Chapter 6: General Discussion and Conclusion

6.1. Treatment of plants and the OTC environment

During the last few decades it has become evident that air pollution in the Highveld are dominated by O$_3$ and SO$_2$. The critical SO$_2$ level for agricultural crops, as determined by UNECE, is 11.4 ppb per winter mean (Bell & Marshall, 2000). In Europe critical levels for gaseous pollutants are used to establish binding protocols for emission reductions on effect-base criteria (Fuhrer & Bungener, 1999). This motivated the effect-orientated pollution research directed towards the mechanisms of SO$_2$ action and the possible range of plant responses to elevated SO$_2$ levels in a South African climate. In our investigation we used open top chambers (OTCs) as method of pollutant application. Closed chambers have many disadvantages, one of them being the high humidity, which causes condensation in the chamber which will affect the deposition of SO$_2$ to the plant. The experimental tool that has been regarded as acceptable for dose-response research, is open-top chambers (Heyneke et al., 2012a). Under favourable CO$_2$ gas exchange and water regime, the main pathway for the uptake of SO$_2$ is through the stomata (Garsed, 1985). The administered dose will however not necessarily represent the true pollutant concentration within the substomatal space which will lead to pollutant related vegetation damage. Plants thus respond to the absorbed pollutant dose (also called pollutant absorbed dose, PAD). The rate at which a pollutant is absorbed by plant surfaces is determined by three transport components, namely (i) the atmospheric transport by turbulent diffusion (atmospheric resistance = $r_{am}$), (ii) molecular diffusion across the leaf boundary layer (boundary layer resistance = $r_{bg}$) and (iii) the diffusion through the stomatal pore i.e. stomatal uptake (resistance imposed by stomatal behaviour = $r_{st}$) (Figure 6.1). Under OTC conditions the $r_{am}$ and $r_{bg}$ is virtually zero due to air movement and elimination of the leaf boundary layer (Fuhrer & Bungener, 1999) and consequently the PAD will thus largely be regulated by the stomatal uptake ($r_{st}$). The OTC experiments was conducted at an air speed of about 2 m.s$^{-1}$ through the chamber which is sufficient to provide low enough values of boundary layer resistance in order to maintain realistic conditions of
exchange of gas molecules between the leaves and the air (Bell & Ashmore, 1986; Bell, 2002). This feature emphasises the uniqueness and effectiveness of OTCs in dose-response studies.

Figure 6.1: Diagram showing the main elements controlling SO₂ flux into plants, namely: $r_{am}$ (atmospheric resistance due to turbulent diffusion), $r_{bg}$ (boundary layer resistance) and $r_{st}$ (resistance due to stomatal behaviour).

### 6.2. Visual effects of SO₂ on crops

In both *Brassica napus* (C₃) and *Zea mays* (C₄), detrimental effects on the leaves, shoot biomass, yield and produce quality occurred in response to elevated SO₂ concentrations. The decrease in chlorophyll content (CCI) was associated with the development of injury symptoms in leaves also found by Hamid & Jawaid (2009) studying the effects of short-term exposure of SO₂ and NO₂ on biochemical parameters of soybeans. Injury symptoms include the occurrence of interveinal lesions on leaf laminas and reductions in crop yield. These
reductions are the cumulative effects of internal disturbances of the biochemistry (Sultana et al., 2000). Damage in the form of necrotic lesions became visible in all SO₂ treatments at 23 days after onset of fumigation with SO₂ enriched air of the C₃ crop, Brassica napus plants. In the C₄ crop, Zea mays, visual symptoms became apparent as chlorotic spots on older leaves after 25 days’ exposure to concentrations of 100 ppb SO₂ and higher (for WW and DS treatments). Visual damage only occurred long after physiological changes became evident. After 30 days’ fumigation, Zea mays plants, exposed to 50 ppb SO₂, started to show very few chlorotic lesions while those exposed to higher SO₂ concentrations (100 and 200 ppb) developed progressively more chlorotic spots, fusing with one another to form chlorotic lesions and in some cases necrotic regions.

6.3. Chlorophyll content index

A decrease in the CCI of the test plants of the C₃ crop, Brassica napus, only occurred in the 50DS treatment, namely a 8.14% decreases compared to the control (CFDS). The CCI of all other WW and DS treatments, however increased relative to their controls (CFDS and CFWW, respectively). These results were difficult to interpret, but the possible explanations were scrutinised and are may be explained as follows: The CCI is a direct indication of the concentration of chlorophyll molecules present in a leaf. Older leaves generally contain more chlorophyll molecules per leaf area than younger leaves (Waldhoff et al., 2002) as was reflected by the increase in CCI. An alternative explanation for the higher CCI in DS treatments could possibly be due to shrinking of the leaves (Linke, 2012). During drought conditions a loss of turgor can be expected in the leaf tissue cells which give rise to more densely packed mesophyll tissue per leaf area. More chlorophyll molecules per cross section are thus being accounted for resulting in a higher measured CCI than would be expected.

After 7 weeks’ fumigation with SO₂, both WW and DS Zea mays test plants showed a decline in CCI relative to their controls (CFWW and CFDS, respectively), with the exception of the 100WW treatment, which increased only slightly. The greatest decrease in CCI was detected in the 200WW treatment, namely a 52.42% decrease relative to the control (CFWW) and a decrease of 14.01% in the 200DS treatment relative to the control (CFDS).
Singh & Khan (2006) reported a 28% decrease in CCI after exposing Blackgram to 200 ppb SO₂ for 3 hours on alternative days for 6 weeks. The decrease in CCI can be attributed to SO₂-related deformation of the structure of the chloroplasts. The structure of the lamellae becomes less distinct and disordered and other membranous structures such as those of the mitochondria can also be damaged which may cause cells and their organelle structures to collapse (Liu et al., 2009).

### 6.4. Impact on photosynthesis

Plants respond to intensities of environmental pollution according to their capacity to tolerate various pollutant concentrations. Our data, using a C₃ and a C₄ crop plant under well watered (WW) and drought stressed (DS) conditions, suggest that though plants did not suffer fatal injuries at all SO₂ concentrations used, their physiology and growth were disrupted. In many studies the first symptoms of injury after exposure to air pollutants occur as foliar necrotic lesions (Al-Rawahy et al., 2007). In the current study the first indications of damage to the photosynthetic apparatus of *Brassica napus* (C₃) and *Zea mays* (C₄) crop plants were reflected as inhibition of leaf gas exchange and the constraints on structure and function of PSII. Inhibition of the photosynthetic capacity occurred as early as one week after onset of fumigation before any visible injury symptoms became apparent. The fluorescence data corroborated the gas exchange data extremely well and provided valuable information for conclusive interpretation of the photosynthetic response of both species to SO₂. The specific site(s) of inhibition in the photosynthetic apparatus seems to be different for the WW and DS treatments in both C₃ and C₄ plants, however, in both treatments inhibition seemed to appear mainly as a result of mesophyll (biochemical) limitation. Even though mesophyll limitation played a more pronounced role in the decline in photosynthesis, stomatal limitation also contributed to the decline in photosynthesis. The possible sites of limitation on photosynthesis are discussed below (Figure 6.2 for C₃ crops and Figure 6.3 for C₄ crops):
6.4.1. Brassica napus (C₃)

Results with regard to photosynthetic gas exchange obtained by analysing the A:Ci response curves, indicated that a larger SO₂-induced reduction in the CO₂ assimilation rate (A₃₇₀) occurred in the WW treatment than in the DS treatment. This phenomenon was reflected by the larger reduction in stomatal conductance (gₛ), Rubisco activity (CE) and RuBP regeneration capacity (Jₘₐₓ) in WW test plants. This may be due to drought stress that might have ameliorated the SO₂-related constraints on the photosynthetic capacity of the test plants due to the decrease in stomatal conductance. This finding proved the second hypothesis set at the outset, of a possible ameliorating effect of SO₂ damage by drought stress to be true.

Figure 6.2: The different sites of inhibition occurring in the chloroplast during photosynthetic carbon reduction cycle in C₃ plants. The numbers in red indicate the main sites of inhibition of photosynthesis due to SO₂, i.e. stomatal limitation (1), decrease in Rubisco activity (2), reduction in regeneration capacity of the acceptor molecule RuBP (3), inhibition due to the uncoupling of the OEC (4), impairment of the flow of electrons in the intersystem electron transport chain, i.e. inhibition of electron transport (5) and inhibition reduction of the end electron acceptors (6) (Smit et al., 2007).
A marked decrease in the slope of the supply function ($g_{CO2}$) of the A:Ci response curve (Figure 4.4) with increasing SO$_2$ concentrations was noticeable in the WW treatments, pointing towards stomatal limitation. This observation was corroborated by the highly significant decrease (53.62%) in stomatal conductance at elevated SO$_2$ concentrations. The decline in stomatal conductance also caused inhibition of the electron transport inter-system. Except for the 10.21% decrease in stomatal conductance in the 100DS treatment, all other drought stress treatments exposed to elevated SO$_2$ concentrations (50DS and 200DS) showed increases in stomatal conductance relative to their control plants (CFDS). Stomatal closure is considered as the earliest response to water deficit, decreasing the CO$_2$ availability at the carboxylation site and consequently the leaf CO$_2$-uptake (Chaves, 1991). It is proposed that after an early partial closure of the stomata, metabolic limitation occurs. The increase in stomatal conductance for the 50DS and 200DS treatments could be due to impairment of the functioning of the stomata. The reason for the impairment may be SO$_2$ exposure and consequential damage to the guard cells and surrounding epidermal cells. Changes in the cell structures due to elevated SO$_2$ concentrations are evident in the micrographs (Figures 4.5 a and b). According to Lawlor (1993) water deficit and loss of turgor override the CO$_2$ response of stomata and that abisic acid may be produced in the chloroplasts of water stressed leaves when assimilation is much reduced. Inhibition at large stomatal conductance when drought is mild is often due to impaired ATP synthesis and thus ATP-limited regeneration of RuBP. Further reduction of stomatal conductance with increasing drought leads to reduced photochemical activity (Flexas & Medrano, 2001). A reduction in Rubisco activity in response to elevated levels of SO$_2$ was reflected as a decrease in the apparent carboxylation efficiency (CE). The greatest decrease in CE in both WW and DS treatments occurred at a fumigation level of 100 ppb SO$_2$ (56.99 and 15.69%, respectively). Decreased Rubisco activity was commonly reported to occur in C$_3$ plants exposed to severe drought stress conditions (Flexas & Medrano, 2001). During fumigation, SO$_2$ molecules are converted into the anions, SO$_3^{2-}$, HSO$_3^-$ and SO$_4^{2-}$. These anions may result in various damages to the chloroplast and play a role in the decline in CO$_2$ fixation (Ghisi et al., 1990). In a study done by N’soukpoé-kossi et al. (1994) on the sites of SO$_3^{2-}$ and HSO$_3^-$ action on the photosynthetic apparatus of sugar maple leaves, it was found that the Calvin-Benson cycle is a valid candidate for the primary site of action of bisulphite.
This anion was found to be the most toxic among the sulphur anions during SO$_2$ metabolism in plants. This toxic anion may partially be responsible for the inhibition of the reaction catalysed by Rubisco. Inhibition in the regeneration capacity of RuBP and the maximum electron transport rate, shown as a decrease in $J_{\text{max}}$, contributed to the decreased photosynthetic efficiency in WW plants exposed to SO$_2$ concentrations of 100 ppb and higher. It was evident from our experimental data that the 100WW treatment was affected more by SO$_2$ than the 200WW treatment. This phenomenon could be attributed to (i) the annuli, dispersing the SO$_2$-air mixture in the OTCs may not have effectively and homogenically disperse the SO$_2$ throughout the chamber in the advanced stages of the experiment when the chamber volume was quite filled with the test plants, especially in the case of *Zea mays* growing tall; (ii) the fact that the 100WW test plants had a severe aphid infestation and had to be treated with malathion. This could have affected the biochemistry of the plants and acted as an additional stress. Haile et al. (1999) studied the physiological changes in *Triticum aestivum* plants induced by Russian wheat aphid injury. They similarly recorded a reduction/change in chlorophyll fluorescence parameters and photosynthetic rates in plants infested with aphids.

Fumigation of *Brassica napus* test plants with elevated SO$_2$ concentrations for three consecutive weeks yielded characteristic OJIP fluorescence transients. Induced photochemical inhibition at various sites in the electron transport chain could be identified by analysing the OJIP fluorescence rise. It is important to note that the inhibition of the photosynthetic electron transport chain of the test plants subjected to drought stress (DS) occurred in a different fashion than in the WW treatment. The details are discussed below.

Normalisation of the raw average fast phase fluorescence transients between $F_0$ and $F_1$ revealed that SO$_2$-induced changes occurred in the multiple turnover phases of WW treatments, opposed to the single- and multiple turnover phases of DS treatments. Increased SO$_2$ concentrations thus elicited inhibition within these phases of the primary photosynthetic events. Another normalisation of the raw average fast phase fluorescence transients ($\Delta V_{OJ}$) revealed a prominent $\Delta V_K$ band in the region of 30 ms in 100DS and 200DS treatments pointing at inhibition on the donor side of PSII (OEC). No prominent bands occured in WW
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treatments, indicating that the inhibition that occurred was not due to inhibition in the single turnover phase. $Q_A \rightarrow Q_A^- \rightarrow Q_A$. $\Delta V_I$-bands appeared within the range of 300 ms in 100WW and 200WW test plants, while very prominent $\Delta V_I$-bands appeared in addition in the 100DS and 200 DS treatments. This phenomenon correlated with the concomitant changes in the specific and phenomenological (per CS) energy fluxes and the performance index ($\text{PI}_{\text{ABS.total}}$), showing that the decrease that occurred in the photosynthetic performance ($\text{PI}_{\text{ABS.total}}$) in SO$_2$ treatments in both WW and DS treatments, was mainly due to inhibition of the reduction of end electron acceptors, indicated as a reduction in $\text{PI}_{\text{RE}}$ (Figure 4.9). Prolonged exposure to increased SO$_2$ concentrations lead to impairment of mostly PSI efficiency in WW test plants, and an impairment of PSI and PSII in DS treated plants. The $\text{PI}_{\text{ABS.total}}$ is a performance index used to evaluate plant vitality. A stimulatory effect was noticed in 50WW in contrast to reductions occurring at higher SO$_2$ concentrations. The latter reductions pointed at a decrease in photochemical efficiency of the photosystems. Looking at the partial processes of $\text{PI}_{\text{ABS.total}}$ of the 100WW and 200WW treatments, revealed that reduction in the photosynthetic capacity may be caused by reduction in (i) electron transport per cross section ($\text{ET/CS}$), (ii) density of active reaction centers per cross section ($\text{RC/CS}$) and (iii) reduction of end electron acceptors, namely Fd and NADP$^+$ ($\text{PI}_{\text{RE}}$). It is evident that the DS treatments are more susceptible to SO$_2$ damage, seen as a strong SO$_2$-dependent decline in $\text{PI}_{\text{ABS.total}}$ (Figure 4.9 b). The greatest decline occurred in the 200DS treatment (61%). Inhibition occurred due to (i) reduction of the number of active reaction centers per leaf cross section area ($\text{RC/CS}$), (ii) reduction in the rate of electron transport per cross section, i.e. leaf area ($\text{ET/CS}$), (iii) reduction of the number of active reaction centers per total photon absorption ($\text{PI}_{\text{RC}}$), (iv) a decline in the efficiency by which reaction centers can trap an exciton ($\text{PI}_{\text{TR}}$), (v) a reduction in the efficiency to transport electrons within the electron transport chain ($\text{PI}_{\text{ET}}$) and (vi) an inhibition in the reduction of end electron acceptors. The greatest contributor to the reduction in $\text{PI}_{\text{ABS.total}}$ however was the decreased ability to reduce the end electron acceptors Fd and NADP$^+$ (21% less than the CFDS control plants) beyond PSI ($\text{PI}_{\text{RE}}$). This phenomenon corresponded to the occurrence of prominent $\Delta V_I$-bands in the $\Delta V_{IP}$ graphs (Figure 4.11). An increase in the maximum trapping flux (trapping per reaction center) and antenna size occurred in DS treatments, opposed to the results obtained with the WW treatments. The increase in trapping flux may be a reaction of
the plant to compensate for the decrease in the quantum efficiency (PI\textsubscript{TR}, i.e. TR/ABS). Yusuf \textit{et al.} (2010) investigated the overexpression of γ-tocopherol methyl transferase gene in transgenic \textit{B. juncea} during abiotic stress conditions induced by salt stress, heavy metal stress and osmotic stress. They found that an increase in abiotic stress resulted in an inhibition in the rate at which end electron acceptors was being reduced, and that a reduction in the pool size of end electron acceptors occurred. In the present study, further analyses of the I-P phase of the fluorescence transients of WW and DS treatments showed similar results indicating that not only the size of the pool of end electron acceptors (Feredoxin and NADP\textsuperscript{+}) at the acceptor side of PSI, decreased in response to an increase in SO\textsubscript{2} concentration, but that the reduction rate also declined. The inhibitory effect was greater in DS treatments than in the WW treatments, which may be due to the additional abiotic stress imposed by drought (DS). It is well known that under drought stress, acidification of the stroma occurs (Berkowitz \& Bibbs, 1983; Yordanov \textit{et al.}, 2003; Lisar \textit{et al.}, 2012). Under drought stress conditions the delicate balance between ROS production and scavenging suffers an upward shift, ROS production being enhanced due to stomatal closure and the concomitant limitation of CO\textsubscript{2} fixation. As a result the antioxidant enzymes could not efficiently detoxify the generated free radicals. This finding corresponds to the data presented by Takahama \textit{et al.} (1992) and Cruz de Carvalho (2008). This finding proved the second hypothesis not to be entirely true, as drought stress did not effectively ameliorate the adverse effects of SO\textsubscript{2} on the photosynthetic electron transport chain.

\subsection*{6.4.2. \textit{Zea mays} (C\textsubscript{4})}

A larger reduction in CO\textsubscript{2} assimilation rate (A\textsubscript{370}) occurred in the WW treatments at elevated SO\textsubscript{2} concentrations than in the DS treatments, as derived from the A:Ci response curves (\textbf{Figures 5.5. a and b}). These results were difficult to interpret and further analysis of the data was needed to shed light on this finding. A\textsubscript{370} values were thus expressed as a ratio of the amount of chlorophyll present (CCI) per 1 cm\textsuperscript{2} leaf section (i.e the photosynthetic rate at atmospheric CO\textsubscript{2} concentrations per 1 cm\textsuperscript{3} excited chlorophyll molecules) for the different SO\textsubscript{2} concentrations (\textbf{Figure 6.4}). Comparing the CO\textsubscript{2} assimilation rate of the WW and DS test plants on CCI basis, it was clear that the WW plants had a higher photosynthetic
Figure 6.3: The different sites of inhibition occurring in the chloroplasts during photosynthetic carbon reduction cycle in C₄ plants. The numbers in red indicate the main sites of inhibition of photosynthesis due to SO₂, i.e. stomatal limitation (1), decrease in PEPc activity (2), reduction in regeneration capacity of the acceptor molecule PEP (3), inhibition due to the uncoupling of the OEC (4), impairment of the flow of electrons within the electron transport chain, (5) and the inhibition of the reduction of the end electron acceptors (6).
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capacity per chlorophyll molecule than those in the DS treatments. This finding was corroborated by the higher PEPc (CE) activity in WW plants, compared to DS plants. The PEP regeneration capacity J$_{\text{max}}$ of DS treatments were higher, which points at a protective role that drought stress played to partly ameliorate the SO$_2$-induced inhibition on the photosynthetic apparatus, due to the decrease in stomatal conductance and consequently less SO$_2$ entering the leaf. Thus, based on some of the gas exchange data of seven weeks fumigation, the hypothesis set at the outset on a possible ameliorating effect of SO$_2$ damage by drought stress proved to be true, but only for test plants fumigated with 50 and 100 ppb SO$_2$. At SO$_2$ a concentration of 200 ppb, drought could not ameliorate the damage done by SO$_2$, proving the second hypothesis not to be true in this regard. Research done on drought-stressed Pinus plants by Panek (2004) focussed on the effect of O$_3$ uptake through the stomata, revealed that stomata open and close in response to changes in their environment such as light intensity, temperature and water stress in order to prevent extensive water loss, maximising carbon uptake which will directly and indirectly affect pollutant uptake. It is reported that at high pollutant concentrations such as SO$_2$ and O$_3$, stomatal closure usually occurs, but at low concentrations stomatal conductance often increases (Robinson et al., 1998). In the present study, it was found that the stomatal conductance of WW Zea mays test plants showed only slight decreases. With added drought stress (DS), stomatal conductance decreased up to 32.11% at the highest SO$_2$ concentration (200DS). These findings were corroborated by the noticeable decrease in the supply function of the A:Ci response curve, pointing at stomatal limitation in response to elevated SO$_2$ concentrations. Drought stress thus leads to more pronounced stomatal limitation and consequential closure of the stomatal aperture. Drought triggers the production of the phytohormone abscisic acid (ABA) which in turn causes stomatal closure and induces expression of stress related genes (Shinozaki & Yamaguchi-Shinozaki, 2007). This stomatal closure may (as seen in the present study) partially ameliorate the damaging effect of SO$_2$ on the biochemistry of the test plants by impairing entry of pollutants into the leaf.

Inhibition of the photochemical events was evident in WW treatments as changes in the characteristic OJIP fluorescence transients compared to the control (CFWW). The SO$_2$-induced constraints on the functioning of photosystem II, the inter electron transport system and the reduction of end electron acceptors, resulted in decreased production of reducing equivalents. Closer examination of the fluorescence transients by obtaining ΔV$_{\text{OJ}}$ and ΔV$_{\text{JP}}$ curves of WW and DS treatments, revealed that inhibition occurred at different sites in the electron transport chain.
Normalised chlorophyll a fluorescence transients ($\Delta V_{OJ}$) revealed a prominent $\Delta V_{K}$ band in the 30 ms region in 200WW test plants, pointing at inhibition on the donor side of PSII (OEC). No prominent bands occurred in the DS treatments, indicating that the inhibition did not occur in the single turnover phase. While no $\Delta V_1$-bands appeared within the range of 300 ms in WW test plants, a SO$_2$ concentration dependent $\Delta V_1$-band occurred in DS test plants corroborating the gas exchange data very well. Thus, in SO$_2$-DS combined treatments concentrations caused inhibitions in the reduction of end electron acceptors which lead to the decrease in CO$_2$ fixation. The PI$_{ABS, total}$ is a performance index used to evaluate plant vitality. In WW treatments PI$_{ABS, total}$ values varied greatly with the largest reductions occurring in the 7th week after the onset of fumigation. DS treatments almost consistently showed an SO$_2$-dependent decrease in PI$_{ABS, total}$ over weeks measured. The data suggest that the combination of DS and high SO$_2$ concentrations causes greater inhibition of the primary photochemical events than in any other treatment. The same phenomenon was seen in a study done by Heyneke et al. (2012b). A stimulatory effect was noticed in the PI$_{ABS, total}$ of WW plants exposed to 50 ppb SO$_2$ (50WW) in contrast to reductions occurring at higher SO$_2$ concentrations. Foliarly absorbed SO$_2$ may directly, or after its oxidation to form SO$_4^{2-}$, enter the S-assimilatory pathway and be reduced to sulphide, incorporated to cysteine and subsequently organic Sulphur compounds and utilized as Sulphur nutrient (De Kok & Tausz, 2001; Tausz et al., 2003; Yang et al., 2006). Plants could thus benefit from low SO$_2$ exposure since it may contribute to the plant’s nutrition (Yang, et al., 2006). This could be the reason for the slight stimulatory effect observed in some treatments. Reduction in PI$_{ABS, total}$ pointed at a decrease in photochemical efficiency of the photosystems. The reduction in PI$_{ABS, total}$ proved to be mainly due to slight reductions in (i) electron transport per cross section (ET/CS), (ii) density of active reaction centers per cross section (RC/CS) and (iii) reduction of end electron acceptors, Fd and NADP$^+$ (PI$_{RE}$). Evaluating the partial processes of PI$_{ABS, total}$ of WW treatments, indicated that reductions in the photosynthetic capacity could be ascribed to reduction in (i) the number of active reaction centers per total photon absorption, (ii) the efficiency by which reaction centers trap excitons, (iii) the efficiency to transport electrons in the electron transport chain, (iv) the number of active reaction centers per leaf cross section area and (v) the rate of electron transport per cross section leaf area (1 cm$^2$). Due to a great reduction in PI$_{ABS, total}$ in the 200WW treatment after 7 weeks’ fumigation, a compensatory increase in maximum trapping flux and antennae size occurred. In a study done by Smit et al. (2009) on the
influence of increased trifluoroacetate (TFA) concentrations on *Zea mays*, increases in the pollutant TFA resulted in decreases in the PI\textsubscript{ABS.total}. The data obtained in the present study correlated with those of Smit *et al.* (2009). Smit (2009) found that the TFA-related decrease in PI\textsubscript{ABS.total} was partly due to a 18% decrease in reduction of end electron acceptors (RE). Further analyses of the I-P phase of the fluorescence transients indicated that not only the size of the pool of end electron acceptors (Fd and NADP\textsuperscript{+}) at the accepting side of PSI decreased in response to an increase in SO\textsubscript{2} concentration, but that the reduction rate also declined at higher SO\textsubscript{2} concentrations.

### 6.4.3. Differential response of *Brassica napus* (C\textsubscript{3}) and *Zea mays* (C\textsubscript{4}) to SO\textsubscript{2} and SO\textsubscript{2} plus drought on photosynthesis

Analysing the influence of SO\textsubscript{2} on the photosynthetic capacity of crop plants, it is was evident that the C\textsubscript{3} and C\textsubscript{4} plants responded differently to the treatment, especially regarding the gaseous exchange. These differences were evident from the data derived from A:Ci response curves (*Figures 4.4 and 5.5*) as well as information provided by the chlorophyll fluorescence analysis.

The basis of the differential response of the C\textsubscript{3} and the C\textsubscript{4} plants lies in the different leaf anatomy and biochemistry of the species. The differing C\textsubscript{3} and C\textsubscript{4} leaf anatomy specialise plants for their specific metabolic requirements. The chloroplasts of bundle sheath cells lack obvious grana, have very little PSII and may synthesise ATP by cyclic photo-phosphorylation. This greatly decreases oxygen accumulation in the bundle sheath and the chances of photorespiration occurring. Compared to C\textsubscript{3} crops, photosynthesis in C\textsubscript{4} crops is more efficient (Lawlor, 1993). The PEP carboxylase reaction acts as an effective CO\textsubscript{2}-pump, supplying CO\textsubscript{2} to the photosynthetic carbon reduction (PCR) cycle in the bundle sheath cells, even during adverse environmental conditions such as drought and exposure to air pollutants. This function allows the plant to photosynthesise efficiently at low stomatal conductance, which also suppresses water loss (Lawlor, 1993; Furbank & Taylor, 1995). This is why, when we look at the photosynthetic capacity of *Brassica napus* and *Zea mays*, the difference in CO\textsubscript{2} assimilation efficiency is evident (*Figure 6.4*). The ratio of the CO\textsubscript{2} assimilation rate at atmospheric CO\textsubscript{2} concentrations (A\textsubscript{370}) and the chlorophyll content index (A\textsubscript{370}/CCI) is shown for the C\textsubscript{3} and C\textsubscript{4} crops, *Brassica*...
B. napus and Z. mays after 4 weeks’ fumigation, respectively. Interesting to note is the role of drought on the CO$_2$ assimilation rate. In the winter crop *Brassica napus*, the DS plants had a higher photosynthetic capacity than the WW plants, indicative of the ameliorating effect of drought on the photosynthetic capacity. In *Zea mays* on the other hand, WW test plants showed a higher photosynthetic efficiency (expressed as $A_{370}$/CCI) than DS test plants. This phenomenon could be explained by the unique anatomical and biochemical differences of C$_3$ and C$_4$ crops. Thus, in *Zea mays* drought did not effectively ameliorate the effect of SO$_2$ in the plants becoming more responsive to SO$_2$. Lawlor (1993) also mentions the possibility of the differences in the C$_3$ and C$_4$ thylakoid structure influencing the overall efficiency of the thylakoid in relation to the demand for ATP and NADPH. The difference in response to SO$_2$ may lie in the different stomatal behaviours of C$_3$ and C$_4$ plants. The decline in photosynthetic capacity is closely related to the stomatal conductance, especially in short-term fumigation experiments (Winner & Mooney, 1980). It was found that plants with more efficient stomatal conductance (such as C$_3$ plants), will

Figure 6.4: The ratio of CO$_2$-assimilation rate (at atmospheric concentration = 370 ppb) and chlorophyll content for WW and DS (→ and ←, respectively) *Brassica napus* (a) and *Zea mays* (b) after 4 weeks’ fumigation with different SO$_2$ concentrations (50, 100 and 200 ppb).
have less tolerance toward SO$_2$ than C$_4$ crops. The greater stomatal conductance will result in more SO$_2$ being taken up by the plant and consequently influence the photosynthetic capacity and ability to metabolise SO$_2$ and CO$_2$. These findings are supported by data collected in the present study. *Zea mays* (C4) test plants clearly showed a better ability to assimilate CO$_2$ than *Brassica napus* (C$_3$) crop plants even at increased SO$_2$ concentrations. Except for the differences in stomatal behaviour, C4 plants also have a biochemical advantage over C$_3$ plants. C4 plants are more tolerant toward SO$_2$, because of PEPc’s high affinity for HCO$_3^-$ and CO$_2$ and HCO$_3^-$. The chance of SO$_2$ to bind with PEPc to substitute HCO$_3^-$ is much smaller compared to the RuBP-CO$_2$ situation (Ziegler, 1973). Because the bundle sheath cells possess no grana, there are no PSII with accompanied oxygen evolution, which means that there is less chance for photochemical inhibition due to active oxygen radical impairment (Lawlor, 1993).

6.5. The effect of SO$_2$ on the antioxidant metabolism

Stress conditions often lead to an increase in ROS and can cause serious damage if not detoxified. Air pollution stress can cause an increase in ROS (Noctor & Foyer, 1998). When SO$_2$ enters the leaf it reacts with the aqueous phase of the apoplast to form sulphuric acid, H$_2$SO$_4$. This molecule may diffuse across the biomembrane barrier of the plasmalemma into the cytosol. Here it is neutralised to form two cytotoxic anions, bisulphite (HSO$_3^-$) and sulphite (SO$_3^{2-}$). These molecules can effectively be detoxified either by reduction (endogenously produced oxidants) or oxidation reactions (Slovik, 1996), as long as air pollutant concentrations are not excessive. Sulphite and bisulphite anions can also be detoxified by means of oxidation to yield sulphate ions (Heber *et al.*, 1995; Takahama *et al.*, 1992; Olzyk & Tingey, 1984). The scavenging of superoxide (O$_2^-$) and other reactive oxygen species, is achieved through antioxidant enzymes (Liu *et al.*, 2009). SOD catalyses the dismutation of superoxide to H$_2$O$_2$ (Blokhina *et al.*, 2003) and POD reduces H$_2$O$_2$ to H$_2$O in the Asada-Halliwell pathway (Hopkins & Hüner, 2009). ROS that is generated within cellular structures may damage proteins, leading to chloroplast deformation and reductions in chlorophyll content and increased lipid peroxidation (membrane destruction). Radical-scavenging reactions must thus be fast enough to out-compete the destructive reactions (Luwe *et al.*, 1993).
Water stress is one of the most important environmental factors that have a negative impact on plant growth and development, which ultimately leads to a reduction in plant production (Jiang & Zhang, 2002). ROS are thought to be involved in the cellular signalling process as secondary messengers to induce antioxidant defences under water stress (Zhu & Scandalios, 1994; Larkindale & Knight 2002). In a study done on Nicotiana plumbaginifolia, SO₂ elicited a defense-reponse in test plants by increasing POD activity (Willekens et al., 1994). Pierre & Queiroz (1981) observed similar results in Phaseolus vulgaris plants that were fumigated with SO₂. After approximately one week large increases were evident in POD activity. The same phenomenon occurred in the present study where SO₂ elicited a defense response by increasing POD activity. This was especially true for well watered (WW) plants. In test plants exposed to a combination of drought and elevated SO₂ concentrations (i.e. DS treatments), an initial SO₂-dependent increase in POD activity was displayed, but after 6 weeks POD activities were equal to, or less than that of control plants. These results point to the adaptation of test plants to the adverse environmental conditions. Adaptation of test plants in the form of partial stomatal closure inhibitits the entry of SO₂ into the stomatal air space, consequently reducing the production of ROS in organelle structures.

Though there was no significant differences in any of the WW or DS treatments, the greatest increase in POD activity overall, occurred in the 200WW treatment after 3 weeks of fumigation. It is assumed that an increase in ROS resulted from the photochemical inhibition of the light phase, probably due to SO₂ toxicity which may lead to oxidative damage. This assumption is supported by our fluorescence data, showing prominent ΔVₖ and ΔV₁ bands in the different spectra which suggest inhibition due to (i) the uncoupling of the OEC or (ii) inhibition of the reduction of end electron acceptors after PSI, respectively (Heyneke et al., 2012b). With an increase in ROS a build up of protective mechanisms occur to prevent oxidative damage (Singh & Khan, 2006). With some plants (100WW test plants fumigated for 2 weeks and 50WW fumigated for 3 weeks) the damage due to ROS was very extensive and consequently the chronic stress imposed by SO₂ could not be compensated for solely by means of scavenging enzymes. Similar results were reported by Liu et al. (2009) working on oxidative stress responses of Arabidopsis thaliana to certain air pollutants. They found that with increases in air pollutant concentrations, accompanying increases occurred in the POD antioxidant enzymes at a threshold value, followed by dose-declining activities at higher pollutant concentrations. At high SO₂
concentrations the toxic ROS load may have exceeded the capacity of the plant’s antioxidant mechanisms. The balance of antioxidant enzymes is crucial for suppressing toxic ROS levels in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms. Abiotic stresses such as elevated SO$_2$ concentrations result in increased antioxidant enzyme actions which in turn lead to detoxification of ROS to water (Apel & Hirt, 2004). The decline in POD activity suggests that the detrimental effects of ROS overpowered the plants’ ability to counteract the cytotoxic compounds.

6.6. Implications on biomass and yield

Yang et al. (2006) did research on the toxic and nutritional effects of sulphur dioxide on Chinese cabbage (Brassica pekinensis) and found that reductions of 26% and 33% occurred in shoot biomass of plants exposed to 100 ppb and 200 ppb SO$_2$, respectively. These findings correspond to those found in the present study. Researchers have long debated the question whether changes in physiology and biochemistry is ultimately manifested in permanent losses in growth, yield or quality (Jones, 1985). The data presented in the current study is evidence of such detrimental effects after prolonged exposure to the air pollutant, SO$_2$.

6.6.1. Brassica napus

Yang et al. (2006) did research on the effect of elevated SO$_2$ concentrations on Chinese cabbage cultivars. They found that a reduction in the functioning of many of the CO$_2$-response parameters was evident at SO$_2$ concentrations greater than 100 ppb, leading to a significant reduction in shoot biomass. These findings correspond to those found in the present study where WW and DS treatments that were exposed to 200 ppb showed a 30.12% and 24.33% reduction in shoot biomass, respectively. Bosac et al. (1994) reported that the most significant finding in their study on the impact of O$_3$ and SO$_2$ on reproductive development in Brassica napus, was that exposure to 200 ppb SO$_2$ for 6 hours was sufficient to promote bud abortion and abscission. These findings correlated with data of the present experiment. The pod weight decreased by 7.99%, 11.85%, and 12.6% in the 50WW, 100WW and 200WW treatments, respectively. It could be speculated that the reduction in pod biomass recorded may thus partly be ascribed to the abscission of flowering buds in response to elevated SO$_2$ concentrations. Lee et al. (1997) did
research on the response of field-grown soybean cultivars under elevated SO$_2$ concentrations. They found that the photosynthetic response to elevated SO$_2$ was non-significant during pre-pod fill stages but that fumigation with 120 ppb SO$_2$ during pod fill stages resulted in a decrease of 17.2% in photosynthetic rate compared to control treatments. Blum (2005) wrote a paper on the influence of drought on the WUE and yield potential. It was found that drought stress reduced the water use and consequently lowered the biomass. An increase in WUE pointed at an improved drought resistance and greater yield under stress. Under stress conditions, one of the strategies that plants have adopted is to slow down growth. The ability to reduce cell division under unfavourable conditions conserves energy and may also limit the risk of hereditable damages (May et al., 1998). A strong SO$_2$-concentration dependent decrease occurred in Brassica napus test plants exposed to elevated SO$_2$ concentrations in combination with a drought treatment (DS). This high correlation ($r^2 = 0.967$) is evidence of a greater sensitivity of DS test plants towards SO$_2$, compared to the weak correlation in WW test plants (Figure 6.5).

![Figure 6.5](image.png)

**Figure 6.5:** Correlation between the decline in shoot biomass and the increase in SO$_2$ concentration for well watered (a) and drought stressed (b) Brassica napus test plants after 5 weeks’ fumigation.
6.6.2. *Zea mays*

In a study conducted by Dodd & Doley (1998) on the influence of SO$_2$ on the biomass of crops revealed that the biomass of rapidly growing plants under conditions of high light intensity (such as the conditions of Southern Africa), are suggested to expend proportionately less energy on detoxification of sulphur metabolites (Olszyk *et al.*, 1986) and allocate proportionately more energy to growth. It can thus happen that plants can still grow a considerable length and because less energy is available to cob formation, the cob-mass and size would proportionately decrease. This was indeed the case with *Zea mays* plants exposed to elevated SO$_2$ concentrations. The effect of a lack of detoxifying abilities and the destructive properties of SO$_2$ are evident in the reduction of cob size in both WW and DS treatments (Figure 5.18). Contradictory to our data on *Brassica napus* and results displayed by Bosac *et al.* (1994), the reproductive organs (cobs) in *Zea mays* test plants exposed to elevated SO$_2$ concentrations (50, 100 and 200 ppb) developed quicker than in plants given only CF air for both WW and DS treatments.

6.7. Concluding remarks

It is important to establish guidelines for ambient air quality standards that are appropriate for agricultural crops. In order to achieve this, reliable dose-response relationships are required for a wide range of crops (Bell & Marshall, 2000). In many papers the sensitivity of different cultivars within crops has been confirmed (Yang *et al.*, 2006), and the importance of cultivar screening in terms of crop resistance towards the damaging effects of air pollutants, as well as potential and indicator species for pollution thresholds are recognised. The presented data complemented the existing standards which are based on protection of human health and cultural heritage. It was clear that reduction in photosynthetic capacity and ultimately marked decreases in crop production and yield can be expected to occur in crops exposed to concentrations of 50 ppb SO$_2$ and above.

The only measurements of plant response that is useful for decision makers in managing air quality are those that have economic meaning for the crop in question. These include measurements of growth, yield and quality (Jones, 1985). If one could anticipate the amount of damage that would occur, then necessary precautions could be made to buffer or prevent damage and loss due to adverse environmental conditions. In this study it was determined that through
indirect measurements such as chlorophyll fluorescence induction (JIP test) and photosynthetic gas exchange, fairly accurate prediction of potential yield reductions/damage is possible, even before the occurrence of visible injury. An unraveling of the responses due to increasing SO₂ concentrations revealed that antioxidant enzyme activities, photosynthetic rate and morphological structures of the leaf laminas can be used as indicators of oxidative stress. The data convincingly demonstrated that SO₂, even at low concentrations, inhibits photosynthesis, proving the first hypothesis at the outset as true. The toxic nature of SO₂ was evident as:

i. Destruction of chlorophyll pigments due to the very reactive HSO₃⁻ and SO₃²⁻ anions derived from SO₂ molecules in *Brassica napus* and *Zea mays* plants (Takahama et al., 1992; Ghisi et al., 1990) (Figure 5.3);  
ii. loss of stomatal functionality. This was reflected is a reduction in the stomatal conductance of WW and DS treatments of both *Brassica napus* and *Zea mays* plants;  
iii. decline in Rubisco (*Brassica napus* – C₃) and PEPc (*Zea mays* – C₄) activity in WW and DS treatments (evident as a decline in the CE in the photosynthetic gas exchange data);  
iv. inability to effectively regenerate RuBP (*Brassica napus* – C₃) and PEP (*Zea mays* – C₄) of WW and DS treatments (evident as a decline in the Jₘₐₓ in the photosynthetic gas exchange data);  
v. inhibition of the primary processes of photochemistry which was the result of:  
   a. uncoupling of the OEC in WW *Zea mays* and DS *Brassica napus* plants;  
   b. inhibition of the reduction of end electron acceptors beyond PSI in WW and DS *Brassica napus* plants, as well as in DS *Zea mays* test plants.  
   c. In *Zea mays* the source of the inhibition of the primary photochemistry as a result of the decline in the reduction of end electron acceptors was investigated by further analysis of the I-P phase. The inhibition in WW and DS treatments was found to be as a result of:  
      i. reduced pool size of electron acceptors and  
      ii. decline in the rate at which end electron acceptors were being reduced;  
vi. marked SO₂-concentration dependent decline in the shoot mass (biomass) and yield in both WW and DS *Zea mays* and *Brassica napus* test plants, respectively.
An increase in H$_2$O$_2$ content due to elevated SO$_2$ concentrations lead to a decrease in chlorophyll content and inhibition of the photosystems which consequentially inhibited the photosynthetic capacity of Zea mays plants. The effectiveness of the antioxidant metabolism to remove H$_2$O$_2$ from mesophyll cells was displayed by the overall decrease in H$_2$O$_2$ content after 9 weeks’ fumigation. This was achieved by the increased scavenging enzyme activity (increased POD activity) that effectively removed the ROS from mesophyll cells.

The additional drought stress, together with elevated SO$_2$ concentrations imposed a double stress on Zea mays test plants and the data is therefore accordingly difficult to interpret. What can be concluded was that the C$_3$ plant, Brassica napus was more adversely affected by elevated SO$_2$ concentrations, reducing photosynthetic assimilation rate greatly. Drought stress (DS) did, however, ameliorate the damaging effect of SO$_2$ on the photosystems in all Brassica napus test plants treated with elevated SO$_2$ concentrations to some extent. Zea mays plants showed greater sensitivity towards elevated SO$_2$ concentrations with the addition of drought as a co-stressor (DS) and amelioration because of stomatal closure was not as effective as with the C$_3$ crop plants. These findings did not convincingly demonstrate that drought stress ameliorates the damaging effect of SO$_2$ on crops. The second hypothesis on the outset of this paper was thus only partially proven to be true, and only at low SO$_2$ concentrations. At higher SO$_2$ concentrations (100 and 200 ppb) the second hypothesis was proven not to be true. It is suggested in other papers (Abedi & Pakniyat, 2010) that the detection of new isozyme bands under drought stress conditions could be used as a biochemical marker to differentiate drought tolerant cultivars under drought stress and, as with the present study determine whether there is a measure of drought-pollutant cross-tolerance in certain crop cultivars, which could mean a revolutionary breakthrough in the agronomical sector. Understanding the physiological and biochemical mechanisms conferring drought tolerance of these species is very important in terms of developing selection and breeding strategies (Abedi & Pakniyat, 2010).
6.8. Recommendations and future perspectives

The research that was carried out on SO$_2$ and drought effects over the last few years has increased our understanding of crops’ differential responses toward SO$_2$. Even though the aims of this research study were adequately reached, there are still some aspects that require further investigation:

- In our research quantifications confirmed a concentration dependent change in the photosynthetic capacity of crop plants, and resultantly a decrease in yield. Investigations of stomatal responses, yield responses and biochemical responses must be extended to include even more studies on other crop species in OTCs.
- We especially need a better understanding of how crops would respond to a combination of air pollutants, since atmospheric pollutants usually appear in parallel with one another in polluted areas. This will provide a realistic, actual response of crops to a polluted area.
- The effects on crop plants need to be quantified under controlled conditions and under field conditions. It is recommended that field trials on bigger scale together with a pollutant monitoring programme, should be introduced into research programmes.

To achieve these aims, we must adopt an interdisciplinary approach should be adopted in which the physical, chemical and growth aspects are investigated concurrently. Only then will it then be possible to answer the many questions still perplexing air pollution scientists.