reproductive phase than the vegetative phase. The decrease in pod yield in S156 (O₃S) relative to the control, was comparable to the decrease reported by Burkey et al. (2005) comparing yield of S156 grown under ambient air containing a seasonal mean of about 50 nmol.mol⁻¹ O₃ to those grown under carbon-filtered air. Flowers et al. (2007) exposing S156 to 60 nmol.mol⁻¹ O₃ in OTCs reported a 77% decrease in seed yield.

Crop yield

Crop yield attributes were assessed as dry weight at the end of the experiment. Various yield components of the sensitive (S156) and resistant (R123) genotypes exposed to elevated O₃ concentrations of 80 nmol.mol⁻¹ for 40 days, which included the reproductive growth phase, were markedly suppressed when compared to their carbon-filtered controls (Table I). The S156 plants displayed a severe reduction in most of the attributes. The total pods per plant, number of seeds per pod, total seeds per plant and total gram of seed per plant were reduced by 34%, 21%, 51% and 55% respectively. All mentioned differences were statistically significant, with p-values of less than 0.001. The moderate decrease of 21% in seeds per pod in S156, indicated that seed initiation was not drastically affected. Although O₃R was less affected in all yield parameters measured, total pods per plant decreased by 16%. The number of seeds per pod, total seeds per plant and total gram of seed per plant, decreased by 21%, 34% and 31%, respectively. It was pointed out that beans (Tingey et al. 2002) and soybean (Morgan et al. 2003) are more vulnerable to O₃ during the reproductive phase than the vegetative phase. The decrease in pod yield in S156 (O₃S) relative to the control, was comparable to the decrease reported by Burkey et al. (2005) comparing yield of S156 grown under ambient air containing a seasonal mean of about 50 nmol.mol⁻¹ O₃ to those grown under carbon-filtered air. Flowers et al. (2007) exposing S156 to 60 nmol.mol⁻¹ O₃ in OTCs reported a 77% decrease in seed yield.

Physiological response

Chlorophyll a fluorescence, difference kinetics and JIP-test

Average chlorophyll a fluorescence transients of dark adapted leaves of the test plants are presented on logarithmic time scale in Figure 2a-c. These transients show the typical O-J-I-P fluorescence rise, starting from an initial level of F₀, up to a maximum Fp = FM, which can be considered as representing the maximum fluorescence yield, since the intensity of the actinic light source of the fluorimeter (600 W.m⁻², peak at 650 nm) is high enough to ensure the closure of all the reaction centres. Important to note is that Fp and Fe of the chlorophyll a fluorescence transients of the test plants were remarkably similar before fumigation commenced, indicating that the plants were physiologically homogenous and active (Figure 2a). The similar and low F₀ value furthermore indicated that the plants were fully dark adapted, i.e. all the reaction centres were fully open (oxidised). After 25 days of fumigation with 80 nmol.mol⁻¹ O₃, a major treatment effect relative to the control was evident in the OIP transients of the O₃S plants. Figure 2b depicts the average fluorescence transients of the test plants normalised between F₀ (50 µs)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total pods</th>
<th>Seeds per pod</th>
<th>Total seeds per plant</th>
<th>Total gram of seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR</td>
<td>36.12 (1.43)</td>
<td>3.78 (0.10)</td>
<td>136.87 (7.07)</td>
<td>34.37 (2.13)</td>
</tr>
<tr>
<td>O₃R</td>
<td>30.12 (1.12)</td>
<td>2.97 (0.13**)</td>
<td>90.00 (6.04**)</td>
<td>23.42 (1.47**)</td>
</tr>
<tr>
<td>FS</td>
<td>38.25 (2.32)</td>
<td>3.25 (0.06)</td>
<td>130.66 (6.60)</td>
<td>31.65 (1.88)</td>
</tr>
<tr>
<td>O₃S</td>
<td>25.25 (0.97**)</td>
<td>2.55 (0.08**)</td>
<td>64.50 (3.20**)</td>
<td>14.19 (0.93**)</td>
</tr>
</tbody>
</table>

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Table I: Mean yield parameters (± standard errors) for different treatments measured after seed maturity. Parameters include, total pods per plant, number of seeds per pod, total seed per plant, and total gram of seed per plant, with * and ** indicating significant differences at p < 0.05 and p < 0.01 respectively, compared to control plants.
and $F_i$ (2 ms) displaying the events of the O-J and J-P phases of the fluorescence rise respectively. The transition from O to J represents the single turnover range of the transient (i.e. $Q_A$ is only reduced once), reflecting mainly photochemical reactions leading to the reduction of the electron acceptor $Q_A$, while the transition J to P reflects the multiple turn-over phase which is strongly affected by the subsequent dark reactions in the ET chain. Figure 2b suggests that the major O$_3$-induced effect occurred in the multiple turn-over events of PSII function of the S156 (O$_3$S) plants, i.e. in the transition J (~2 ms) to P (~330 ms) (Figure 2b).

Further analysis of the fluorescence transients comprised normalisation of the average fast phase chlorophyll a fluorescence transients of the different treatments between the steps O (50 μs) and J (2 ms) as $V_{OJ} = (F_t - F_o)/ (F_J - F_o)$, as well as between steps J (2 ms) and $F_M$ (peak), as $V_{JP} = (F_t - F_j)/(F_P - F_J)$. The normalised fluorescence transients of the control (FR) were then subtracted from the normalised fluorescence transients of the different treatments to obtain difference kinetics ($\Delta V = V_{treatment} - V_{control}$) respectively. A gain factor of 6 was used to visualise these differences. The two difference kinetics were then plotted as $\Delta V_{OJ}$ and $\Delta V_{JP}$ respectively ($\Delta V_{OJ}$ left and $\Delta V_{JP}$ right in Figure 2d). The positive $\Delta V_{OJ}$ band (at about 300 μs) appearing in the O$_3$S plants is the consequence of an increase in fluorescence (Figure 2d, left), probably due to the short-lived accumulation of reduced electron carriers such as pheophytin (Pheo$^-$), which, in turn, is caused by the dissociation of the OEC (oxygen evolving complex),

![Figure 2: Fast phase chlorophyll a fluorescence kinetics of intact leaves of the different treatments and genotypes after 25 days exposure to 80 mol.mol$^{-1}$ O$_3$](image-url)
resulting in an imbalance between the electron flow from the OEC to the RC and towards the acceptor side of PSII. Uncoupling of the OEC enables an alternative internal electron donor such as ascorbate or proline (instead of H$_2$O) to donate electrons to PSII (Strasser 1974; De Ronde et al. 2004; Toth et al. 2007; Nagy et al. 2012). Such a condition causes a short-lived increase in the reduced Pheo$^{-}$ concentration, creating a $\Delta V_{\alpha}$-band appearing between 100 and 300 ms. The $\Delta V_{\alpha}$-band can also occur due to an increase of the functional PSII antenna size (Strasser et al. 2004). On the other hand the O$_3$R plants displayed a negative $\Delta V_{\alpha}$-band indicating that in this case the OEC function was not negatively affected relative to the control during vegetative growth, confirming its superb resistance.

A positive $\Delta V_{\alpha}$-band also became visible between 2 ms and F$_{\alpha}$ (Figure 2d, right) in the O$_3$S plants after 25 days of exposure to O$_3$. The $\Delta V_{\alpha}$-band provides information on the activation state of ferredoxin NADP$^+$ reductase (FNR) and on the possible inhibition of reduction of end electron acceptors such as NADP$^+$ and Fd due to a higher reduced state of the pool mixture of plastoquinone, cytochrome$^{-}$b/f and plastocyanin (Yusuf et al. 2010). Note that again O$_3$S was not effected in this regard.

To further elucidate the effect of O$_3$ on the I-P part of the fluorescence curves, the averaged raw fluorescence curves were normalised between the steps O (50 ms) and I (30 ms) and presented as relative variable fluorescence $V_{\text{os}} = (F_0 - F_I)/ (F_0 - F_O)$ vs logarithmic time (Figure 2c). The O-I part of the transient represents the single turnover photochemical event $Q_a \rightarrow Q_a^-$ as well as the beginning of reduction of the intersystem electron carriers, while the I-P part of the transient represents only the PSI-driven reduction of end-electron acceptors, namely Fd and NADP$^+$ (Luo et al. 2006). The marked effect of O$_3$ on the O-I and I-P phases of the transient of O$_3$S is depicted by Figure 2c. To reveal the hidden information, two different normalisations of the I-P phase of the transients were used and are presented in Figure 2e. The insert represents only the part $V_{\text{os}} \geq 1$ of the normalised curve of Figure 2c, plotted in the 30–1000 ms time range (log scale). For each curve, the maximum amplitude of the fluorescence rise reflects the size of the pool of the end electron acceptors at the PSI acceptor side (Yusuf et al. 2010). It is evident that a 10% decrease in the pool size occurred in the O$_3$S plants, opposed to a 3% increase in the O$_3$R plants (Figure 2e, insert). In the main plot, fluorescence data were normalised between the steps I (30 ms) and F (peak), as $V_{\text{ip}} = (F_0 - F_I)/(F_0 - F_O)$, and plotted on a linear scale in the 30–400 ms range. This normalisation, where the maximal amplitude of the rise was fixed at unity, facilitated a comparison of the reduction rates of the end electron acceptors pool for the different treatments; their half-times are shown by the interception of the curves with the horizontal dashed line drawn at $V_{\text{ip}} = 0.5$ (half-rise). The overall rate constant for reduction of e-end acceptors can be extrapolated by the inverse of the half-time (Yusuf et al. 2010). No significant changes in the relative reduction rate could be seen in the $V_{\text{ip}}$ kinetics of end electron acceptors of the different treatments (Figure 2e). Note that Figure 2e (main plot) can be seen as Michaelis-Menten enzyme kinetics $V$ versus $S$, where the velocity is given by the relative variable fluorescence $V_{\text{ip}}$ and the substrate $S$ as the light dose given as the product of light intensity and illumination time.

The comparison of Figure 2c and 2e shows that the different phases OJ,JI, and IP of the transient behave differently and carry therefore different information.

The fluorescence transients depicted in Figure 2b and 2c, were in addition analysed by the JIP-test (Strasser et al. 2004) to derive 10 structural and functional parameters of PSII function, quantifying the photosynthetic behaviour of the test plants 25 days after fumigation commenced. The values of the parameters are expressed relative to the control (FR) and plotted in a multi-parametric radar plot (Figure 3). In the O$_3$S plants a 13.3% ($p \leq 0.01$) decrease occurred in the number of active reaction centres per absorption (RC/ABS), while a simultaneous 10.8% ($p \leq 0.01$) increase occurred in the apparent antenna size (ABS/RC). The increase in ABS/RC may be due to a compensation strategy to offset the decrease in RC/ABS, resulting in no change in TR$_{ABS} = (F_I/F_o)$ in O$_3$S (Strasser et al. 2004). The decrease in RC/ABS indicates that the size of the PSII units was affected, but in some of them the reaction centres have been deactivated, hence the corresponding units contributed towards light absorption but not photochemistry (increase of apparent antenna size), or that the size of PSII units with active reaction centres increases (Luo et al. 2006). The 8.07% ($p \leq 0.01$) increase in the maximum trapping flux (TR$_{RC}$) of the O$_3$S plants suggests that changes took place both in the fraction of RCs transformed to non-Q$_A$-reducing RCs and in the functional antenna size (Yusuf et al. 2010). The decrease of 9.53% ($p \leq 0.01$) in the electron transport per cross-section (ET$_{CS}$) in the O$_3$S plants can be attributed to a decrease of 9.31% ($p \leq 0.01$) in the density of PSII reaction centres per excited cross-section (RC/CS$_0$). This finding corresponds to the report of Guidi et al. (2010) that down-regulation of PSII activity occurred in beans exposed to acute O$_3$-stress as protective measure against the generation of active oxygen species.

The O$_3$-induced changes relative to the control (FR) in the specific (per RC) and phenomenological (per CS) energy fluxes of the O$_3$S plants were reflected by the 53.5% ($p \leq 0.01$) decrease in the PI$_{rad}$, the latter which provides a measure of the potential of the whole photosynthetic ET chain converting light energy into redox energy (Figure 3). The decreases in the potential of the four partial processes of photosynthesis relative to the control, were as follows: absorption [$\gamma_{RC}/(1-\gamma_{RC}) = \text{RC/ABS}$]: 13.7%, trapping [$\phi_{ip}/(1-\phi_{ip})$]: 14.5%, electron transport [$\psi_{ip}/(1-\psi_{ip})$]: 20.8%, reduction of end electron acceptors [$\delta_{ip}/(1-\delta_{ip})$]: 22.9%. The large decrease in the potential of the reduction of end-electron acceptors in O$_3$S (Figure 3), strongly corroborated the data obtained of the difference kinetics of variable fluorescence
transients displaying the prominent $\Delta V_I$-band (Figure 2d). In contrast to the 53.5% decrease in $P_{\text{Itotal}}$ in O3S, O3R showed an increase of 18.4% ($p \geq 0.01$), which was mainly due to the higher density of RC/CS and the increased number of active RC/ABS, indicating a stimulatory effect (Figure 3). It should be noted that the parameter $\varphi_{P_0} = F_v/F_M$, which is the only fluorescence parameter used in some studies on plant stress effects, was the most insensitive of all parameters. $F_v/F_M$ also proven to be insensitive to drought stress in cotton (Luo et al. 2016).

The changes in structure and function of the photosynthetic apparatus caused by O3 in the S123 (O3S) plants confirmed that considerable injury occurred at the membrane level, specifically regarding the photosystems and redox components of the thylakoids. This could be ascribed to inadequate antioxidant capacity in O3S in contrast to O3R, which proved to be well protected.

**Photosynthetic gas exchange**

It is accepted that the most reasonable standard for comparing and characterising the status of the photosynthetic apparatus on the basis of gas exchange is a CO2 response curve of the intact leaf (Lange et al. 1987). All the measurements shown were done on comparable physiologically active leaves of the different genotypes and treatments after 25 days of exposure. To determine the effect of O3 on the photosynthetic gas exchange, several photosynthetic gas exchange parameters were derived from the $A_{\text{CO}_2}$ response curves (Figure 4) and are shown in Table II. The actual assimilation rate (ambient conditions, $A_{\text{sat}}$), occurs where we find the simultaneous solution of the demand function and the supply function, i.e. the operational point (Figure 4). The $A_{\text{sat}}$ value of the O3S test plants decreased by 63% ($p < 0.01$), while the corresponding small decrease of 3% ($p \geq 0.01$) measured in $A_{\text{sat}}$ of the O3R plants showed that O3 had almost no effect on $A_{\text{sat}}$ in the R123 genotype at ambient CO2 level, corroborating the fluorescence data (Plast). Note that $A_{\text{sat}}$ in FS was 16% higher compared to FR, although this phenomenon was not supported by the $P_{\text{Itotal}}$ value (Figure 3). This finding is in accordance with the data of Flowers et al. (2007) showing that S156 has a higher inherent capacity in clean air as either R123 or R331, but was unable to translate the extra capacity into seed yield. The drastic inhibition of $A_{\text{sat}}$ (CO2 assimilation capacity) in the O3S plants could be ascribed to the decrease in the CO2 saturated rate of photosynthesis ($J_{\text{max}}$), which indicated that O3 had an inhibitory effect on the regeneration capacity of RuBP. $J_{\text{max}}$ of the sensitive O3S plants in fact decreased by 61%. This finding concurs with the explanation of Von Caemmerer and Farquhar (1984), that ‘RuBP-regeneration capacity’ is more sensitive to stress than Rubisco activity as RuBP regeneration involves the whole biochemistry necessary for photosynthetic carbon assimilation except for one enzyme namely Rubisco. On

![Figure 3: Fractional change in selected functional and structural parameters of PSII relative to FR (closed triangles and broken line of regular polygon). O3R = open circles, FS = closed circles, O3S = open triangles.](http://www.satnt.ac.za)
The carboxylation efficiency (CE; initial slope of the A:Ci response curve at Ci ≤ 200 μmol.mol⁻¹) of the O₃S plants decreased by a significant 75% (Table II). The CE expresses the rate of CO₂ assimilation in terms of the effective Ci and the capacity of the system to assimilate CO₂. Farquhar et al. (1980) showed that at low CE, CO₂ assimilation follows the Michaelis-Menten kinetics and is determined by the RuBP saturated rate of the enzyme: the lower the carboxylation capacity, the less steep the slope of the demand function. The FR and O₃R test plants displayed exactly the same CE value (Figure 4 and Table 2), indicating that O₃ had no inhibitory effect on the carboxylation efficiency of the resistant plants exposed to elevated 80 nmol.mol⁻¹, corroborating the findings of Izuta et al. (1996) working with beech seedlings, that elevated ambient O₃ inhibits both CE and Jᵢ₃₆₀. An ozone-induced decline in Rubisco activity in sensitive plants was also reported by Pell et al. (1997).

The 14% increase in Cᵥ₃₆₀ of the O₃S test plants, opposed to the 9% decrease in O₃R, served as further confirmation that the large decrease in Aᵥ₃₆₀ in O₃S was due to mesophyll limitation rather than stomatal limitation. This fact is corroborated by the 90% increase in Γ (representing the CO₂ compensation concentration) and large decrease (77%) in CE in O₃S plants compared to no decrease in O₃R. The supply function (gₗₒ₂) in O₃S decreased by 38% (p < 0.05). The supply function expresses the rate of CO₂ assimilation in terms of the difference in concentration between Ci and Cᵥ₃₆₀ (the driving force for inward movement of CO₂) and the prevailing stomatal conductance (gₗₒ₂), however, according to Farquhar and Sharkey (1982) reduced stomatal conductance is rarely the main cause of reduced assimilation rates. Accordingly the moderate 21% increase in Γ calculated for O₃S, confirmed that although stomatal limitation played a role, the reduction in A was mainly due to mesophyll limitation. The O₃R plants in contrast displayed a small increase of 9% in Γ. A corresponding decrease of 51% occurred in the water use efficiency (WUE) of O₃S compared to the moderate 7% decrease in O₃R. This decrease in WUE occurred in spite of a reduction in transpiration rate (E). VanLoocke et al. (2012), exposing soybean to different O₃ concentrations in the range 40 to 120 nmol.mol⁻¹ in the Soybean Free Air Concentration Enrichment (SoyFACE)

**Table 2:** Mean values (± standard errors) of photosynthetic gas exchange parameters of leaves of R123 and S156 genotypes (n = 4 plants per treatment) 25 days after commencement of exposure to filtered air (F) and 80 nmol.mol⁻¹ ozone (O₃) respectively. Symbols: Aᵥ₃₆₀ = rate of CO₂ assimilation at Ci = 360 μmol.mol⁻¹; Aᵥ₃₆₀ = intercellular CO₂ concentration at Ci = 360 μmol.mol⁻¹; Aᵢ₃₆₀ = rate of CO₂ assimilation at Ci = 360 μmol.mol⁻¹; CE = carboxylation efficiency; Jᵢ₃₆₀ = minimum rate of CO₂ assimilation; Γ = CO₂ compensation concentration; Λ = percentage stomatal limitation of photosynthesis; WUE = water use efficiency. Asterisks * and ** indicate significant differences at p < 0.05 and p < 0.01, respectively, compared to the control plants (FR).

<table>
<thead>
<tr>
<th></th>
<th>FR</th>
<th>O₃R</th>
<th>FS</th>
<th>O₃R</th>
</tr>
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<tbody>
<tr>
<td>E (μmol·m⁻²·s⁻¹)</td>
<td>5.3 (1.12)</td>
<td>4.66 (0.48)</td>
<td>4.90 (0.86)</td>
<td>3.70 (0.63)</td>
</tr>
<tr>
<td>Aᵥ₃₆₀ (μmol·m⁻²·s⁻¹)</td>
<td>11.45 (1.2)</td>
<td>11.12 (1.12)</td>
<td>13.76 (1.38)</td>
<td>4.92 (1.27)**</td>
</tr>
<tr>
<td>Cᵢ₃₆₀ (μmol·m⁻¹)</td>
<td>295.75 (19.34)</td>
<td>270.0 (18.24)</td>
<td>256.16 (18.48)</td>
<td>292.2 (26.61)</td>
</tr>
<tr>
<td>Aᵢ₃₆₀ (μmol·m⁻²·s⁻¹)</td>
<td>14.87 (1.24)</td>
<td>14.98 (0.76)</td>
<td>16.07 (0.94)</td>
<td>5.97 (0.85)**</td>
</tr>
<tr>
<td>gₗₒ₂ (μmol·m⁻²·s⁻¹)</td>
<td>259 (80.97)</td>
<td>203.25 (25.96)</td>
<td>274.66 (14.71)</td>
<td>165.2 (50.45*)</td>
</tr>
<tr>
<td>CE (μmol·m⁻²·s⁻¹)</td>
<td>0.056 (0.002)</td>
<td>0.056 (0.002)</td>
<td>0.061 (0.003)</td>
<td>0.014 (0.003)**</td>
</tr>
<tr>
<td>Jᵢ₃₆₀ (μmol·m⁻²·s⁻¹)</td>
<td>20.25 (0.56)</td>
<td>20.17 (0.31)</td>
<td>20.95 (1.27)</td>
<td>7.87 (1.51)**</td>
</tr>
<tr>
<td>Γ (μmol·m⁻¹)</td>
<td>98 (4.57)</td>
<td>101.6 (5.64)</td>
<td>76.4 (2.78)</td>
<td>145.8 (6.35***)</td>
</tr>
<tr>
<td>Λ (%)</td>
<td>22.99</td>
<td>25.23</td>
<td>14.37</td>
<td>17.73</td>
</tr>
<tr>
<td>WUE (μmol·mmol⁻¹)</td>
<td>2.97 (0.23)</td>
<td>2.75 (0.24)</td>
<td>2.99 (0.13)</td>
<td>1.46 (0.55*)</td>
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facility, reported that evapotranspiration decreased linearly with [O₃] and that WUE decreased by about 50% which was attributed to O₃-induced limitation of the ability to take up water. Similarly Clebsch et al. (2009) reported a significant reduction in WUE of Phaseolus vulgaris Pinto exposed to O₃ in OTCs at concentrations comparable to those used in our study. They attributed the decrease to a larger decrease in A relative to a moderate decrease in E.

**Chlorophyll content index (CCI)**

After 30 days of exposure to 80 nmol.mol⁻¹ O₃ the O₃S plants showed a 21.8% decrease in the CCI when compared to the FS plants and a 24% decrease compared to FR (Figure 5), reflecting the 10% decrease that occurred in the density of RCs (Figure 3). On the contrary the O₃R plants showed a 19% increase in the CCI supporting the 18.4% increase in PIₜₚₑₚ relative to FR measured in these plants after 25 days exposure (Figure 3). A correlation existed between PIₜₚₑₚ and the CCI, both indexes displaying an O₃-induced decrease in O₃S and a moderate increase in O₃R relative to the control. The positive correlation between chlorophyll content and quantum efficiency of photosynthesis is a phenomenon often reported (Nyachiro et al. 2001). The 19% increase found in CCI in O₃R plants, corresponded to the O₃-induced increase found in the chlorophyll content of winter wheat in an OTC experiment by Zheng et al. (2005). According to Guzy and Heath (1993) ozone tolerant bean varieties appear to resist chlorophyll loss through mitigation by antioxidants such as ascorbate and non protein sulphydryl. In our investigation the increase in CCI of the O₃R plants was however not reflected in the seed yield. It however must be borne in mind that the values of the yield parameters are the result of the full 40 days of O₃ exposure.

**Summary and Conclusions**

Exposing the test plants to 80 nmol.mol⁻¹ ozone for the full growth cycle up to seed yield showed that the stress imposed was within the physiological capacity of the test plants. This was also supported by the minimal visual effect after 25 days and moderate decrease in yield after 40 days of exposure, respectively, displayed by the resistant (R123) genotype.

Our approach of parallel measurement of chlorophyll a fluorescence and photosynthetic gas exchange allowed correlation of complementary information of indirect and direct signals on the photosynthetic response of the test plants. Our data convincingly demonstrated that the photochemical processes of the S156 test plants were not stable against O₃. It is known that O₃-induced altered plasma membrane protein function can serve as initial signals of O₃-responses (Castillo and Heath 1990). It is assumed that thylakoid membranes containing the multi-molecular peptide complexes comprising the photosynthetic electron-transport chain of susceptible plants would be equally subjected to early O₃ damage. Our data suggests that R123 may be protected by features of their antioxidant metabolism that scavenge O₃-derived ROS (Fiscus et al. 2005). Through analysis of direct chlorophyll fluorescence transients by the JIP test, we could strongly demonstrate that all four domains of the photosynthetic electron transport, namely light absorption (ABS) the PSI electron donor side, electron transport between PSII and PSI, and the PSI electron acceptor side, generating redox energy (reducing equivalents) driving the Calvin-Benson cycle and secondary metabolism, was detrimentally affected in S156. By utilisation of the whole fluorescence transient, data were processed indicating that in S156, O₃ exposure caused dissociation of the OEC resulting in an imbalance between the electrons flowing from the OEC to the RC and towards the acceptor side of PSII (positive ∆Fᵥ, band). In addition a strong, positive ∆Fᵥ band appeared in the difference fluorescence kinetics, pointing at a decreased activation state of NADP⁺ reductase and the possible decrease in the reduction of end-electron acceptors (Fd and NADP⁺).

In depth analysis of the I-P phases of the fluorescence transients revealed that in S156 a ~10 % decrease relative to the control (and relative to R123) occurred in the pool size of end-electron acceptors. The inhibitory effect of O₃ on photosynthetic electron transport of S156 was also accompanied by a 24% decrease in the chlorophyll content index (CCI) measured after 30 days of exposure. R123 proved its resistance to O₃ by not displaying the mentioned effects.

Given the profound inhibitory effects on the functioning of the photosynthetic ET chain in S156 shown by our data and taking into account that the Calvin-Benson cycle is driven by the reducing equivalents generated by these reactions, we concluded that both the light and dark processes and the interplay between them are attacked by O₃. The large (63 %)
decrease of $J_{max}$ in S156, corresponding to the maximum rate of RuBP regeneration and assumed to equal the maximum rate of coupled photosynthetic electron transport (Long and Hallgren 1987), strongly pointed at O$_2$-induced disruption of the interaction between the Calvin-Benson cycle in the stroma and the ET chain in the thylakoids. What’s more, the sensitivity of RuBP-regeneration to O$_2$ in S156 is an indication that several enzymes of the Calvin-Benson cycle are affected. The large decrease in CE showed that Rubisco was inhibited, either by de-activation, decreased synthesis or degradation.

Although the PI$_{final}$ is an index of photosynthetic potential based on light absorption and measured in the dark-adapted state, it was found to correlate very well with physiological parameters during the vegetative growth stage. Our study demonstrated an excellent correlation between PI$_{final}$ and CO$_2$ assimilation parameters ($A$, $J_{max}$, CE) measured in the treated plants the following day. Analysing direct fluorescence transients by the JIP test provided a wealth of information on the functionality of the OEC (oxygen evolving system), accumulation of electron carriers such as Q$_A^*$ and reduction of end electron acceptors of the photosynthetic electron transport chain.

Our data confirm the remarkable O$_2$-resistance of Ri23 during the vegetative growth stage. The extreme sensitivity displayed by S156 supports its suitability as bio-indicator species as alternative to the clover system as was suggested by Burkey et al. 2005.

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Authors’ contribution
CCWS conducted the experiments and wrote a concept article. GHJK designed the experiments and protocol and wrote the final article. RJS contributed to the presentation and interpretation of the chlorophyll $a$ fluorescence data. JMB contributed to the finalising of the figures.

References
Luo, H.H., Tsimilli-Michael, M., Zhang, Y.L., Zhang W-F. 2016. Combining gas exchange and chlorophyll a fluorescence measurements to analyze the photosynthetic activity of drip-irrigated cotton under different soil water deficits. Journal of Integrative Agriculture, 15(6), 1256–1266.