Influence of SO_2 fumigation on growth, photosynthesis, lipoxygenase and peroxidase activities of soybean (*Glycine max*), in open-top chambers.

S Lindeque

12850446

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Supervisor: Dr. J.M. Berner

Co-supervisor: Prof. G.H.J. Krüger

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ABSTRACT

INFLUENCE OF SO_2 FUMIGATION ON GROWTH, PHOTOSYNTHESIS, LIPOXYGENASE AND PEROXIDASE ACTIVITIES OF SOYBEAN (*GLYCINE MAX*), IN OPEN-TOP CHAMBERS

Air pollutant exposure poses a health risk to humans and impacts negatively on agriculture. High levels of air pollution resulted in extensive crop damage and yield reduction in Europe and USA. The Highveld region in South Africa, a very important area for maize and soya production, has already been declared an air pollution hot spot, with SO_2 being the most concerning air pollutant. Most of the SO_2 over the Highveld originates from the burning of coal for power generation. Developing countries, such as South Africa, are highly dependent on agriculture for food security and high levels of air pollution pose serious risks to the agricultural industry. Currently very little information is available on the effects of air pollution on crop production in South Africa.

This study aimed to establish exposure-response relationship for SO_2 on soybean and the quantification thereof on the morphological, physiological and biochemical characteristics. Two soybean cultivars were used, namely: LS 6164 and PAN 1666. The plants were fumigated for 7 hours, 7 days a week with 0 (carbon filtered control; CF), 25, 75 and 150 ppb SO_2. The effect of SO_2 was investigated on the growth, photosynthetic capabilities, photosynthetic gas exchange, peroxidase activity and lipoxygenase activity of the cultivars.

Foliar injuries and interveinal chlorosis were visible with increasing levels of SO_2 as well as a decrease in biomass accumulation, especially in root biomass; a more prominent feature of LS 6164. The number of nodules of both cultivars decreased insignificantly as the levels of SO_2 increased. The number of pods per plant and the average weight of 30 seeds indicated a downward trend with an increase in SO_2 concentration. The chlorophyll content of PAN 1666 was lower compared to LS 6164.

PAN 1666 had the largest reduction in stomatal conductance at 150 ppb SO_2 fumigation.

The photosynthetic vitality index indicated that LS 6164 was more sensitive to SO_2 inhibition from 25 ppb SO_2 and higher, whereas PAN 1666 mostly became sensitive to SO_2 from 75 ppb SO_2. A decrease in the ability to absorb light energy, the trapping of excitation energy to transfer electrons beyond Q_A^-, and the reduction of end electron acceptors all contributed to the decline in the vitality index.

Sulphur content increased significantly in the 75 ppb and 150 ppb treatments of both cultivars. Induced peroxidase and lipoxygenase activity was seen in both cultivars, especially at higher concentrations of SO_2 treatments. PAN 1666 had a higher rate of peroxidase and lipoxygenase activity compared to LS 6164.

The implication for SO_2 on crop production in the highly industrial Highveld area was demonstrated to be potentially of great concern. The dose-response relationships plotted for OJIP parameters emphasized that SO_2 is an inhibitor of photosynthesis and phytotoxic of nature. Both cultivars experienced limitations from 75 ppb, especially at the 150 ppb SO_2 concentration. From these results it appears that PAN 1666 is more adapted to SO_2 compared to LS 6164 and levels of 75 ppb SO_2 and higher become toxic to these plants.

Key words: Air pollution, biomass, chlorophyll *a* fluorescence, chlorophyll content, gas exchange, *Glycine max*, lipoxygenase, peroxidase, soya, sulphur dioxide, stomatal conductance, sulphur content.

OPSOMMING

DIE INVLOED VAN SWAEL DIOXIDE BLOOTSTELLING OP GROEI, FOTOSINTESE, LIPOKSIGENASE EN PEROKSIDASE AKTIWITEITE VAN SOJABONE (GLYCINE MAX) IN OOP-TOP-KAMERS

Blootstelling aan lugbesoedeling hou ‘n gesondheidsrisiko vir die mens in en het ook ‘n negatiewe uitwerking op die landboubedryf. In die VSA en Europa is dit bevind dat hoë vlakke van lugbesoedeling omvangryke skade aan kropgewasse en afname in opbrengs veroorsaak. Die Hoëveldstreek in Suid-Afrika, ‘n belangrike gebied vir koring- en sojaproduksie, is alreeds verklaar as ‘n lugbesoedeling gevaarsone. Swael dioxide, wat sy oorsprong in die verbranding van steenkool vir energie opwekking het, is die belangrikste oorsaak van lugbesoedeling. Ontwikkelende lande, soos Suid-Afrika, is afhanklik van landbou vir voedselsekuriteit. Huidiglik is daar beperkte inligting rakende die invloed van lugbesoedeling op kontantgewasse beskikbaar en gevolglik is die invloed op die ekonomie onseker.

Die doel van hierdie studie is om die verhouding tussen SO_2 blootstelling en die reaksie daarvan op sojabone te bepaal, asook die kwantifisering van die effek op die morfologiese, fisiologiese en biochemiese eienskappe. Twee sojaboon kultivars is vir die studie gebruik, nl. LS 6164 en PAN 1666. Die plante is daagliks vir 7 ure blootgestel aan 25, 75 en 150 dpb (dele per biljoen) SO_2. Die effek van SO_2 op groei, fotosintetiese vermoëns, fotosintetiese gaswisseling asook peroksidase en lipoksigenase aktiwiteit is ondersoek.

Blaarskade en tussenaarse chlorose was sigbaar gedurende die toename van SO_2 vlakke asook ‘n afname in biomassa, veral wortel biomassa, wat meer prominent in die geval van LS 6164 was. Die hoeveelheid nodules van beide kultivars het onbetekenisvolle afname getoon met toenemende SO_2 vlakke. Die hoeveelheid peule per plant and die gemiddelde massa van 30 sade het ‘n afwaartse tendens, met toenemende SO_2 konsentrasies aangetoon. Die chlorofil inhoud van PAN 1666 was laer in vergelyking met LS 6164. PAN 1666 het die grootste afname in stomatale geleiding aangedui.

Die fotosintetiese vitaliteitsindeks het aangedui dat LS 6164 meer sensitief was vir SO_2 inhibisie vanaf 25 dpb SO_2 en hoër, terwyl PAN 1666 hoofsaaklik sensitiwiteit vanaf 75 dpb SO_2 aangetoon het. ‘n Afname in die vermoë om ligenergie te absorbeer, vasvang van opwekkingsenergie, die oordrag van elektrone verder as Q_A^- en die reduksie van eind-elektron akseptore het almal bygedra tot die afname in die vitaliteitsindeks.

Swael inhoud het beduidende toename in die 75 dpb en 150 dpb behandelings getoon van beide kultivars. Geïnduseerde. peroksidase en lipoksigenase aktiwiteit is by beide kultivars gevind, veral by 150 dpb SO_2 behandelings. PAN 1666 het ‘n hoër koers getoon vir peroksidase sowel as lipoksigenase in vergelyking met LS 6164.

Die moontlike implikasies van SO_2 besoedeling op landbougewasse in die hoogs geïndustriële Hoëveld-gebied is kommerwekkend. Die dosering-reaksie verhouding gemeet vir OJIP parameters beklemtoon dat SO_2 fotosintese belemmer en van nature fitotoksies is. Beide kultivars het beperkings ondervind vanaf 75 dpb, maar veral by die 150 dpb SO_2 behandeling. Na aanleiding van hierdie resultate wil dit voorkom asof PAN 1666 meer aangepas het by SO_2 in vergelyking met LS 6164, en dat vlakke vanaf 75 dpb SO_2 en hoër toksies is vir hierdie plante.

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I hereby declare that this thesis presented for the degree Master in Environmental Science (*M. Env. Sci),* at the North-West University (Potchefstroom Campus), is my independent work and has not previously been presented for a degree at any other university or faculty.

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A_{360}	CO_2 assimilation rate at normal atmospheric CO_2 concentration (360 µmol mol^{-1})
A_0	CO_2 assimilation rate at an intercellular CO_2 concentration of 360 µmol mol^{-1} or above, where no stomatal limitation is present
A_{max}	Light saturated rate of photosynthesis
ABS/CS_M	Phenomenonological energy flux (per excited cross section of leaf) for light absorption
ABS/RC	Specific energy flux (per PSIIreaction centre) for light absorption
C_a	Atmospheric CO_2 concentration
CCI	Chlorophyll content index
CCM-200	Chlorophyll meter
CE	Carboxylation efficiency
CF control	Carbon filtered control
Chl	Chlorophyll
C_i	Intercellular CO_2 concentration
CIRAS	Automatic infrared analyzer
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ET	Electron transport
EVAP	Evapo-transpiration
HANDY-PEA	Handy Plant Efficiency Analyser
HSO_3^-	Bisulphite
H_2O	Water
H_2O_2	Hydrogen peroxide
H^+	Hydrogen ion
J_{max}	Maximum CO_2 assimilation rate at saturating CO_2 concentration
$_l$	Relative stomatal limitation of photosynthesis
ℓ	Percentage stomatal limitation of photosynthesis
LOX	Lipoxygenase

LIST OF ABBREVIATIONS

Lλ =	Reference value length
L_n	Length of the central leaf on trifoliate leaf
L_{n+1}	Length of the central leaflet on trifoliate leaf
M	Moles
mM	Millimoles
mmol / m^2s	Millimoles per meter-squared seconds
ms	Milliseconds
n	Indicate the number of the trifoliate leaves equal to or just longer than the reference leaf
O_2	Oxygen
OEC	Oxygen Evolving Complex
OTC's	Open Top Chambers
O_3	Ozone
PEA	Plant Efficiency Analyser
PEP	Phosphoenol pyruvate
PI	Plastochron index
PLC	Photosynthetic leaf chamber
PAN	Peroxyacetyl nitrate
POD	Peroxidase
PQH_2	Dihydroplastoquinone
ppb	Parts per billion
PQ	Plastoquine
PSI	Photosystem I
PSII	Photo system II
PUFA	Polyunsaturated fatty acid
PVPP	Polyvinylpolypyrrollidone
P_{2G}	Overall grouping probability within PSII antenna
Q_A	Primary bound quinone
Q_A^-	Primary bound quinone in reduced state
Q_B	Secondary quinone acceptor
Q_B^-	The reduced (one electron) secondary quinone acceptor

LIST OF ABBREVIATIONS

Q_BH_2	The protonated secondary quinone acceptor
Rpm	Revolutions per minute
Γ	CO_2 compensation concentration
ROS	Reactive oxygen species
RuBP	Ribulose 1, 5-bisposphate
Rubisco	Ribulose 1, 5-bisposphate carboxylase / oxygenase
SH	Sulfhydryl
SOD	Superoxide dismutase
SO_2	Sulphur dioxide
S^{2-}	Sulphide
SO_3^{2-}	Sulphite
SO_4^{2-}	Sulphate
UV	Ultra violet
2 e^-	Electron donor
Δ	Difference

CHAPTER 1
Introduction

Increases in the levels of air pollutants poses a danger to all living organisms as well as to the environment they live in (Anon, 2008). Though the effects of air pollutants on humans are well known, the impact on crops and vegetation is less known. The causes of air pollution are both human-generated (anthropogenic) and natural (biogenic), but it is the human-generated contribution that is of particular concern (Hopkins & Hűner, 2004:476).

Air pollutants are described as the introduction of gases, dust, fumes or odours in harmful amounts that can cause harm when released into the air (Anon, 2008). These amounts can be harmful to the health or comfort of humans and animals or can cause damage to plants and materials (Vallero, D. 2007:413; Kampa & Castanas, 2008:362). Primary pollutants are introduced directly into the atmosphere such as carbon monoxide from car exhausts and sulphur dioxide (SO_2) from the burning of coal. Secondary air pollutants can arise if primary pollutants in the atmosphere undergo chemical reactions, especially in the presence of UV light. Examples of secondary air pollutants include photochemical smog, ozone and acids (Stedman, 2000:86).

The most important biogenic source of air pollution results from volcanic eruptions, forest fires and the natural decomposition of organic substances (Hopkins & Hűner, 2004:477). During volcanic eruptions, immense quantities of sulphur dioxide, carbon dioxide and hydrogen fluoride are released into the air. Another great contributor to air pollution is forest fires that emit carbon monoxide, sulphur dioxide, nitrogen dioxide, and particulate matter. There are many other biogenic sources of air pollution like wind erosion, pollen dispersal, evaporation of organic compounds and natural radioactivity (Vallero, 2007:313). However, these sources are usually not significant and because they are part of the natural environment is no major concern to us. The most relevant sources of anthropogenic air pollutants are the incineration of fossil fuels to produce energy (heat and electricity), major industrial processes (like metallurgy industry or cement/construction industry), transportation and agricultural systems. The four major groups of gaseous air pollutants based upon their historical importance,

concentration, and overall effects on plants and animals are sulphur dioxide (SO_2), oxides of nitrogen (NO_x: NO, NO_2), carbon dioxide (CO_2), ozone (O_3) and PAN (peroxyacetyl nitrate) (Hopkins & Hüner, 2004:477; Srivastava, 1998:525). The majority of these air pollutants are the direct result of the combustion processes in large power plants and piston engines (Vallero, 2007:313).

The growth and prosperity of the human population intensifies the desire to maintain a higher lifestyle and this causes an increase of the quantity of energy and materials needed by each individual. The Industrial Revolution of the mid-19th century along with the amazing technological advances introduced new sources of air pollution. The worldwide levels of air pollutants present in the atmosphere increased especially during the 20^{th} century because of increases in population, transportation, industrialization and urbanization (Srivastava, 1998:525). The increase in energy consumption and technical evolution, which coincided with the industrial revolution, is the primary causes of manmade air pollution. This demand is associated with ecologically unplanned industrialization, uncontrolled urbanization and deforestation (Renuga & Paliwal, 1995:59).

Sulphur dioxide is one of the most phytotoxic by-products of fossil fuel burning and has become an unrelenting aspect of atmospheres in industrialized countries (Emberson, 2003:3; Winner & Mooney, 1980:290). South Africa has one of the largest industrialized economies in the Southern hemisphere and the only industrialized region on the African continent (Josipovic, 2007:6). Approximately 90% of South Africa's scheduled emissions of industrial dust, SO_2 and NO_x are observed on the Highveld plateau, which represents a large portion of this industrial infrastructure (Josipovic, *et al.*, 2011:1). The Highveld represents 90% of South Africa's total SO_2 emissions (Fig 1.1) (Josipovic, 2007:6).

The predicament of air pollution in the Highveld area was emphasized when it was declared a national air pollution hot spot (South Africa, 2007). Sources of the air pollution include a range of industrial and mining activities. The Mpumalanga Highveld region is home to five of the largest coal-fired power plants in the world. The three main power stations, Malta, Duvha and Arnot produce 860 tons of SO_2 per km^2 per year. The area consist of 31 106 km^2 which extends from the eastern parts of

Gauteng to Middelburg in the Mpumalanga province and to the edge of the escarpment in the south and east (Lourens, *et al*., 2011:1). This area is associated with diverse anthropogenic activities such as gold and platinum mines, mineral mines, coal mines; coal based electrical power generation, many informal settlers using wood fuel as well as agricultural activities (Lourens, *et al*., 2011:1). These anthropogenic activities contribute to elevated levels of inorganic gaseous air pollutants such as SO_2; NO_2; O_3 and volatile organic compounds (Lourens, *et al*., 2011:1).

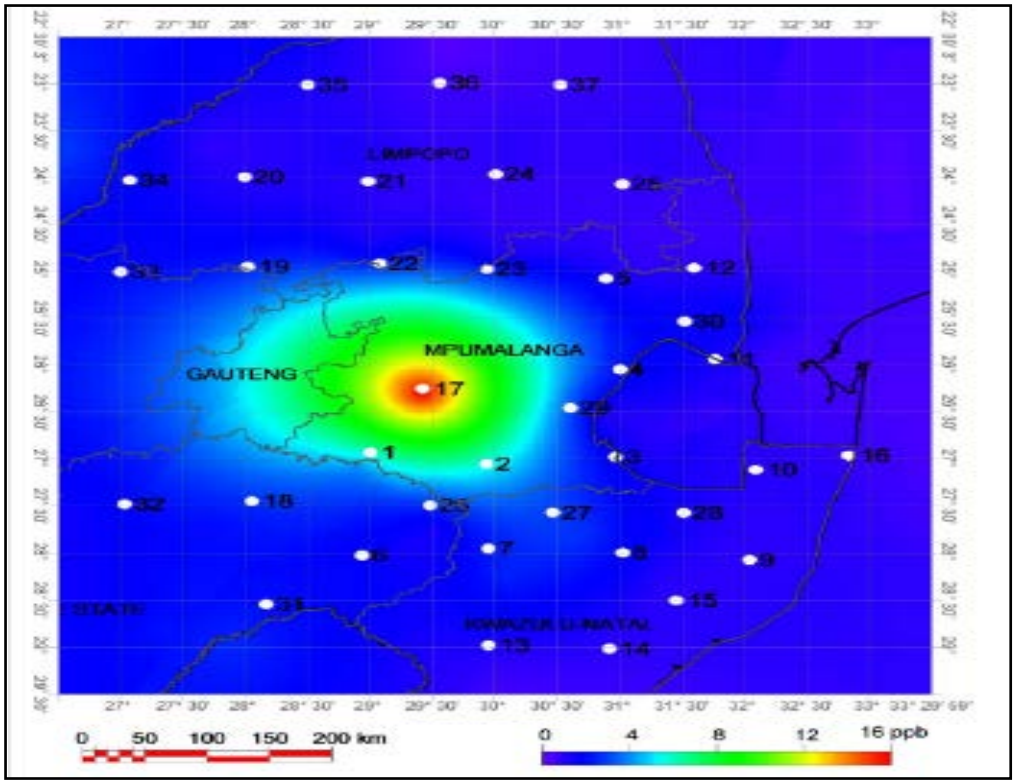

Figure 1.1: Average concentration of SO_2 (ppb) from January 2005 to May 2006: The SO_2 concentrations are represented through colour indices where red represents the largest concentration of 16 ppb over the Highveld area (Josipovic, *et al*., 2007:11).

The growing economy and the increase in South Africa's population increase the demand for electricity. The SO_2 concentrations over the Highveld mostly range between 10 and 50 ppb, but at the point of origin the SO_2 concentrations exceeds 60 ppb (Zunckel, *et al*., 2000:2797; Josipovic, *et al*., 2007:11 & 2010:181). The maximum dry deposition rates for sulphur is more than 10 kg $S.ha^{-1}.a^{-1}$ and the maximum wet deposition rates for sulphur ranged between 1 and 5 kg $S.ha^{-1}.a^{-1}$ over the central Highveld (Zunckel, *et al*., 2000:2797). In South Africa the concentration of SO_2 peaked especially during winter, when more power is needed and wood fires are made to keep

warm (Lourens, *et al*., 2011:8; Josipovic, *et al.*, 2010:181). To generate electricity Eskom used 247 million tonnes of coal during 2009 and due to increasing demand 374 million tonnes of coal will need to be produced by 2018 (Prinsloo, 2009). In the Mpumalanga province 84% of South Africa's coal mining operations can be found (Anon, 2011). Sulphur dioxide emissions in the Highveld total 1 622 233 tons per year, 99% of the total SO_2 originates from industrial sources of which 82% originate from coal power generation (South Africa, 2011).

Air pollution has been recognized as a cause of vegetation injury over the past few centuries. Exposure to high concentration of air pollutants can result into injuries of agricultural crops (Griffiths, 2003:1). Due to the negative impact of SO_2 on plant community structure as well as plant metabolism, SO_2 can be considered as a chronic environmental stressor for vegetation (Winner & Mooney, 1980:290). The extend of these injuries can fluctuate from visible markings to reduced growth as well as to the yielding capacity of major agricultural crop species (Hopkins & Hűner, 2004:478).

Air pollution has become an extremely serious problem for the modern industrialized world (Rai, *et al*., 2011:78). Data for long-term fumigation of SO_2 are incomplete and long-term experiments are necessary to establish the impact of air pollution on vegetation. Air pollution negatively impacts crop production in the United States (Muller *et al*., 2011:1649), Switzerland (Fuhrer & Bungerer, 1999:355), Sweden (Pleijel, *et al*., 1991:151), Germany (Adaros, *et al*., 1990:162), China (Wang *et al*., 2007:394), Pakistan (Wahid, 2006:304), Japan (Kobayshi, *et al*., 1995:109), Malaysia (Ishii, *et al*., 2004:205) and Thailand (Ariyaphanphitak, *et al*., 2005:179). The impact that these gasses have on agricultural crops depends on the concentration of the pollutant and the species. At lethal dosages, severe morphophysiological aberrations such as yellow, brown or necrotic patches or bleaching of the leaves occur. With chronic dosages symptoms can only be detected on metabolic and enzymatic levels (Darrall, 1989:1).

The effect of air pollution on various crop species has been explored in several countries. In Turkey the effect of refinery pollution increased non-enzymatic foliar defence mechanisms, but decreased the total chlorophyll content, SH-compounds, ascorbic acid. The levels of proline, carotenoids and lipid peroxidation increased when

plants were exposed to air pollution (Deniz & Duzenli, 2006:71). In Switzerland and various other regions of North-West-Europe established that the ozone causes visible injury on crops and has negative effects on long term changes in growth, biomass, crop yield, reproduction, competitiveness and vitality (Fuhrer & Bungener, 1999:355). In China the distribution and effects of ground-level ozone were examined and found the yield and biomass of certain crops to be reduced (Wang, *et al*., 2007:394). In Pakistan the influence of atmospheric pollutants on agriculture in developing countries were explored and they concluded that the yield and photosynthetic rate were significantly decreased (Wahid, 2006:304). The same effects were observed in Europe (Mills, *et al*., 2011:592).

The impacts of air pollution on crop production in South Africa are still poorly understood. Only a few superficial studies have been conducted and the results have so far been inconclusive. However, we do know that air pollutants have a very detrimental effect on grain crops if the levels become too high. Taking the current air pollution problem over the Highveld, and the planned expansion of coal-fired power stations into account, air pollution will become a more serious threat to agriculture in South Africa, especially over the Highveld.

Air pollutants, like SO_2, enter plants by making use of the same pathway as CO_2. Upon entry SO_2 dissolves in the apoplastic fluids and sulphite ions (SO_3^{2-}) are produced, which can be detoxified at low concentrations and be used as a sulphur source (Emberson, 2003:16). However, at higher concentrations it can cause stomatal closure. Closing of the stomata can protect plants from further exposure to the pollutant, but physiological damage to plants have already occurred (Vallero, 2007:397).

In developing countries food security is of primary concern and the implications of air pollution on crop yield is of great concern. South Africa is rich in agricultural activities and it is therefore vital and of great importance to understand the impact of air pollutants on crops. The reduction in subsistence economically important crops could have social and economic consequences (Zunckel & Tienhoven, 2002:2). Environmental stress is known to cause significant crop loss, at least in part due to oxidative damage (Ali & Alqurainy, 2006:187).

About half of South Africa's soybean is planted in the Highveld (Mpumalanga Business, 2009). This crop has a high input cost and is sensitive to environmental changes. The Bureau for Food and Agricultural Policy made a projection in 2010 that due to increasing yield as well as the growing demand for animal protein feed, by the year 2020 the hectares of soybeans planted in South Africa would be 605 000. This will cause production to possibly triple to 1.62 million tons by then (Esterhuizen, 2010).

To date there is limited information available on the effects of air pollution, especially SO_2, on soya production. There is also very little knowledge available of the plants' natural ability to deal with acute levels of air pollution. In this study we intended to establish exposure-response relationship for long-term SO_2 fumigation in two cultivars of soybean (PAN 1666 and LS 6164) and secondly to investigate the effects of SO_2 on the photosynthetic capability of the soybean plants.

CHAPTER 2
Literature overview

2.1 Chemical properties of SO_2

Sulphur dioxide (SO_2) is a colourless gas consisting of one atom of sulphur (S) and two atoms of oxygen (O_2) at atmospheric temperature and pressure. It has a suffocating, choking odour and it is toxic to humans and at concentrations as low as 8 ppm will produce coughing. Sulphur dioxide is over twice as dense as air. The density of SO_2 is 2.618 g/L at 25ºC and 1 atm. When compressed and cooled, sulphur dioxide forms a colourless liquid, which at atmospheric pressure boils at -10°C and freezes at -75.5°C. Liquid SO_2 is heavier than water, having a specific gravity of 1.436 at 0°C. As a vapour, SO_2 is heavier than air, with a relative density of 2.2636 when compared to air at atmospheric pressure and a temperature of 0°C. When heated about its critical temperature, 157.12°C, SO_2 can only exist as a vapour regardless of pressure. Generally, undiluted (dry) sulphur dioxide is not corrosive to ordinary metals; however, when small amounts of moisture are present, sulphur dioxide will attack most metals (Lewis, 1992:1104).

2.2 Uptake of SO_2

The leaves of plants are directly exposed to air pollutants and the air pollutants enter the plant via the stomata on the leaves (Cross, *et al.*, 1998:1241). Sulphur dioxide enters the leaves through the same diffusion pathway as carbon dioxide (Hopkins & Hűner, 2004:477). Although most of the SO_2 enters the leaves through the stomata, SO_2 are also deposited at significant rates to wet surfaces, from here it may dissociate to form sulphite (SO_3^{2-}) or bisulphite (HSO_3^-), which can react with the cuticular waxes on the leaf surface. The cuticle can be damaged to such an extent that a certain amount of SO_2 can enter through the damaged cuticle which will cause the oxidation of SO_2 to occur in the mesophyll tissue in plant cells (Emberson, 2003:15; Winner, *et al*., 1985:119). Resistance to SO_2 upon entrance through the stomata is low due to its high solubility and rapid dissociation (Yang, *et al*., 2006:237).

When SO_2 diffuses through the stomatal opening it dissolves in the apoplastic space, chloroplasts of the mesophyll cells oxidize as well as reduce SO_2 through light dependant reactions (Renuga & Paliwal, 1995:60). The conversion by means of photo-oxidation of HSO_3^- and SO_3^{2-} to the less toxic SO_{4-}^{2-} sets in motion the formation of reactive oxygen species (ROS) (Renuga & Paliwal, 1995:60). The photosynthetic transport mediates the detoxification of SO_3^{2-} and lead to the formation of O_2^-, OH and H_2O_2. Once SO_2 is hydrated divalent sulphurous acid is formed which is neutralized into HSO_3^- and SO_3^{2-}. The chemical reaction and production of ROS due to SO_2 absorption (Rai, *et al.*, 2011:85) are illustrated by the following cascade of reactions.

$SO_2 + H_2O \rightarrow HSO_3^- + H^+$

$HSO_3^- + {}^{\cdot}O_2 + 2H \rightarrow HSO_3$ (bisulphate radical) $+ 2{}^{\cdot}OH$

$HSO_3^- + {}^{\cdot}OH + H \rightarrow HSO_3^- + H_2O$

$HSO_3^- + O_2 \rightarrow SO_3^{2-} + O_2^{\cdot -} + H^+$

$HSO_3^- + {}^{\cdot}OH \rightarrow SO_3^{2-} + H_2O$

$2HSO_3^- \rightarrow SO_3^{2-} + HSO_3^- + H^+$

$SO_3 + H_2O \rightarrow SO_4^{2-} + 2H^+$

${}^{\cdot}O_2^- + {}^{\cdot}O_2^- + 2H^+ \rightarrow O_2 + H_2O^2$

${}^{\cdot}OH + {}^{\cdot}OH \rightarrow H_2O_2$

Figure 2.1: The chemical pathway for SO_2 in plants (Rai, *et al.*, 2011:85).

According to Arora, *et al*., (2002:1230) the sulphurous acid may also be converted to sulphuric acid. Neutralization of divalent sulphurous acid depends on the pH of the apoplast or cytosol. Sulphur dioxide exposure causes a shift in the cytoplasmic pH as acidification occurs (Arora, *et al*., 2002:1227). Proton concentration of the cytoplasm is a critical aspect of regulating cellular activity (Karuppanapandian, *et al*., 2011:715). The buffering capacity of the cellular fluids plays a great role in the impact of SO_2 (Emberson, 2003:15). Metabolism will be affected if cellular mechanisms cannot compensate for the decrease in pH.

According to Takahama, *et al*., (1992:261) detoxification of HSO_3^- and SO_3^2 is achieved either by oxidation to SO_4^{2-} or reduction to sulphide (S^{2-}) and incorporation of the reduced sulphur into organic compounds such as amino acids for example cysteine. At low concentrations HSO_3^- and SO_3^{2-} are effectively detoxified by plants and SO_2 can serve as a sulphur source for plants (Zeiger, 2006). The accumulation of HSO_3^- and SO_3^{2-} can disrupt the balance between incompletely oxidized sulphur compounds and the sulphydryl groups that can be found in glutathione and cysteine essential for structural integrity of protein (Malhotra & Hocking, 1976:232). Sulphite (SO_3^{2-}) is a nucleophilic agent that is able to attack numerous substrates through opening S—S bridges (this reaction is called sulphitolysis) and is therefore responsible for the inactivation of enzymes and proteins (Lang, *et al*., 2007:447). The HSO_3^- and SO_3^{2-} anions are cytotoxic (Takahama, *et al*., 1992:261).

Plants control the internal SO_3^{2-} concentration through controlling the uptake of the gas by the laminar boundary layer, the cuticle or the guard cells and the rate of its metabolic conversion by supplying into the sulphur assimilation stream for production of cysteine, or reoxidation into SO_{4-}^{2-} (Lang, *et al*., 2007:447). Sulphite is oxidized to non-toxic SO_{4-}^{2-} and O_2 in the chloroplasts. Chloroplasts are also the site for the formation of organic sulphur compounds (Garsed, 1985:89).

Sulphur is necessary in general metabolism of vegetation because it is an important component of amino acids, proteins as well as certain vitamins (Li & Yi, 2012:46; Malhotra & Hocking, 1976: 227). Therefore SO_2 uptake from the atmosphere can be used to meet a plants sulphur requirements, however if the concentration of SO_2 rises above a critical level fundamental cellular processes will consequentially be disrupted (Malhotra & Hocking, 1976:227). At ambient temperature SO_2 is highly soluble in water and therefore dissolves completely when it comes in contact with plant moisture. When SO_2 dissolves in water, three types of chemical substances are formed: sulphurous acid (H_2SO_3), bisulphite (HSO_3^-), and sulphite (SO_3^-). The concentration of each of these substances depends upon the pH of the solution in which they dissolve. Sulphur dioxide is also capable to act as both a reducing and an oxidizing agent (Malhotra & Hocking, 1976:228).

The SO_3^{2-} anions are highly reactive and cause a lot of cell damage (Kubo *et al.*, 1995:479). The pH for cytoplasm of most plants is about 7.2 and in these conditions HSO_3^- and SO_3^{2-} will theoretically exist at 50% each (Malhotra & Hocking, 1976:228). HSO_3^- is considered to be the most phytotoxic of these chemical species.

2.3 Damaging effects of SO_2

The physical and the biochemical background to the phytotoxicity of SO_2 can be ascribed to the negative consequences of acidification of tissues upon the dissociation of SO_2 and/or the direct reaction of the formed (bi)sulphite with cellular constituents and metabolites. The impact of SO_2 on plant functioning is ambiguous, since SO_2 may both act as toxin and nutrient (De Kok *et al*., 2002b:201). Plants may even benefit from elevated levels of atmospheric sulphur gases since they contribute to plants sulphur nutrition and exposure may result in enhanced yields, especially when sulphate is deprived in the root environment (De Kok *et al.*, 2000:41). The contribution of SO_2 as a sulphur source for biomass production depends on the duration of the sulphate deprivation.

Injuries to plants are caused when unmanageable levels of air pollutants disrupt the plants' metabolism, resulting in the altering of their appearance and ultimately lowering the agricultural productivity of crops (Heath, 2007:1). The impact of air pollutants can be divided into two classes' namely direct and indirect injury. Direct injury involves the direct uptake of the air pollutant resulting in associated damages in biochemical and physiological processes and this is a major concern. Indirect injury involves for example the acidification of soils. Various physiological processes such as photosynthesis, respiration, carbon allocation along with stomatal function are influenced by air pollutants (Darrall, 1989:1; Malhotra & Hocking, 1976:227). Major impacts of SO_2 on vegetation are visible as foliar injury, altered plant growth and forest decline. When the amount of sulphur (in the form of SO_2) taken up by the plant through the leaves, exceeds the sulphur requirements of the plant toxic symptoms will appear. These symptoms include chlorosis and necrosis, growth inhibition as well as cell death (Li & Yi, 2012:46; Malhotra & Hocking, 1976:228). Bifacial intercostals necrosis and tissue collapse is characteristic when a plant's metabolic pathways are overwhelmed with coping with excess sulphur (Malhotra & Hocking, 1976: 228).

2.4 The assimilation of SO_2

The toxicity of SO_2 can be classified as direct as well as indirect due to its metabolic derivatives SO_3^{2-} and HSO_3^- (Hopkins & Hűner, 2004:477). The toxic effect of SO_2 is consequently a result of H^+; HSO_3^- and SO_3^{2-} which are produced after the entry of SO_2 through the stomata (Renuga & Paliwal, 1995:60). The SO_3^{2-} can directly enter the sulphur reduction pathway and be reduced to sulphide, incorporated into cysteine, and subsequently into other sulphur compounds (Fig. 2.1). Sulphite may also be oxidized to sulphate, extra- and intracellularly by peroxidases or non-enzymatically catalyzed by metal ions or superoxide radicals and subsequently being reduced and assimilated again. Excessive absorbed SO_2 is presumably transferred into the vacuole as SO_4^{2-}. The foliar uptake of H_2S appears to be directly dependent on the rate of H_2S metabolism into cysteine and subsequently into other sulphur compounds (De Kok et al., 1998:51, 2000:41, 2002a:1, 2002b; Figure 2.2). There is strong evidence that *O-acetyl*-serine (thiol)lyase is directly responsible in the active fixation of atmospheric H_2S by plants. Plants are able to transfer from sulphate to foliar absorbed SO_2 or H_2S as sulphur source (De Kok, 1990:125, De Kok et al., 1998:51, 2000:41, 2002a:1, 2002b:201, Yang et al., 2002:255) and levels of 0.06 $\mu l.l^{-1}$ appear to be sufficient to cover the sulphur requirement of plants (Yang et al., 2002:255; Buchner et al., 2004:3396). There is an interaction between atmospheric and pedospheric sulphur utilization. For instance, H_2S exposure resulted in a decreased activity of APS reductase and a depressed sulphate uptake in *Brassica oleracea* (Westerman et al., 2000:443, 2001:425; De Kok et al., 2002b:201). However, H_2S solely affected the expression of the different sulphate transporters in the shoot, but not in the roots (Buchner et al., 2004:3396).

Sulphur dioxide exposure results in tissue damage and the release of stress ethylene in both photosynthetic and non-photosynthetic tissue (Arora, *et al*., 2002:1230; Ali & Alqurainy, 2006:193). The sensitivity of photosynthesis toward SO_2 exposure is evident both between and even within species; this might be as a result of genetic as well as environmental factors during and prior of fumigation. (Darrall, 1989:11; Hopkins & Hűner, 2004:477).

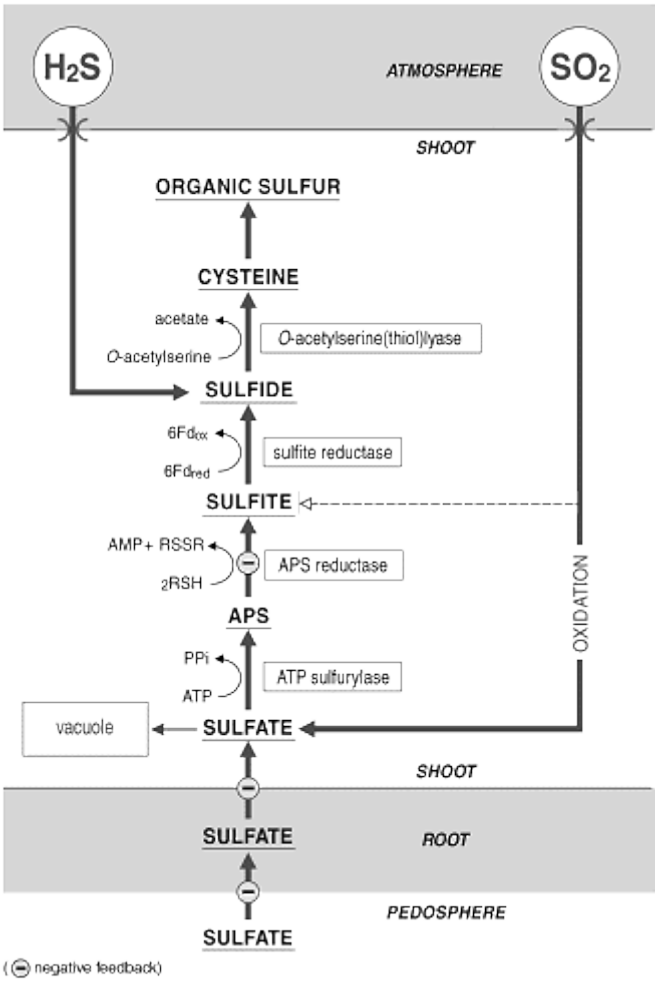

Figure 2.2 SO_2 and H_2S metabolism in plant shoots: Metabolism of SO_2 and H_2S in the plant shoots and possible sites of feedback inhibition of sulphate uptake (APS, adenosine 5'-phosphosulfate; Fd_{red}, Fd_{ox}, reduced and oxidized ferredoxin; RSH, RSSR, reduced and oxidized glutathione; De Kok *et al*., 2002a:1).

Sulphur dioxide at low concentrations can have positive effects on growth as a well as physiological characteristic of plants in particular when plants are growing in sulphur deficient soil; this is possible through normal sulphur metabolism (Malhotra & Hocking, 1976:228). Sulphur is an essential macronutrient for plants and enhances the development of nodules and also affects carbohydrate metabolism (Li & Yi, 2012:46). The sulphate can be metabolised to fulfil the demand for sulphur as a nutrient, through detoxifying SO_3^{2-} and HSO_3^- (Hopkins & Hűner, 2004:478). However an increase in uptake of SO_2 inhibits plant growth, photosynthesis and bio-productivity. Sequentially these inhibitions can bring about an increase in susceptibility to disease as well as

disorganization of cellular components (Renuga & Paliwal, 1995:60). Many of the cell biochemical processes are effected due the SO_2 interfering with structure and permeability of cellular membranes and enzyme activity (Malhotra & Hocking, 1976:229). The destructive effects of SO_2 are due to an accumulation of SO_3^{2-} or sulphate (SO_{4-}^{2-}) (Ali & Alqurainy, 2006:193). When the concentration of polluting gasses becomes too high to be detoxified, injury will be unavoidable and crop yield will decline (Zeiger, 2006:1).

2.5 The production of reactive oxygen species (ROS) during stress conditions

When plants are exposed to either biotic or abiotic stress conditions, it often coincides with the production of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). These increases in ROS have been proposed to be a central component of a plants adaptation to both biotic and abiotic stresses. Under these conditions, ROS can perform different roles like exacerbating damage or signalling the activation of defence responses (Dat *et al*., 2000:779).

It is imperative to understand the concept of reactive oxygen species (ROS) to comprehend the possible implications since it is one of the major factors that affect a plant's productivity in case of environmental stress (Kim, *et al*., 2007:909). Oxidative stress is defined as a shift in the balance between pro-oxidative and antioxidative reactions (Bartosz, 1997:47). Normal cell metabolism in plants results in production of ROS but under stress conditions the sense of balance is disturbed between production and elimination (Karuppanapandian, *et al.,* 2011:709). The major source of ROS in plant tissue is the photosynthetic electron transport system. Chloroplast generates highly active oxygen species by direct donation of excitation energy, or electrons, to oxygen from the photosynthetic electron transport chain. (Arora, *et al*., 2002:1227).

Reactive oxygen species can be described as a free radical with at least one unpaired electron in its outer orbit that is capable of independent existence (Salway, 2006:49). Stress induces the over production of ROS which are very unstable and short-lived; they cause cellular damage by rapid reactions with adjacent molecules (Salway,

2006:49; Karuppanapandian, *et al*., 2011:710). Reactive oxygen species have an important role in inducing protection mechanisms during biotic as well as abiotic stress (Van Breusegem, *et al*., 2001:406). During abiotic stress ROS are essentially produced in organelles with highly oxidizing metabolic activities or with sustained electron flows for example chloroplasts and mitochondria (Van Breusegem, *et al*., 2001:406). The biological consequences of ROS lead to a variety of irreparable metabolic- and physiological dysfunctions that can lead to cell death. (Scandalios, 2005:997). The capability of plants to regulate the equilibrium between oxidants and antioxidants determines if it survives. Plants have the capability to manage these ROS by eliminating them with an efficient ROS-scavenging system (Van Breusegem, *et al*., 2001:406).

Under stress conditions the generation of toxic oxygen species are increased and plants are more susceptible to photo-inhibition (Arora, *et al*., 2002:1227). This is due to damage to the photosynthetic apparatus and will ultimately cause severe cellular damage and leaf chlorosis (Van Breusegem, *et al*., 2001:406). Under normal unstressed circumstances superoxide anion is formed by photo reduction of O_2 by photosystem I (PSI) and photosystem II (PSII) (Srivastava, 1998:526). Once O_2 undergoes one-electron reduction the free radical superoxide is formed. This molecule is charged and can't cross the membranes (Srivastava; 1998:526). Hydrogen peroxide is produced when superoxide anions is further reduced to OH^{1-} and O_2. It is one of the most reactive ROS and known effects are oxidation of proteins, DNA, steroidal compound as well as peroxidation of the unsaturated lipids in cell membranes to form unstable hydroperoxides (Ali & Alqurainy, 2006:199). Free radicals can cause cell damage through lipid peroxidation, inactivation of enzymes and other functional proteins (Bartosz, 1997:49). The formation of ROS, in the extra-cellular fluid, due to O_3 exposure attack unsaturated components of the membranes and therefore causes a loss in membrane function (Fuhrer & Bungener, 1999:356).

Damage to biological systems, as a result of SO_2 exposure, is most probably due to the detoxification of SO_3^{2-} to SO_4^{2-} (Li & Yi, 2012:46). In the chloroplasts, SO_2 oxidation can be initiated by superoxide generated from the photosynthetic electron transport chain. This process can induce the production of ROS and sulphur trioxide radicals. (Klopfenstein *et al*., 1997:326). Oxidative detoxification is possible due to

peroxidases which can oxidize SO_3^{2-} to SO_{4-}^{2-}, provided hydrogen peroxide is available as a reducible substrate (Takahama, *et al*., 1992:261). Sulphite oxidation to $SO_4^{2-}$is initiated by light and is mediated by photosynthetic electron transport (Ali & Alqurainy, 2006:193). The oxidation of SO_3^{2-} gives rise to the formation of O_2^- (Ali & Alqurainy, 2006:193). Loss of photosynthetic function is probably due to the inhibition of the activity of SH-containing, light-activated enzymes of the chloroplast (Arora, *et al*., 2002:1228, Ali & Alqurainy 2006:193).

To counteract the toxicity of ROS, plants have efficient antioxidative defence systems in place to detoxify ROS (Vanová, *et al*., 2002:1227). The antioxidative defence mechanism of higher plants consists of enzymes, low molecular weight compounds (among them peptides, vitamins, flavonoids, phenolic acids, alkaloids, etc.), and integrated detoxification chains. Enzymatic defences in plants include enzymes capable of removing, neutralizing, or scavenging oxy-intermediates.

2.6 The role of peroxidase (POD)

Peroxidases (PODs) are involved in many physiological processes in plants, involving responses to biotic and abiotic stresses and the synthesis of lignin. Plant PODs are haem-containing enzymes which catalyse the single one-electron oxidation of several substrates at the expense of H_2O_2: $2RH + H_2O_2 \rightarrow 2R^- + 2H_2O$ (Scandalios, 2005:1000). In chloroplasts peroxidases scavenge H_2O_2 whereas in the apoplast peroxidases are concerned in the formation of cross-linking's within cell wall constituents and can either be bound or soluble (Takahama,*et al*., 1992:261).

The involvement of PODs during plant stress has been demonstrated during several biotic and abiotic stress conditions. Plants that are able to withstand stress conditions are able to induce POD activity to higher levels that are normally expressed in plant tissue.

Peroxidases (PODs) in plants and their involvement in defence mechanisms against air pollutants have been suggested on occasion but the role of POD during air pollution stress is however poorly understood (Kim, *et al*., 2007:909). Peroxidases are

detoxifying enzymes that can be found in several cellular compartments and is of great value with screening sensitivity (Takahama, *et al.*, 1992:261).

2.7 The role of lipoxygenase (LOX)

Lipoxygenases (LOX) are a family of non-heme iron-containing enzymes that catalyze the hydroperoxydation of polyunsaturated fatty acids (PUFA); singlet oxygen ($^1O_2$) and/or superoxide (O_2^-) (Ali & Alqurainy, 2006:209; Karuppanapandian, *et al*., 2011:710). The catalyzation of hydroperoxidation of PUFA is a response to stress but is also another possible source of ROS species (Karuppanapandian, *et al*., 2011:714). In plants, linolenic and linoleic acids are the majority ordinary substrates for LOX (Porta, 2002:15). Lipoxygenase transform PUFAs to lipid hydroperoxides (LOOHs) with a reaction called lipid peroxidation, these lipid-peroxidation products represent biological signals and a non-specific response (Spiteller, 2003:6). Lipid peroxidation is a natural metabolic process under normal aerobic conditions and is an important consequence of ROS action on membrane structure and function (Ali & Alqurainy, 2006:211). It was found that lipid peroxidation and the subsequent chlorophyll bleaching are effects of SO_2 on plants (Bartosz, 1997:56). Lipoxygenase plays a primary role in generating peroxidative damage in membrane lipids through peroxidation reactions on plasma membrane lipids, and therefore decrease lipid unsaturation and membrane fluidity (Mao, *et al*., 2007:403).

The involvement of LOX in defence reactions became evident as a response to environmental stress and pathogen exposure (Spiteller, 2003:8; Blée, 2002:317). The resistance of tobacco to fungal infections was found to be dependent on a specific inducible LOX (Blée, 2002:317). Lipoxygenase is important for the synthesis of products such as jasmonic acid that is of great importance in defence against insects and pathogens (Blée, 2002:317). Lipoxygenase enzymes cause peroxidative damage to the membrane lipids resulting in a decrease of lipid unsaturation as well as membrane fluidity.

2.8 Influence of SO_2 on stomatal behaviour

The response of stomata to air pollution needs to be explored for the reason that the stomata manage water lost from plants as well as the exchange of gasses between the interior of the leaves and the atmosphere (Black, 1985:96). Stomata are responsible for gas exchange and conductance is the term used to describe the effectiveness of gas diffusion (Winner, *et al*., 1985:119). If the control over water relations are influenced the cellular turgor, growth and effectiveness of important physiological and biochemical processes will experience change (Black, 1985:96).

Sulphur dioxide causes direct damage to leaves of crop plants and trees when it enters the stomata. There is evidence of acclimation to pollutant exposure by stomatal closure (in response to injury to the photosynthetic processes) and by enhanced rates of respiration for repair and detoxification processes (Darrall, 1989:18). Stomatal responses to SO_2 are different and depend on a variety of factors. There are a variety of theories how SO_2 can cause either an increase or a decrease in stomatal conductance. Alterations in stomatal response can result in change the transpiration rate, SO_2 absorption as well as CO_2 assimilation (Winner, *et al*., 1985:119). Both stomatal closing as well as an increased stomatal opening as a result of exposure to air pollutants can have a negative effect on plants. An increased opening will increase transpiration and this can lead to water stress and will therefore have a restriction on growth (Black, 1985:114). If stomatal closure occurs photosynthesis will be reduced as a result.

2.9 Influence of SO_2 on assimilate distribution

Air pollutants can cause a change in assimilate distribution (Darrall, 1989:18). In the determination of plant productivity it is essential to take the processes of carbohydrate production, distribution and utilization in account (Darrall, 1989:18). The rate of carbohydrate production during photosynthesis are influenced by the resistance of gas exchange between mesophyll cells and atmosphere; carbon fixation facilitated by ribulosebiphosphate carboxylase as well as the regeneration of carbon fixing enzymes (Darrall, 1989:3). The changes in carbohydrate distribution can be the result of a reduction in photosynthetic carbon fixation and a greater demand for assimilate at the

source. The reduced translocation of carbohydrates to the roots of plants will give rise to a smaller root mass to utilize accessible soil moisture (Darrall, 1989:26).

2.10 Influence of SO_2 on photosynthesis

The chloroplast and photosynthesis has been observed to be the primary site of SO_2 injury (Hopkins & Hűner, 2004:478). Sulphur dioxide is known to cause the disruption of the chloroplast membrane, plasmalemma and other membranes, inhibit enzymes and generally disturb overall metabolism of plants (Hopkins & Hűner, 2004:478). The photosynthetic rate at any time is regulated by several physiological factors. These factors include stomatal conductance; biochemical integrity of organelles, membranes, enzymes as well as leaf nutrient content (Winner,*et al*., 1985:118). The membranes of chloroplasts are fragile structures that can easily be disrupted by SO_2 (Malhotra & Hocking, 1976:229). Sulphur dioxide and CO_2 compete for binding sites on carbon-fixing enzymes for example ribulose 1, 5-diphosphate carboxylase which is central to photosynthetic CO_2 fixation (Malhotra & Hocking, 1976:230; Winner, *et al*., 1985:120). HSO_3^- compound reduce photosynthetic CO_2 fixation in isolated chloroplasts by the inhibition of PEP carboxylase and NADH malate dehydrogenase (Malhotra & Hocking, 1976:232).

The balance between light harvesting and energy utilization are disturbed by environmental stress and will lead to an extended half-life of singlet chlorophyll (^{1}Chl) (Karuppanapandian, *et al*., 2011:710). The ^{1}Chl may form triplet chlorophyll (^{3}Chl) which when reacting with ground state triplet oxygen ($^3O_2$) will lead to the formation of $^1O_2$. Singlet oxygen that arise in the chloroplast will in all likelihood react with membrane proteins and lipids close to the site of production (Karuppanapandian, *et al*., 2011:710). The production of $^1O_2$ in the chloroplast will most probably affect the reaction centre of the photosystem II (PSII) (Karuppanapandian, *et al*., 2011:710). Damage at photosystem II can also occur when the absorption of excitation energy exceeds the capacity of dissipitation by the plant (Ranieri, *et al*., 1999:920). The inhibitory effect of SO_2 on photosynthesis is possibly as a result of in activation of electron carriers (Malhotra & Hocking, 1976:231).

Sulphur dioxide decreases the photosynthetic capacity of mesophyll cells and their ability to fixate CO_2 (Winner, *et al*., 1985:121). Bisulphite compounds most likely inhibit CO_2 fixation by interfering with chloroplast membrane by affecting other transport systems associated with the chloroplast membranes during photosynthesis (Malhotra & Hocking, 1976:229). Chloroplast are greatly affected by SO_2 and the degradation products generated and is known to result in an impairment of the chloroplast functionality through a loss of net CO_2 assimilation, decline in photosynthetic electron transport rate and the inhibition of dark reaction of photosynthesis (Ranieri, *et al*., 1999:920). The strong oxidizing capabilities of absorbed SO_2 influence cell and organelle membranes, disulfide bonds of enzymes as well as chlorophyll (Winner, *et al*., 1985:120).

The oxidation of SO_2 produces sulphuric acid which acidify the cytoplasm and therefore a shift in the cytoplastic pH. The increase in acidification loads the cytoplasm with the SO_4^{2-} anion, which in elevated concentrations inhibits photosynthetic reactions (Renuga & Paliwal, 1995:60). The proton concentration in cytoplasm is one of the most important factors regulating enzyme activity (Arora, *et al*., 2002:1230; Ali & Alqurainy, 2006:193). The acidification occurs due to a reaction with water to form sulphurous acid which may then be converted into sulphuric acid. In the chloroplast a thylakoid bound reductant in PSI and reduced ferredoxin photo produce superoxide through auto-oxidation. The presence of SO_3^{2-} causes the O^{2-} initiated aerobic chain oxidation of SO_3^{2-} to yield a larger extent of O^{2-}, hydrogen peroxide (H_2O_2) and OH^{-1} than those formed in its absence (Renuga & Paliwal, 1995:60). These active O_2 molecules are highly reactive and lead to oxidative damage when accumulated. Electron transport capacity controls the regeneration of carbon-fixing enzymes (Darrall, 1989:1).

The oxidation of SO_3^{2-} is initiated by light and is mediated by photosynthetic electron transport (Karuppanapandian, *et al*., 2011:715). The oxidation of SO_3^{2-} to SO_4^{2-} in the chloroplast also gives rise to the formation of O_2^- (Karuppanapandian, *et al*., 2011:715). A loss of photosynthetic function follows caused by inhibition of the activity of SH-containing light-activated enzymes of the inhibition of the activity of SH-containing light-activated enzymes of the chloroplast (Karuppanapandian, *et al.*, 2011:715). The exposure to SO_2 results in tissue damage and consequently ethylene

release in the photosynthetic and non-photosynthetic tissue (Karuppanapandian, *et al*., 2011:715).

2.11 Problem statement

Air pollution levels over the Highveld area in South Africa are reaching levels that are problematic. There is already a decrease found in soybean yield that is ascribed to the high levels of air pollution. South Africa is dependent on agriculture for food supply and therefore it is it of great significance to quantify the effect of air pollution on agricultural crops.

2.12 Objectives

- Quantifying the effect of SO_2 on growth, photosynthesis, lipoxygenase and peroxidase activities of *Glycine max*.
- To establish the exposure-response relationship of SO_2 in *Glycine max* and the critical levels that lead to a reduction in yield.
- Establish the genotype variation of *Glycine max* in relation to SO_2 air pollution.

2.13 Hypothesis

Increasing SO_2 levels will lead to a decrease in growth and photosynthesis of *Glycine max*. Tolerance towards SO_2 in both cultivars is expected to be related to their ability to express antioxidant enzymes namely lipoxygenase and peroxidase.

Chapter 3
Material and Methods

The North-West University (School for Biological Sciences, Potchefstroom) has a battery of twelve open-top chambers (OTC's) where the impact of air pollutants are studied on plants. The OTC system is a scientifically accepted method to study the effects of air pollution and is used worldwide. The facility consists of twelve open-top chambers each connected to analysers which enables us to accurately fumigate the plants. The design and operation of the specific OTC system used has been previously published (Heyneke, *et al*., 2012a). In order to quantify the effect of SO_2 on soybeans morphological, physiological and biochemical investigations were included. Destructive as well as non-destructive methods were used to evaluate the effect of SO_2 on the plants. The SO_2 concentrations (of 25 ppb, 75 ppb and 150 ppb) used in this study were requested by the Protein Research Foundation. The data was processed by means of excel 2007, while graphs were constructed by making use of SigmaPlot 10. The standard error was used in the graphs to indicate the standard deviation of the sampling distribution. Analyses of variance (ANOVA) were carried out to establish the statistical significance of the data accumulated by means of Statistica 10.

3.1 Plant material and cultivation

Two commonly planted soybean cultivars namely PAN 1666 and LS 6164 were used in this study. The seeds of PAN 1666 and LS 6164 were planted in a soil mixture of 2:1:1 (soil : sand : vermiculite) in 16 dm^3 pots. Roughly 25 mg of a six month slow release fertilizer (Osmocote) containing 14N:9P:15K:2MgO (Plantacote® pluss, Aglukon Spezialdünger, GmbH & Co.KG, Heerdter Landstraβe 199, D-40549, Düsseldorf, Germany) were added to each pot.

Figure 3.1.1: The open top chambers: The series of open-top chambers (OTC's) on the premises of the North-West University, Potchefstroom, which were used to fumigate the soybean plants with SO_2.

Irrigation was achieved by placing glass fibre wicks (Thoenes Dichtungstechnik GmbH, Germany) at different levels in the pots. The glass fibre wicks were cut at specific lengths (120 cm x 1 and 60 cm x 3) and layered clockwise, at different evenly spaced depths within the pots to guarantee consistent wetting of the soil (Fig. 3.1.2). The protruding ends went through the drainage holes in the base of the pots into water reservoirs (plastic pots). The content of soil water was maintained through capillary action. Water reservoirs were filled daily and simplified by connection of reservoirs to a water tap through a network of PVC tubing.

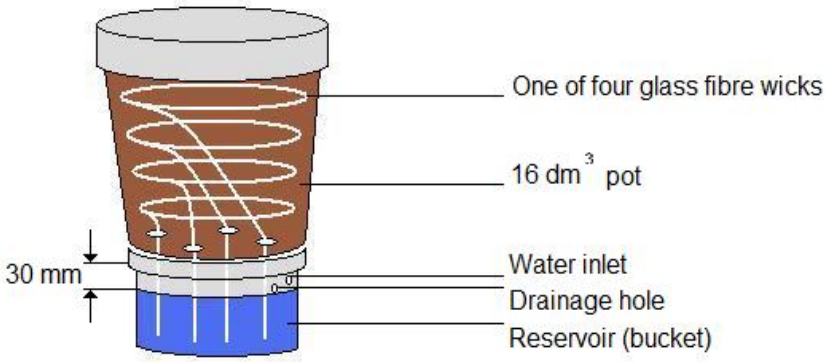

Figure 3.1.2: The water irrigation system: Schematic representation of the water irrigation system used to control the water supply to soil through glass fibre wicks. These wicks were sufficient in providing a reasonable water supply to the plants in the Open-Top-Chambers.

3.2 Experimental design and SO_2 fumigation

Sulphur dioxide fumigation started 47 days after emergence of the 3rd trifoliate leaf and continued until the plants reached maturity. The soybeans were fumigated for 7 hours daily, 7 days a week at 0, 25; 75 and 150 ppb SO_2. For the 0 ppb SO_2 treatment, the air entering the chambers was first filtered through a purafill filter to remove all SO_2 that might be present in the air. Two open-top chambers were allocated for each SO_2 concentration and eight pots were placed in each chamber, four pots with one plant per pot for each cultivar. The SO_2 levels in the chambers were measured at 15 min intervals in each chamber by a SO_2 analyser.

3.3 Morphological parameters

3.3.1 Plant growth and development

The vegetative development of the soybean plants were quantified by measuring the plastochron index (PI) three times a week. This is an easy non-destructive method where variations as a result of germination time and growth rate are eliminated and creates a linear scale for the measurement of development based on morphology (Erickson & Michelini, 1957). All trifoliate leaves with central leaflet exceeding a reference 25 mm (L_{ref}) were counted. Measurements were taken of the length of the youngest central leaflet longer than or equal to 25 mm as well as the length of the central leaflet shorter than 25 mm on the next trifoliate leaf. The plastochron index of each plant was calculated using the subsequent formula:

$$PI = n + \frac{\log Ln - \log\lambda}{\log Ln - \log(Ln + 1)}$$

Where n indicates the number of the trifoliate leaves equal to or just longer than the reference leaf, λ is the reference value length (25 mm were used in this study), L_n is the length of the central trifoliate leaf and L_{n+1} is the length of the central leaflet on trifoliate leaf. L_{n+1} is by definition shorter than λ (Smit, *et al*., 2009:630).

3.3.2 Foliar injury

After four weeks of SO_2 fumigation leaves were photographed to document acute and chronic foliar damage inflicted by the pollutant.

3.3.3 Shoot length

After plants had been exposed to four weeks of SO_2 fumigation the height of the shoots of the plants were measured and documented. The shoot length was measured from the surface to the tip of the shoot.

3.3.4 Biomass accumulation

When plants reached seed maturity, plants were removed from pots. The shoots and roots were separated where after pods and nodules were harvested. The separated shoots and roots were cleaned to remove access soil. Both shoots and roots were oven dried at 80˚C for 48 hours.

3.3.5 Yield parameters

At harvest time the pods were separated from plants. The pods per plant were counted as well as the nodules on plants from different treatments. Seeds were removed from the pods and the weight of 30 seeds was determined for each treatment.

3.4 Physiological measurements

3.4.1 Chlorophyll content index

The chlorophyll content index (CCI) was measured on intact leaves at 15, 29 and 37 days after SO_2 fumigation commenced. Chlorophyll content index was measured with a hand-held chlorophyll meter (CCM-200, Opti-Sciences, Inc., USA). The chlorophyll content index was recorded by taking 6 measurements on the central leaflet on the fourth fully expanded trifoliate leaf of 4 plants.

3.4.2 Measurement of photosynthetic gas exchange

Photosynthetic CO_2 gas exchange was measured with an automatic infrared gas analyzer (CIRAS 2, PP Systems, Hertz, UK) on the central leaflet on the fourth fully expanded trifoliate leaves of four different plants per treatment. These measurements were taken after 4 and 6 weeks after SO_2 fumigation commenced. The CIRAS makes it possible to measure the rate of photosynthesis gas exchange and transpiration simultaneously. Photosynthetic gas exchange and transpiration are interdependent due to the fact that the route for intake of CO_2 and the loss of water is mainly the same.

Light intensity of the CIRAS cuvette was maintained at 1200 µmol photons $m^{-2}.s^{-1}$ to guarantee activation of Rubisco and the leaf temperature was kept at 26°C during measurements. Carbon dioxide assimilation rate (A) against intercellular CO_2 concentration (C_i) curves was generated by increasing the CO_2 concentration with 3 minute increments from 0 to 1000 µmol mol^{-1}, in the sequence 360, 25, 50, 75, 100, 200, 360, 500, 700 and 1000 µmol mol^{-1}. A CO_2 dependency curve ($A:C_i$) present several of the most significant key parameters of photosynthetic gas exchange analysis. The interpretation of the CO_2 response of photosynthesis was done with the assistance of the model of C_3 photosynthesis developed by Farquhar and Sharkey (Farquhar & Sharkey, 1982:318).

3.4.3 Fluorescence measurements

Chlorophyll *a* fluorescence induction kinetics was measured weekly on the central leaflet of the fourth fully expanded trifoliate of four different plants per treatment using the Handy Plant Efficiency Analyser (HANDY-PEA, Hansatech Instruments Ltd., Kings Lynn, Norfolk, UK). Fluorescence measurements were taken 2 hours after sunset when leaves were dark adapted. Each fluorescence induction transient was provoked homogenous by red light (peak at 650 nm) at a fluence rate of 2000 µmol photons.$m^{-2}.s^{-1}$ (sufficient excitation intensity to ensure complete closure of PSII reaction centres to obtain true maximal fluorescence intensity). The fluorescence signal, emitted at 720 nm, was recorded for one second, with 10 µs time resolution from 10 µs to 0.3 ms, every 1 ms (3-30 ms), every 10 ms (30-300 ms) and every 100 ms (300 ms to 1 s) on

a 4 mm diameter area of a dark-adapted leaf. The fluorescence transients were analysed according to the equations of the JIP-test (Strasser, *et al*., 2004:326; Strasser, *et al*,. 2007:325; Tsimilli-Michael, *et al*., 2008:76).

The initial fluorescence, F_O, was measured at 50 µs (O-step) when all the reaction centres of PSII are open, i.e. when the primary acceptor quinone Q_A is fully oxidized. Fluorescence intensity F_J was measured at 2 ms (J-step), fluorescence intensity F_I at 30 ms (I-step), and maximal fluorescence intensity F_M was measured at 300 ms (P-step), when the excitation energy is high enough to ensure the closure of all the reaction centres of PSII, i.e. the full reduction of all reaction centres. To deduce information and computations from the O-J-I-P transient's normalizations and computations were performed using the Biolyzer 4HP software (ver. 4.0.30.03.02). The O-J-I-P transients were analysed according to the equations of the JIP-test (Strasser, *et. al*., 2004:326). The differences in relative variable fluorescence were calculated for the O-P phase (ΔV_{OP}), O-J phase (ΔV_{OJ}), J-P phase (ΔV_{JP}), and K-I phase (ΔV_{KI}). To calculate the difference in relative variable fluorescence (ΔV) at each step, the normalized data of the treatments ($V_{treatment}$) were subtracted from the normalized data of the control ($V_{control}$; $\Delta V = V_{treatment} - V_{control}$). Normalization for each data set was performed from the following equation $V = (F_t - F_{min})/(F_{max} - F_{min})$.

Table 3.4.3.1: Explanation of O-J-I-P parameters: Explanation of the technical fluorescence parameters of the O-J-I-P curves and the selected JIP-test parameters.

Data necessary for the calculation of the JIP parameters	
Area	Area between fluorescence curve and maximum fluorescence (F_M)
T_{Fmax}	The time needed to reach F_M.
F_0	First reliable fluorescence value after the onset of actinic illumination at 50 µs ($F_{50µs}$).
F_J	Fluorescence intensity at the J-step measured at 2 ms (F_{2ms})
F_I	Fluorescence intensity at the I-step measured at 30 ms ($F30_{ms}$)
F_P	Fluorescence intensity at the P-step measured at 300 ms ($F30_{ms}$)
F_M	Maximal fluorescence intensity of the OJIP curve ($F_P = F_M$)
F_v/F_O	$(F_M - F_O)/F_O = k_P/k_N$
$(\Delta V/\Delta t)_0$ or M_0	Slope of the normalized curve at the origin of the fluorescence rise. It is a measure of the rate of the primary photochemistry. $M_0 = 4(F_{300} - F_0)$ expressed per 1 ms
Technical fluorescence parameters	
V_J	Relative variable fluorescence at 2 ms. $V_J = (F_{2ms} - F_0)/(F_M - F_0)$
V_I	Relative variable fluorescence at 30 ms. $V_I = (F_{30ms} - F_0)/(F_M - F_0)$
S_M	Normalised area. Measures the energy needed to close all reaction centres. Area/$(F_M - F_0)$
N	Number of turnover of Q_A. Indicates how many times Q_A is reduced in the time-span from 0 to T_{Fmax}. $N = S_M.M_0.(1/V_J)$.
Quantum efficiencies or flux ratios or yields	
φ_{Po} or TR_0/ABS	Trapping probability, or Quantum yield efficiency. Expresses the probability that an absorbed photon will be trapped by the PSII reaction centre. $\varphi_{Po} = [1 - (F_0/F_M)] = F_V/F_M$
φ_{Eo} or ET_0/ABS	Quantum yield for electron transport (ET).$\varphi_{Eo} = [1 - (F_0/F_M)] .\Psi_0$
φ_{Ro} or RE/ABS	Quantum yield for the reduction of end electron acceptors at the PSI acceptor side (RE).$(1 - V_I)/(1-V_J).\varphi_{Eo}$
Ψ_{E0} or ET_0/TR_0	Expresses the probability that an excitation trapped by the PSII reaction centre enters the electron transport chain. $\Psi_0 = 1 - V_J$
k_P	Photochemical de-excitation constant. $k_P = (ABS/CS)k_F[1/F_0)Area/(F_M - F_0)(1/F_M)]$
k_N	Non-photochemical de-excitation constants, summing up k_H (for heat dissipation) and k_F (for fluorescence emission). $k_N = (ABS/CS)\ k_F\ (1/F_M)$
Specific fluxes or specific activities (expressed in arbitrary units)	
ABS/RC	Effective antenna size of an active reaction centre. Expresses the total number of photons absorbed by chlorophyll molecules of all reaction centres divided by the total number of active reaction centres.M_0 . (1 / VJ) . (1 / φ_{Po})
TR_0/RC	Maximal trapping rate of PSII. Describes the maximal rate by which an excitation is trapped by the reaction centres.M_0 . (1 / VJ)
ET_0/RC	Electron transport in an active reaction centre.M_0 . (1 / VJ) . Ψ_0
DI_0/RC	Effective dissipation in an active reaction centre. (ABS / RC) - (TR_0 / RC)

Subscript "0" indicates that the parameter refers to the onset of illumination

Table 3.4.3.1: Explanation of O-J-I-P parameters continues:

Phenomenological fluxes or phenomenological activities	
ABS/CS	Absorption per cross-section (CS). ABS/CS chlorophyll content, or F_0 (CS_0) or F_M (CS_M) ABS / CS_{Chl} = Chl / CS or ABS / CS_0 F_0 or ABS / CS_M F_M
TR_0/CS	Trapping per cross-section. φ_{P0} . (ABS / CS)
ET_0/CS	Electron transport per cross-section. φ_{P0} .Ψ_0 . (ABS / CS)
DI_0/CS	Dissipation per cross-section. (ABS / CS) - (TR_0 / CS)
Performance indexes	
PI_{ABS}	Performance index for energy conservation from excitation to the reduction of intersystem electron acceptors. (RC / ABS) . [φ_{P0} / (1 - φ_{P0})] . [Ψ_{E0} / (1 - Ψ_{E0})]
$PI_{ABS, total}$	Performance index for energy conservation from excitation to the reduction of PSI end acceptors. (RC / ABS) . [φ_{Po} / (1 - φ_{Po})] . [Ψ_{E0} / (1 - Ψ_{E0})] . [δ_{R0}/ (1-δ_{R0})]
Driving force	
DF_{ABS}	log [PI_{ABS}]
$DF_{ABS,total}$	log [$PI_{ABS, total}$]

Subscript "0" indicates that the parameter refers to the onset of illumination

3.4.4 Stomatal conductance

Stomatal conductance was measured with a steady state leaf porometer (SC-1, Decagon Devices, Pullman, WA) six days after SO_2 commenced. Stomatal conductance is a function of the degree of opening, size and density of the stomata and therefore of great importance. The stomatal conductance was measured by putting the conductance of a leaf in series with two known conductance elements. The humidity measurements between the conductance elements are measured. In other words the vapour flux from the leaf surface to the atmosphere was measured. A fixed diffusion path was clamped to the surface of the leaf and afterwards the vapour pressure gradient in the diffusion path and the known vapour conductance through the fixed path was used to determine the vapour flux. Millimoles per meter-squared seconds (mmol / m^2s) are the unit used for it is a unit of conductance.

Stomatal conductance measurements were done on the lower (abaxial) epidermis on the central leaflet of the forth trifoliate leaflet of *Glycine max* plants. The consistent reading on this leaf was done to minimize variability in readings and to assure comparable readings for all treatments.

3.4.5 Sulphur content

Sulphur dioxide analysis were done by the Institute of Biodiversity (Johann Heinrich von Thünen-Institute (vTI); Federal Research Institute for Rural Areas, Forestry and Fisheries Bundesallee 50 38116 Braunschweig Germany). The sulphur content was expressed as the percentage sulphur in powdered dry (oven dried at 85°C) leaves. Automatic sulphur analyzer is a combined instrument analyzing carbon, nitrogen or sulphur.

3.5 Biochemical measurements

3.5.1 Leaf sampling and protein extraction

Whole trifoliate leaves of *Glycine max* were sampled around midday and immediately frozen in liquid nitrogen and stored at -20˚C for subsequent analyses. Extraction of enzymes was performed according to Rao, *et al.,* (1997:137). One gram of frozen leaf tissue was ground in 10 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 100 mg acid washed sand, 1 mM Methylenediaminetetra-acetic acid (EDTA) and 1% (m/v) polyvinylpyrrolidone (PVP). After centrifugation, (25 000 g, 20 min) the supernatant was used for LOX and POD assays.

3.5.2 Determination of protein concentration

The soluble protein content of the supernatants was determined according to the dye-binding assay technique of Bradford (1976). The reaction mixture contained 100 µL enzyme extract; 100 µL distilled water and 800 µL Bradford reagent. For the protein standard 100µL of a 0.5 $\mu g.\mu L^{-1}$ γ-globulin solution was used. The absorbance was measure at 595 nm.

3.5.3 Determination of lipoxygenase (LOX) activity

The LOX reaction mixture consisted of 1 mL of a 0.1 M sodium citrate phosphate buffer (pH 6.2), 50 µL enzyme extract and 150 µL of 2.5 mM linoleic acid. The continuously change in absorbance was measured at 234 nm for 15 min at 30˚C with a double beam spectrophotometer equipped with an electronic temperature control unit.

Lipoxygenase was expressed as nmol HPOD.mg^{-1} protein.min^{-1} (Grossmann & Zakut, 1979; Ocampo *et al*., 1986)

Preparation of the linoleic acid substrate (2.5mM linoleic acid in 0.15% Tween 20): Linoleic acid (400 µL), 768 µL Tween 20 and 40mL methanol were added into a round bottomed flask and subjected to rotary evaporation at 60˚C until dry. The residue was redissolved in 500 mL 0.05 M sodium phosphate buffer (pH 9). The entire volume was divided into 5mL aliquots and stored in air tight bottles at -20˚C. During transfer to the air tight bottles, nitrogen gas was bubbled through the content of round-bottomed flask and into the small bottles before adding the aliquots. The substrate was used once and stored on ice during experiments.

3.5.4 Determination of peroxidase (POD) activity

The peroxidase assay mixture consisted of 40 mM potassium phosphate buffer (pH5.5), 5 mM guaiacol and 8.2 mM H_2O_2. The continuous change in absorbance was measured at 470 nm for 180 seconds at 30°C. Specific POD activity was expressed as nmol tetraguaiacol.mg^{-1} protein.min^{-1} (Zieslin & Ben-Zaken; 1991:147).

Chapter 4
Results and discussion

4.1 Morphological parameters

4.1.1 Plastochron index

The age and growth of the plants were assessed to assure that comparisons between different treatments are reliable. The growth rate was documented according to the plastochron index (PI) of *Glycine max* plants (Fig. 4.1.1). The PI confirmed that all plants used in the study were on the same growth stage at the onset of SO_2 fumigation (Fig. 4.1.1).

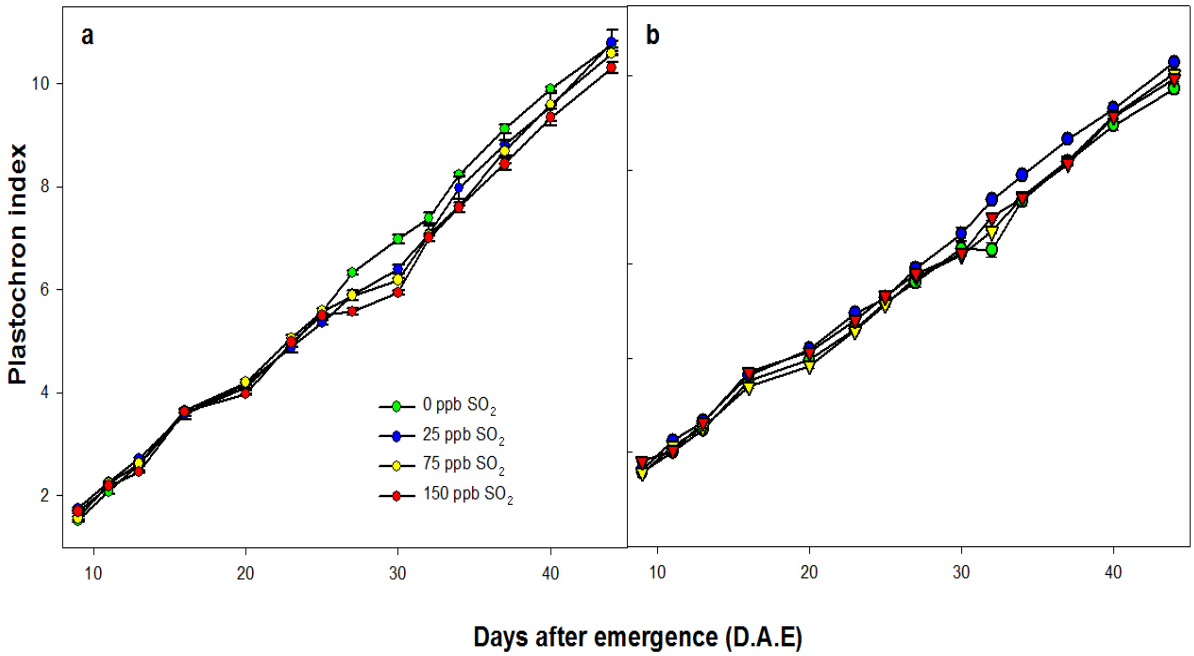

Figure 4.1.1: Plastochron index: Plastochron index of *Glycine max* plants (a) PAN 1666 and (b) LS 6164. The PI indicates that all plants that were grown in the OTCs, were at the same stage of development, before fumigation commenced at 18 days after emergence. (n = 4)

After 30 days of SO_2 exposure, there were no significant ($p > 0.05$) differences between the different treatments. Plastochron index of the PAN 1666 plants decreased after 30 days of fumigation for the 25 ppb, 75 ppb and 150 ppb treatments

with 8.5%, 11.3% and 14.8% respectively in comparison to the CF control (0 ppb treatment) (0 ppb treatment) (Fig. 4.1.1.a). However the plastochron index of LS 6164 had an increase of 5% for the 25 ppb treatment, while the 75 ppb and the 150 ppb treatments decreased with 2.2% and 1.6% respectively in comparison to the CF control (0 ppb treatment) (Fig. 4.1.1.b).

The plastochron index was used in previous studies to quantify vegetative development, where a higher plastrochron index resulted in more rapid vegetative development (Van Heerden, *et al*., 2004:243). The growth rate of PAN 1666 was affected after 30 days of SO_2 fumigation and a SO_2-concentration dependant inhibition was observed. It seemed that after 30 days the growth rate stabilized again between different levels of SO_2 exposure and no further mentionable changes were observed. The exponential growth of all the plants could also be observed in these results.

4.1.2 The effect of SO_2 on foliar injury

After four weeks of SO_2 fumigation leaves from both *Glycine max* cultivars were photographed to illustrate visible damage. The *Glycine max* plants exposed to no SO_2 conditions had a healthy appearance (Fig. 4.1.2.a) while those exposed to SO_2 developed signs of SO_2 damage. The visible symptoms became evident as tan-coloured spots, and similar visible observations were made for both cultivars. Foliar injury progressed with increasing SO_2 levels and was more prominent on older basal leaves.

Acute as well as chronic foliar injury occurred with SO_2 fumigation and was noticeable at 4 weeks of fumigation when photos were taken. Chronic damage was visible as marginal and interveinal chlorosis (yellowing) although the affected areas remained turgid (Fig. 4.1.2.b). Acute SO_2 injury was visible as white interveinal necrosis with slight dark pigmentation at the margins of necrotic areas (Fig. 4.1.2.c). Pigmentation was present before necrotic areas became noticeable. The light brown necrosis progressed from the edge of leaves as indicated in both Fig. 4.1.2.c and Fig. 4.1.2.d.

In Figure 4.1.2.d the overall visible SO_2 damage on *Glycine max* plants at 150 ppb SO_2 was prominent. Both acute and chronic foliar injury symptoms were present

simultaneously, however the light brown necrosis was particularly noticeable. It seemed that the older leaves were more affected and damaged than the younger leaves of the plant, most probably for the reason that they were longer exposed to SO_2. The younger apical leaves mostly had visible interveinal chlorosis.

Figure 4.1.2: Foliar SO_2 injury: Leaves of *Glycine max* plants after four weeks of SO_2 fumigation. (a) *Glycine max* plants with no visible foliar damage can be seen. (b) Interveinal chlorosis on *Glycine max* plants indicating chronic foliar damage, (c) *Glycine max* plants with acute SO_2 foliar damage, visible through the light brown necrosis progressing from the edge of the leaves. It is observable in this photograph (d) that the older leaves at the bottom have more chlorosis and necrosis than the younger leaves at the top of the shoot.

Foliar damage was prominent on plants exposed to high concentrations of SO_2 as expected. Necrotic as well as chlorotic injury symptoms were noticeable as a result of SO_2 fumigation in both cultivars. The foliar injuries concluded in previous studies confirm the results observed in this study (Heyneke, *et al*., 2012b:16). At full stage of development acute SO_2 injury symptoms on broadleaved plants consisted of bifacial, marginal and/or interveinal necrosis and chlorosis where the colour of necrosis varies depending on the plant species (Rai, *et al*., 2011:84).

Visible injury such as bleaching and necrosis at leaf margins and intervienal chlorosis can be attributed to the disruption of chloroplast membranes and the breakdown of chlorophyll (Hopkins & Hűner, 2004:478). The previous statement can be confirmed with the corresponding decrease in measured chlorophyll content index values (Fig. 4.2.1.1). The bleaching effect of SO_2 on plant leaves was confirmed on citrus and this effect can extend across veins in case of SO_2 damage (Darley, *et al*., 1956:9). SO_2 injury on broadleaved trees was observed to be intervienal or marginal chlorosis or necrosis. Foliar injury seen as marginal and interveinal chlorosis were also observed in a previous study of SO_2 on *Glycine max* (Heyneke, *et al*., 2012b:16) and is a further confirmation of the findings of acute and chronic foliar injury that correlated with the decrease in chlorophyll content in this study.

4.1.3 Shoot length

The effects on shoot length after four weeks of SO_2 fumigation are represented in Fig. 4.1.3.1, whereas the visible differences between treatments can be seen in Fig. 4.1.3.2 and Fig. 4.1.3.3. The shoot growth of PAN 1666 and LS 6164 increased insignificantly ($p > 0.05$) by 2.2% and 11.2% at 25 ppb SO_2 treatment was observed respectively in comparison to the CF control (0 ppb treatment) (Fig. 4.1.3.1). There was a decrease at 75 ppb and 150 ppb for both cultivars, PAN plants decreased by 4.1% and 14.2 % respectively for 75 ppb and 150 ppb (the largest reduction in shoot length), while LS 6164 decreased with 6.1% and 9.7% were observed. None of these findings however were significant ($p \leq 0.05$).

The observations made in relation with a reduction in plant growth and development is correlated with the shoot-to-root ratio (Fig. 4.1.4.2). The difference in shoot length is

visible when plants are compared to each other (Fig. 4.1.3.2 and Fig 4.1.3.3). The CF control (0 ppb treatment) (0 ppb treatment) plants of both cultivars appeared to have the most successful growth. The PAN 1666 plants appeared to have an overall shorter, bushier appearance compared to the LS 6164 plants.

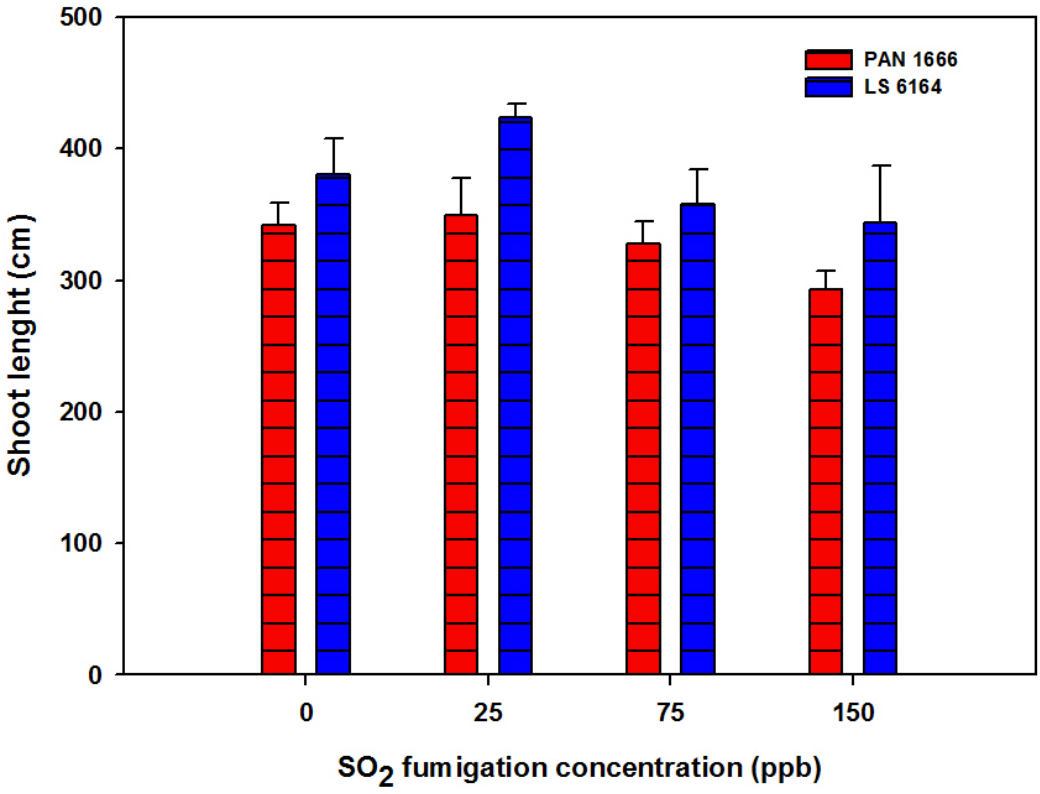

Figure 4.1.3.1: Shoot length: The effect of different SO_2 concentration on the height of the plants after four weeks of SO_2 fumigation for both LS 6164 and PAN 1666 plants. (n = 4)

After 4 weeks of SO_2 fumigation a visible indication of SO_2-concentration dependant inhibition in shoot growth was observable in both cultivars, even though the shoot length measured did not have significance ($p > 0.05$). Plant height was previously observed to be restricted when exposed to an increasing salinity-alkalinity concentration (Deng, *et al*., 2010:52; Zhang & Deng 2012:881). In trials where plants were exposed to osmotic stress, the reduction in growth was correlated to a reduction in the rate of photosynthesis and consequently reduced carbon assimilation under stress (Zhang & Deng, 2012:882). This change in growth occurred even though the growth rate (as indicated by the plastochron index) did not point towards strong

concentration dependence. The reduction of shoot length was in agreement with the results of biomass as well as photosynthetic activity.

Figure 4.1.3.2: Visible comparison between LS 6164 treatments: LS 6164 *Glycine max* plants after four weeks of SO_2 fumigation. In the figure from the left 0 ppb, followed by the 25 ppb, 75 ppb and 150 ppb treatments.

Figure 4.1.3.3: Visible comparison between PAN 1666 treatments: PAN 1666 *Glycine max* plants after four weeks of SO_2 fumigation. In the figure from the left 0 ppb, followed by the 25 ppb, 75 ppb and 150 ppb treatments.

4.1.4 The effect of SO_2 on biomass accumulation

The vegetative response to SO_2 fumigation was evaluated through biomass measurements. The shoot dry mass decreased with increasing SO_2 concentration (Fig. 4.1.4.1.a). There were however no significant difference ($p>0.05$) between the CF control (0 ppb treatment) and the 25 ppb treatment of PAN 1666. The 75 ppb and 150 ppb treatments did show a significant decline in biomass of 24.8% ($p \leq 0.05$) and 24.4% ($p \leq 0.05$) respectively from the CF control (0 ppb treatment). A significant decline in shoot dry mass was furthermore observed at the 75 ppb and 150 ppb SO_2 treatments of LS 6164. The 25 ppb treatment was insignificantly lower by 9.6% ($p > 0.05$), while the 75 ppb and 150 ppb treatments were significantly 31.4% ($p \leq 0.05$) and 26.3% ($p \leq 0.05$) lower than the CF control (0 ppb treatment). The 75 ppb treatment of LS 6164 had the lowest shoot biomass. There were however no significant difference between the 75 ppb and 150 ppb treatments of either cultivar.

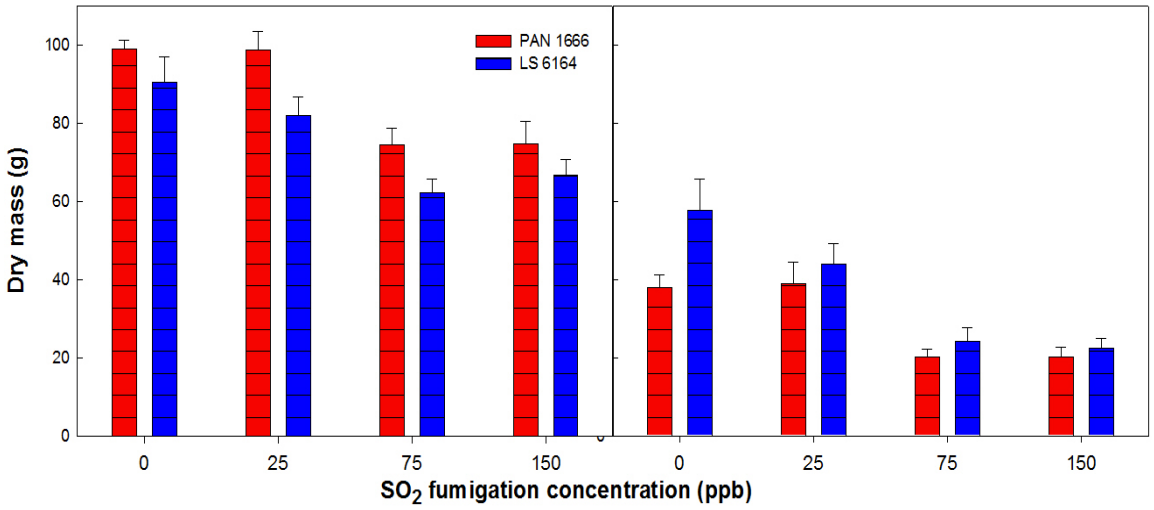

Figure 4.1.4.1: Shoot and root dry biomass: The effect of different levels of SO_2 on (a) shoot biomass and (b) root biomass at the harvest of LS 6164 as well as PAN 1666 *Glycine max* plants. (n = 8)

The dry mass of the *Glycine max* roots decreased as the SO_2 concentration increased. The dry mass of PAN 1666 plants decreased significantly with 47.2% ($p \leq 0.05$) and 47.1% ($p \leq 0.05$) respectively for 75 ppb and 150 ppb treatments in comparison to the CF control (0 ppb treatment). The 25 ppb treatment had a slight insignificant increase of 2.2% ($p > 0.05$) when compared to the CF control (0 ppb treatment). The dry mass of LS 6164 plants decreased for the 25 ppb, 75 ppb and 150 ppb SO_2 treatments correspondingly with 23.9% ($p > 0.05$), 58.1% ($p \leq 0.05$) and 61.2% ($p \leq 0.05$), in comparison to the CF control (0 ppb treatment). The 150 ppb treatment of LS 6164 had the lowest root biomass, which caused the highest shoot-to-root ratio to occur for this treatment. It was observed that the CF control (0 ppb treatment) of LS 6164 had a lower root biomass compared to the CF control (0 ppb treatment) of PAN 1666.

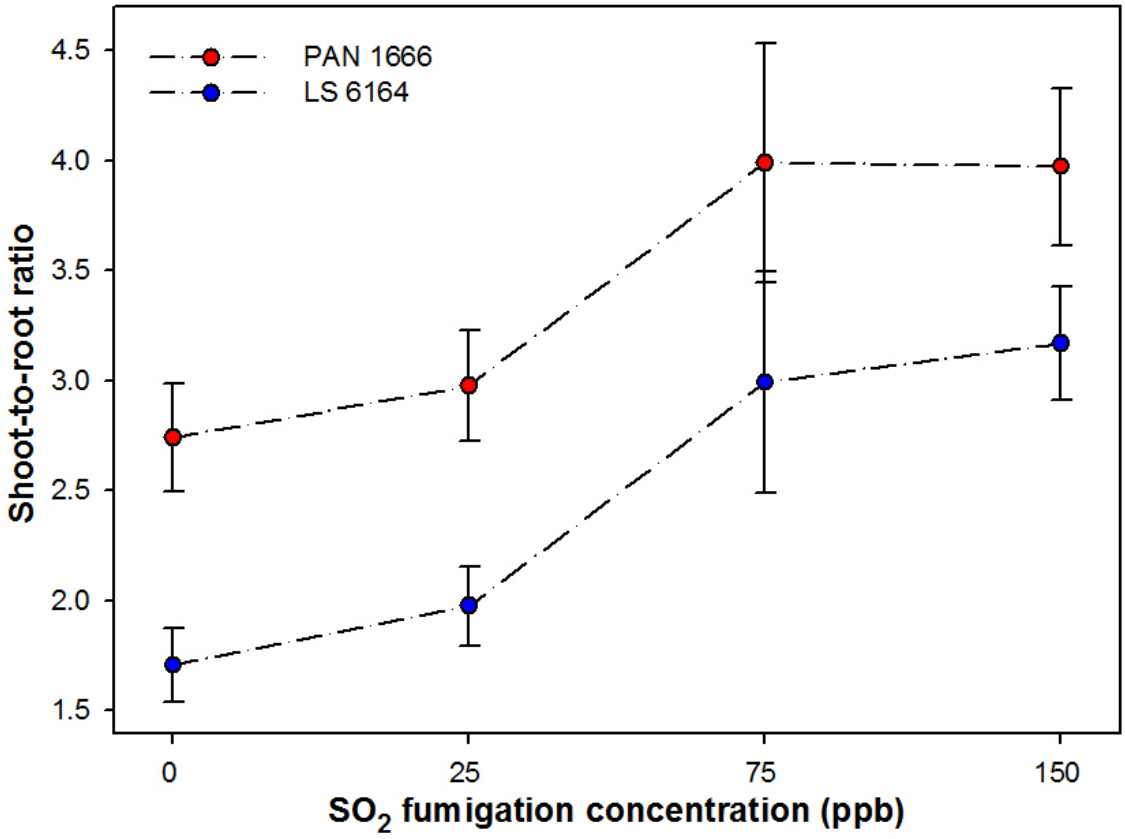

Figure 4.1.4.2: Shoot-to-root ratio: The effect of different levels of SO_2 on shoot-to-root ratio at harvest. (n = 8)

The shoot biomass decreased less compared to the root biomass for the 25 ppb, 75 ppb and the 150 ppb treatments. The greater decrease in root biomass was the basis for an increase in shoot-to-root ratio. This indicates that root biomass reductions occurred prior to that of shoot biomass. An increase in the shoot-to-root ratio was observed in both cultivars with increasing SO_2 levels (Fig. 4.1.4.2). The ratio indicates that the overall decrease in total biomass was primarily as a result of decreasing root mass. PAN 1666 had an increase of 8.64% ($p > 0.05$), 45.6% ($p > 0.05$) and 45.0% ($p > 0.05$) for the 25-, 75- and 150 ppb treatments respectively. LS 6164 had increases of 15.7% ($p > 0.05$), 72.3% ($p \leq 0.05$) and 85.7% ($p \leq 0.05$) with corresponding increases in SO_2 concentrations. Even though the shoot and root biomass were significantly influenced individually by the 75 ppb and 150 ppb treatments of the LS 6164 cultivar, these were the only treatments that indicated a significant decrease of shoot-to-root ratio in respect to the CF control (0 ppb treatment). The shoot-to-root

data correlated with observations of reduction in plant growth and development where the root growth was inhibited much more than shoot growth in both cultivars.

In the case of biomass accumulation, a SO_2 concentration dependant inhibition was observed for shoot, root, shoot-to-root ratio and nodules. All of the biomass accumulation measurements indicated the LS 6164 cultivar to have the most inhibition. The decreasing dry weight can be contributed to the decrease observed in stomatal conductance which suggests that the assimilatory area is smaller (Morgan, *et al*., 2003:1320). Changes in the shoot-to-root ratio have previously indicated a decrease in allocation of carbohydrates to roots, as a response to SO_2 as well as other air pollutants (Darrall, 1989:21). Root growth is known to be limited due to a decrease in carbohydrate allocation (Darrall, 1989:22).

The fact that root biomass was more inhibited in comparison to shoot biomass and the resulting increase in shoot-to-root ratio correlated with findings of Smit, *et al*. (2009:627), where the effect of trifluoroacetate was examined, as well as findings of Heyneke, et al., (2012b:9) where the effect of SO_2 was studied. The change in shoot-to-root ratio has been suggested to be an adaptive mechanism to maintain the relative growth rate, even with reduced carbohydrate (carbohydrate production is measured as net photosynthesis) availability (Darrall, 1989:22). The inhibition of root growth may have an effect on photosynthesis by inducing nutrient shortages which may contribute to a decrease in the amount and activity of important photosynthetic enzymes (Smit, *et al*., 2009:627). Decreases in the activity of photosynthetic enzymes will cause a reduction in chlorophyll content index (CCI) and carboxylation efficiency (CE) (Smit, *et al*., 2009:267). The shoot-to-root ratio demonstrated that shoot growth was benefited to root growth and root growth was more severely inhibited. The increase in shoot-to-root ratio is an indication of a decrease in carbohydrate translocation due to an increase in SO_2 concentration. The destructive consequence of SO_2 on the photosynthetic apparatus can, to a certain extent explain the negative effect of SO_2 on biomass (Rao & De Kok, 1994:283).

Plant growth is reduced in general under environmental stress conditions (Van Breusegem, *et al*., 2001:409). The limited root growth (smaller surface area) will probably reduce water uptake under conditions of limited water supply, and reduce

nutrient uptake as well. Leaf development is favoured by altering the shoot-to-root ratio, this is a possible adaptive mechanism within plants to sustain the relative growth rate, in circumstances of reduced carbohydrate availability.

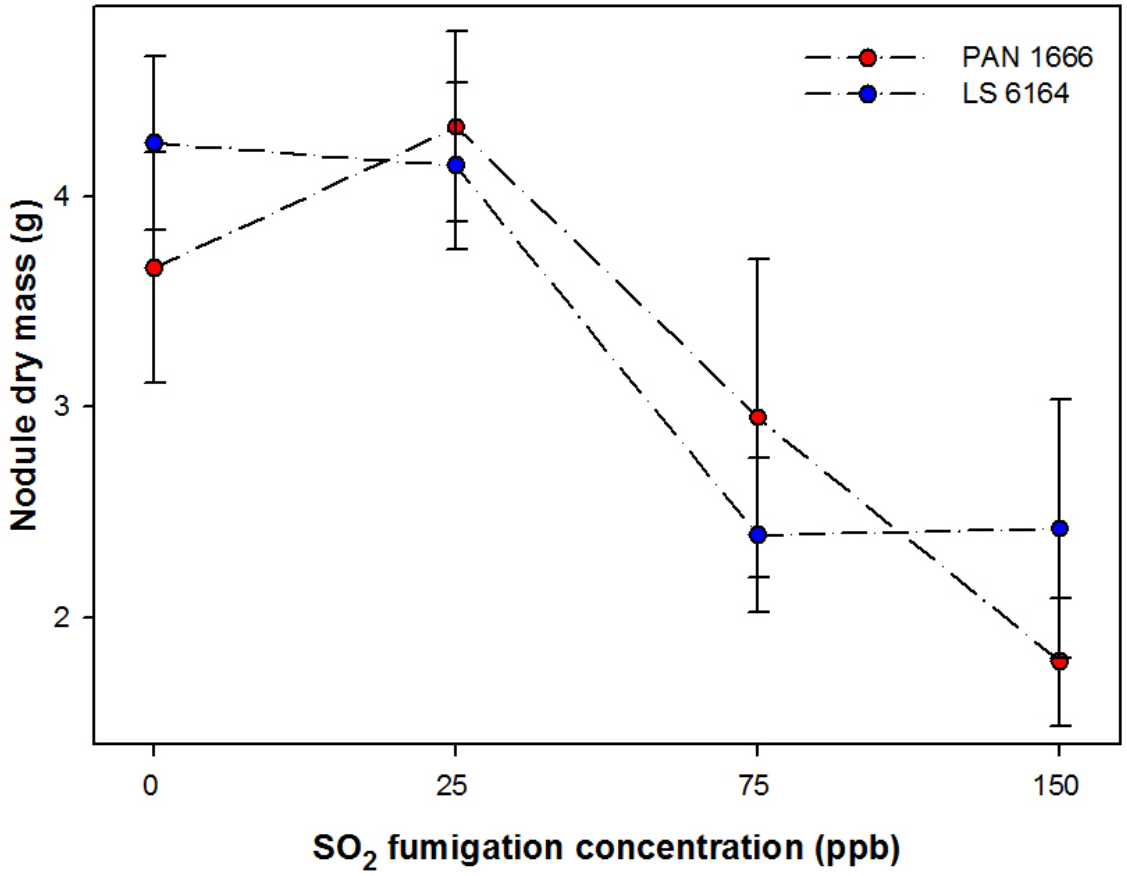

Figure 4.1.4.3: Nodule biomass: The effect of different levels of SO_2 on nodule biomass at harvest. (n= 8)

The nodule biomass decreased insignificantly in LS 6164 plants by 2.6%; 43.7% and 43.0% for the 25 ppb; 75 ppb and 150 ppb treatments respectively (Fig. 4.1.4.3). PAN 1666 *Glycine max* plants had an insignificant increase of 18.2% at 25 ppb, but decreased insignificantly with 19.4%, and 51.0% for the 75 ppb and 150 ppb treatments respectively. The decrease in nodule weight was insignificant for both cultivars, although PAN 1666 had higher decrease, whereas LS 6164 had a higher decrease in root biomass. The decrease in nodule biomass can most probably be attributed to the decrease in root mass.

The differences in dry nodule mass were insignificant, however a concentration dependant relationship was observed. Nodules are enlarged, multi cellular structures which are formed due to a symbiotic association on the roots of host plants (in this case *Glycine max*) (Hopkins & Hűner, 2004:169). The decrease in nitrogen-fixing nodules revealed that the symbiotic relationships with plants exposed to SO_2 are affected as a result of SO_2 stress. An indirect connection exists between photosynthesis and nodule bacteria (Malhotra & Hocking, 1976:234).

4.1.5 The effect of SO_2 on number of pods

A decreasing relationship between yield and SO_2 concentration was observed (Fig. 4.1.5.1 and Fig. 4.1.5.2).The number of pods per plant decreased insignificantly in both cultivars with increasing SO_2 concentration. At 25 ppb a slight insignificant increase of 0.6% was observed where with the 75 ppb and 150 ppb treatments an insignificant decrease of 4.5% and correspondingly 16.0% for the PAN 1666 plants was observed. LS 6164 also demonstrated an insignificant decrease of 10.4%, 18.4% and 21.2% in the number of pods for 25 ppb, 75 ppb and 150 ppb treatments respectively (Fig. 4.1.5.1). The lowest number of pods was observed for the 150 ppb treatment of the LS 6164 cultivar.

A similar trend was observed by a previous study done on three wheat varieties, where a decrease in the number of grains per ear were observed and it was concluded that this contributed to the overall decrease in yield due to air pollutants (Wahid, 2006:308).

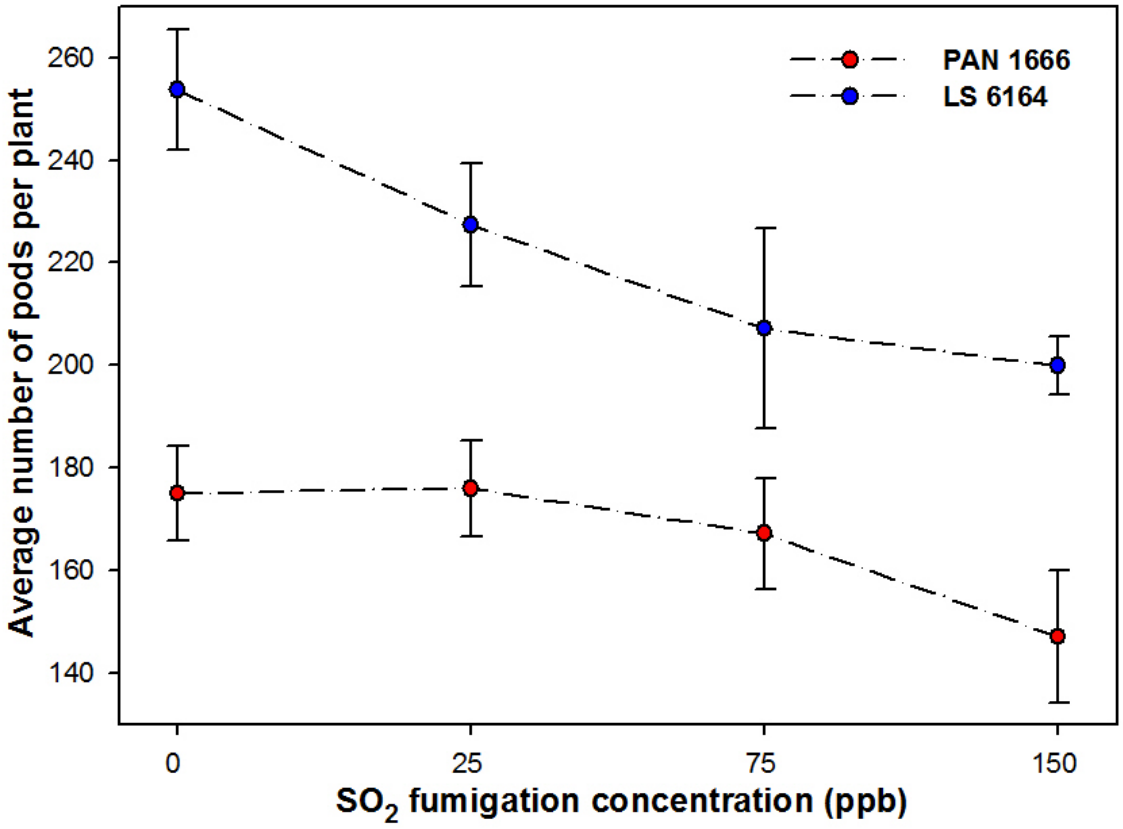

Figure 4.1.5.1: Number of pods per plant at harvest: The effect of different levels of SO_2 on the number of pods per plant at harvest. (n = 8)

Both cultivars demonstrated a decrease in seed mass as the SO_2 concentration increased (Fig. 4.1.5.2). The average weight of thirty seeds decreased by 3.0%, 48.2% and 56.4% respectively for 25 ppb, 75 ppb and 150 ppb for PAN 1666. For LS 6164 a decrease of 3.9%, 38.9% and 43.3% correspondingly to 25 ppb; 75 ppb and 150 ppb treatments. These results were in comparison to their respective CF control (0 ppb treatment)s. The lowest average weight of thirty seeds was observed for the 150 ppb of the LS 6164 cultivar.

A reduction in seed weight was previously established along with the fact that the influence on seed weight varied between different species (Wahid, 2006:308). The findings of Wahid compares to the results observed between the LS 6164 and PAN 1666 cultivars due to SO_2 fumigation.

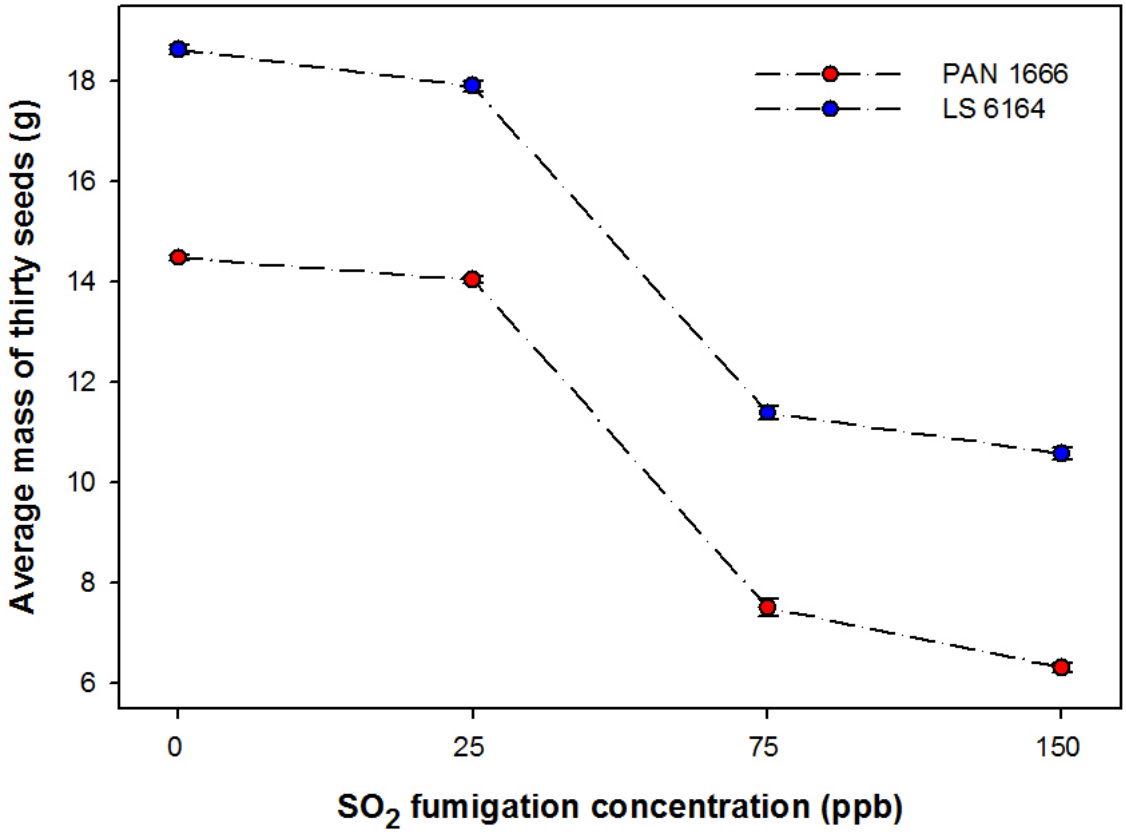

Figure 4.1.5.2: Average seed weight: The effect of different levels of SO_2 on average seed weight of 30 seeds at harvest.

The percentage oil in the seeds of LS 6164 *Glycine max* plants increased with 6.3%, 7.2% and 10.8% for 25 ppb; 75 ppb and 150 ppb treatments (Fig. 4.1.5.3). In the case of PAN 1666 the oil percentage of the seeds decreased with 2.5% at 25 ppb and increased with 10.5% for the 75 ppb as well as 10.0% for the 150 ppb treatment. The percentage protein content of seeds, decreased in LS 6164 plants by 6.1% for the 25 ppb, 3.3% for the 75 ppb and 4.9% for the 150 ppb treatment. The protein content in PAN 1666 seeds increased by 2.4% for the 25 ppb and 0.2% for the 75 ppb treatment, while the 150 ppb SO_2 had a decrease of 1.9%. The ratios of oil-to-protein were calculated in percentage CF control (0 ppb treatment). In the seeds of LS 6164 as well as PAN 1666 the ratios indicated concentration dependant increases. The oil-to-protein ratio of the LS 6164 at 25 ppb, 75 ppb and 150 ppb treatments increased by 13.2%, 10.8% and 16.6% respectively. The seeds of PAN 1666 had oil-to-protein ratios of 10.3% and 12.1% higher compared to the CF control (0 ppb treatment) plant, while the 25 ppb treatments had a ratio of 4.8% lower compared to the CF control (0

ppb treatment). The highest oil-to-protein ratio was observed for the 150 ppb treatment of the LS 6164 cultivar.

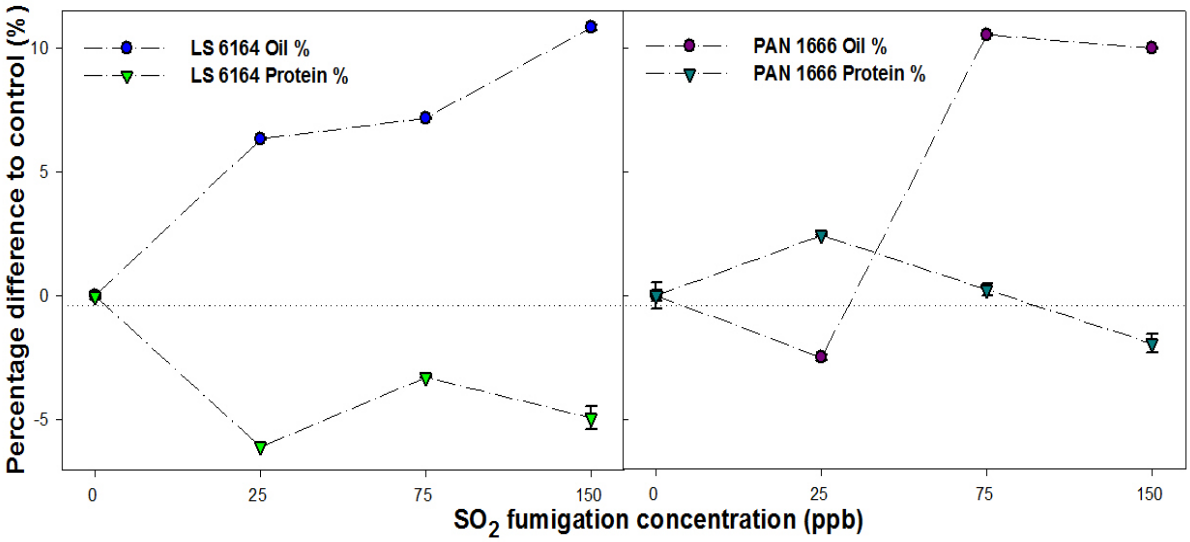

Figure 4.1.5.3: Percentage oil and protein present in seeds: The effect of different levels of SO_2 on the percentage oil and protein content in seeds at harvest. (a) LS 6164 and (b) PAN 1666. (n = 8)

The oil content of the seeds revealed an increase for both cultivars where the protein content revealed a decrease for LS 6164 as well as PAN 1666. It can be concluded that the nutritional value per seed was influenced due to the exposure of SO_2. A decrease in protein content might be explained by an enhanced rate of protein denaturation (Tripathi & Gautam, 2007:131).

The effect of air pollutants on the seed quality of crops, is an aspect that is seldom examined, however the common finding is that there is an existing inverse relationship between seed oil and protein content (Grunwald & Endress, 1988:283). It was previously observed that SO_2 levels do not significantly alter the protein level but the oil levels (Grunwald & Endress, 1988:284). An increase in the oil-to-protein ratio was thus concluded as a result of SO_2 fumigation and is confirmed in observations made in this study. It is probable that the pods oxidize the SO_3^{2-} to the less toxic SO_4^{2-} and consequently protect the seeds from SO_2 exposure (Grunwald & Endress, 1988:287).

4.2 Physiological responses

4.2.1 *Chlorophyll content index*

The chlorophyll content was significantly affected by SO_2 and this response is most certainly related to the inhibition of photosynthesis. A decrease in chlorophyll content occurred in both cultivars at all three time intervals, on the leaves situated on the 4th node. A SO_2-concentration dependant decrease in chlorophyll content was observed (Fig. 4.2.1.1).

The chlorophyll content decreased significantly for both the PAN 1666 and the LS 6164 for the 150 ppb and 75 ppb SO_2 treatments, in comparison to the CF control (0 ppb treatment). After 15, 29 and 37 days of fumigation, the 150 ppb treatment were 14.0% ($p \leq 0.05$), 32.1% ($p \leq 0.05$) and 38.7% ($p \leq 0.05$) lower, while the 75 ppb treatment was 9.4% ($p > 0.05$), 10.4% ($p \leq 0.05$) and 16.7% ($p \leq 0.05$) lower compared to the CF control (0 ppb treatment) of the PAN 1666 cultivar. A significant difference was observed between the 75 ppb and 150 ppb treatment after 37 days of fumigation. The chlorophyll content for LS 6164 after 15, 29 and 37 days of fumigation, the 150 ppb treatment was 11.0% ($p > 0.05$), 26.7%($p \leq 0.05$) and 23.3%($p \leq 0.05$) lower, while the 75 ppb treatment was 6.3% ($p > 0.05$), 17.7% ($p \leq 0.05$) and 10.5% ($p \leq 0.05$) lower compared to the CF control (0 ppb treatment). Significant differences were observed between the 75 ppb and 150 ppb treatment after 29 and 37 days of fumigation. The 25 ppb treatment only had a significant ($p \leq 0.05$) decrease in respect to the CF control (0 ppb treatment) after 37 days.

The decrease in chlorophyll content was predicted by the visible foliar injury and was unmistakable for both cultivars. The PAN 1666 cultivar had the most reduction in chlorophyll content for the 150 ppb SO_2 treatment after 37 days of fumigation, while LS 6164 had the lowest chlorophyll content after 29 days for the 150 ppb SO_2 fumigation. PAN 1666 had a lower chlorophyll content with increasing SO_2 concentration compared to LS 6164 after 15, 29 as well as 37 days of fumigation, even though a higher reduction of PSII efficiency was observed for LS 6164 .

Sulphur dioxide and its oxidation products, SO_3^{2-} and HSO_3^- are known to interfere with the structure of the cellular membranes and enzyme activity, especially at high concentrations. The fragile nature of chloroplast membranes causes them to be vulnerable to disruption and the breakdown of chlorophyll. It has been reported that high levels of SO_2 will lead to a decrease in chlorophyll content for example, a statistical decrease has been observed in total chlorophyll content in plants around refineries in Turkey (Deniz & Duzenli, 2006:75). In previous studies winter wheat *(Triticum aestivum)* exposed to 138 ppb SO_2 had a decrease in chlorophyll content of 42% compared to plants exposed to ambient CO_2 (Rao & De Kok, 1994:283), these results are in compliance to the effect of SO_2 on chlorophyll content. A decrease in chlorophyll content due to abiotic stress was also observed by Yusuf, *et al*., (2010:1433). Salinity-alkalinity is another stressor that has been observed to decrease chlorophyll content significantly (Deng, *et al*., 2010:55). A significant decrease in chlorophyll content was also observed in maize when exposed to acid mine drainage (Pan, *et al*., 2010:984).

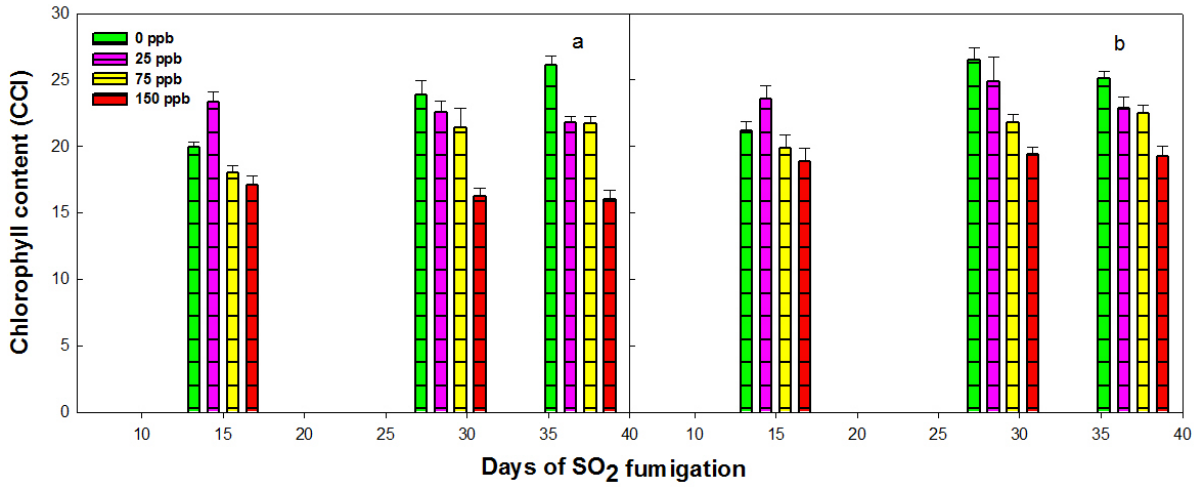

Figure 4.2.1.1: Chlorophyll content index: The effect of SO_2 fumigation on the chlorophyll content index of (a) PAN 1666 and (b) LS 6164 soybeans measured after respectively 15 days; 29 days and 37 days of treatment. (n = 24)

Photo-oxidation of SO_3^{2-} to SO_4^{2-} and the subsequent formation of ROS in the chloroplasts are known to damage chloroplast and can contribute to the reduction in chlorophyll content. It has been observed that *Eucalyptus hybrids* grown at sites with high air pollution exposure experienced a considerable loss in total chlorophyll; this

finding has been used to support the argument that the primary site of air pollution attack is the chloroplast (Tripathi & Gautam, 2007:130). The partial denaturation of the chloroplast and decreasing pigment content, in the cells of leaves, have been previously implicated in decreasing chlorophyll content (Tripathi & Gautam, 2007:131). Chlorophyll content can also be influenced by a nutrient deficiency, due to the reduction in the root system of the plants. The decreases in chlorophyll content are confirmed by the visible necrosis on leaves this will influence the photosynthesis process of the plants.

4.2.2 Gas exchange

Measurements of the response of net photosynthesis to leaf internal CO_2 concentration (A:Ci curves) are widely used for ecophysiological studies (Flexas, *et al*., 2007:1533). The perception of the responses of leaf photosynthesis to environmental factors, is greatly dependant on the interpretation of photosynthetic gas exchange and mainly the relationship between net CO_2 assimilation and C_i which is the intercellular CO_2 mole fraction (Meyer & Genty, 1998:947). The underlying biochemistry of a leaf can be investigated by measuring the response of CO_2 assimilation rate to intercellular CO_2 partial pressure. Varying the intercellular CO_2 concentration allows to separate the influence of stomata from the underlying biochemical limitations. For green plants to photosynthesize at an optimal rate, a constant supply of CO_2 is needed. The CO_2 diffuses into the leaves when the stomata are open and water vapour escapes from the leaf. Therefore the conductance of stomata influences both the photosynthetic rate as well as transpiration.

Changes in the net rate of CO_2 assimilation reflect changes on the stomatal conductance and on the mesophyll capacity for photosynthesis (Von Caemmerer & Farquhar, 1984:320). An A:C_i curve analysis can lead to the determination of the carboxylation rate, ribulosebiphosphate (RuBP) activity, quantification of the maximum carboxylation capacity (V_{max}) as well as the maximum capacity for electron transport rate (J_{max}) (Flexas, *et al*., 2007:1533). The model of Farquhar *et al*. (1980:318) is based on the hypothesis that the A:Ci curve can be mainly assimilated to Michaelis–Menten kinetics where A represents the velocity of an enzyme-mediated reaction and C_i the substrate concentration at the site of the enzyme (Flexas, *et al*., 2007:1534).

This model is based on the relative uniform distribution of transpiration over the leaf area and for this reason may not be valid in the case of a decrease of stomatal conductance (Meyer & Genty, 1998:956). Invalidation in this case is due to the impairment of estimation of C_i (Flexas, *et al*., 2007:1534).

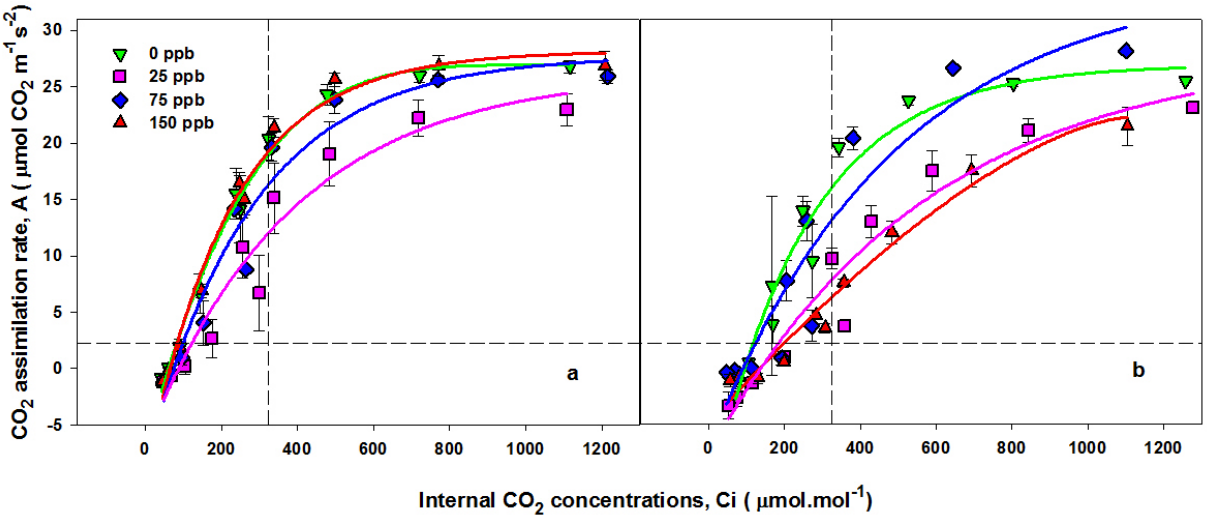

Figure 4.2.2.1: Average CO_2 response curves: Average CO_2 response curves (A:Ci) for intact leaves of (a) PAN 1666 and (b) LS 6164 *Glycine max* plants after 6 weeks of exposure to SO_2 and filtered air measured at 1200 µmol photons.m^{-2}.s^{-1} to ensure full activation of Rubisco. (n = 2)

The CO_2 compensation point (□) is where the net efflux of CO_2 is equal to zero, even with open stomata. This means that the CO_2 fixed by photosynthesis is the same amount as the CO_2 produced by respiration (Küppers & Häder, 1999). A lower CO_2 compensation point reflects low levels of photorespiration. Higher CO_2 compensation point indicates higher CO_2 production as a result of higher photorespiration. The CO_2 compensation point for PAN 1666 increased for the 25 ppb and 75 ppb treatments by 23.3% and 4.1% respectively in comparison to the CF control (0 ppb treatment) (Fig. 4.2.2.2.a). The 150 ppb treatment of PAN 1666 however had a decrease of 4.9%. The CO_2 compensation point for LS 6164 increased with the 25 ppb, 75 ppb and 150 ppb treatments with 52.7%, 2.7% and 45.0% respectively in comparison to the CF control (0 ppb treatment) treatment.

The assimilation rate reflects the maximal rate of compared photosynthesis when there is no stomatal restriction (A_0). The A_0 of PAN 1666 decreased for all treatments by 27.5% (25 ppb), 10.8% (75 ppb) and 0.2% (150 ppb). All of the SO_2 treatments had a decrease for A_0 in respect to the CF control (0 ppb treatment) of 48.0% (25 ppb), 13.6% (75 ppb) and 57.85% (150 ppb). In comparison to the CF control (0 ppb treatment) only LS 6164 25 ppb and 150 ppb treatment had significant differences, while no significant differences were observed in PAN 1666.

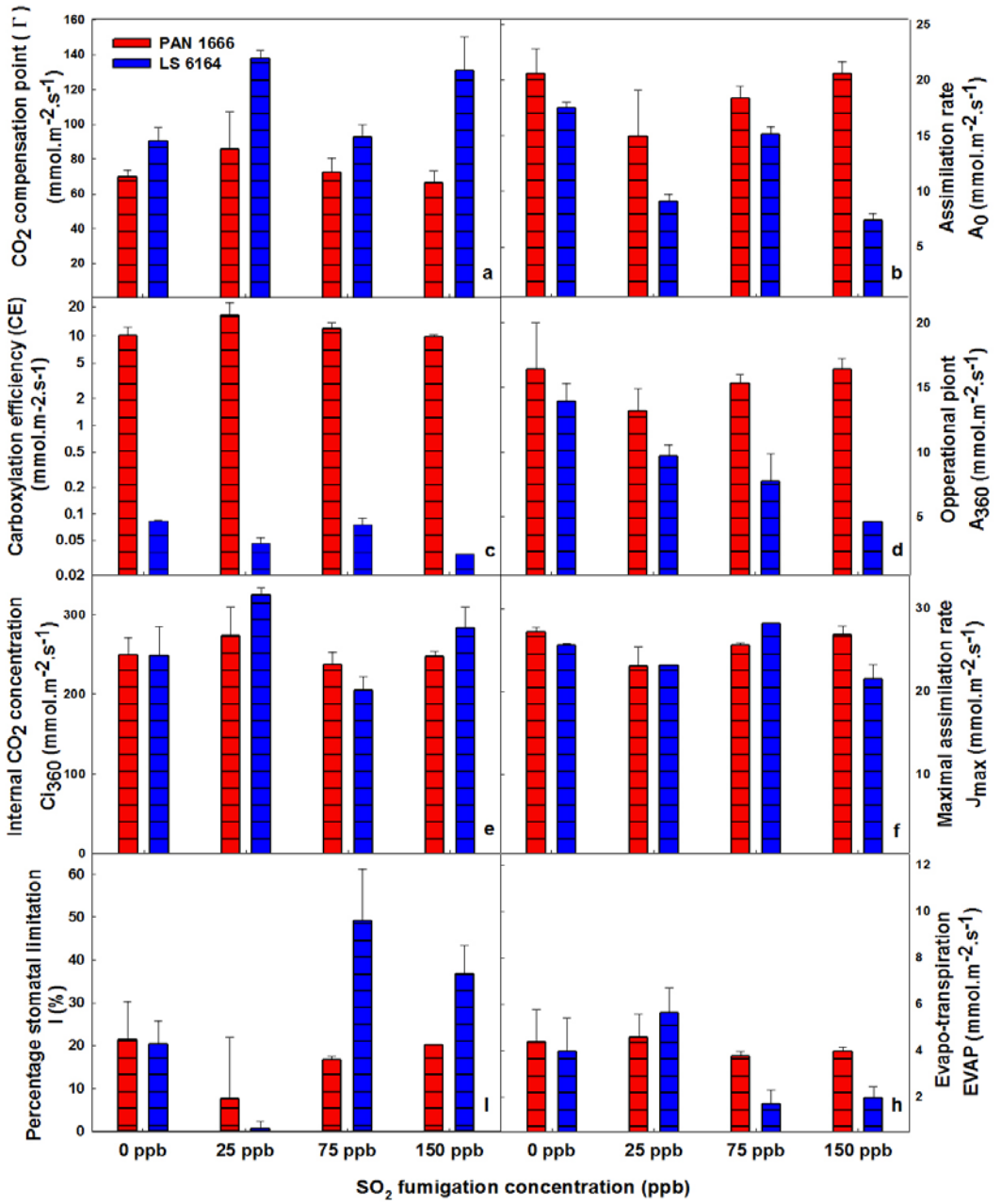

Figure 4.2.2.2: Mean values of gas exchange parameters: Mean values (± standard errors, SE, n = 2) of gas exchange parameters measured in intact leaves of PAN 1666 and LS 6164 *Glycine max* plants (n=2 plants per SO_2 treatment) 6 weeks after SO_2 fumigation commenced. Symbols: (a) Γ, CO_2 compensation point; (b) A_0, rate of CO_2 assimilation at Ci = 360 µmol.mol^{-1}; (c) CE, carboxylation efficiency: (d) A_{360}, rate of CO_2 assimilation at C_a = 360 µml.mol^{-1}; (e) Ci_{360}, (f) J_{max}, maximum rate of CO_2assimilation; (g) ℓ, percentage stomatal limitation of photosynthesis; h) EVAP, the transpiration rate at Ca = 360 µmol.mol^{-1}. (n = 2)

The initial linear slope represents the demand function for CO_2 by photosynthesis and is determined by the induction state of the photosynthetic apparatus as well as the apparent carboxylation efficiency (CE) (Küppers & Häder, 1999). The carboxylation efficiency reflects the rate of CO_2 assimilation in terms of effectiveness and the capacity of the system to assimilate CO_2. The advantage of using the demand function is that without damaging the leaf, information on the mesophyll can be provided (Küppers & Häder, 1999). The CE is a known in-*vivo* indicator of Rubisco activity and has previously been confirmed with an in-*vitro* comparison where a linear relationship was observed between the in-*vivo* and in-*vitro* measurements (Von Caemmerer & Farquhar, 1984:322). Rubisco catalyzes the first major step of carbon fixation. The capacity to regenerate Rubisco depends on the capacity of electron transport to produce ATP and NADH (Farquhar & Sharkey, 1982:319). The CE for PAN 1666 decreased slightly (3.3%) with the 150 ppb treatment, while an increase in the CE of 65.2% and 19.5% were observed for the 25 ppb and 75 ppb treatments (Fig. 4.2.2.2.c). The 25 ppb and 75 ppb treatments however had corresponding increases of 65.2% and 19.5% in comparison to the CF control (0 ppb treatment) for CE. The CE for the LS 6164 plants were 45.1%, 9.6% and 58.0% lower for the 25 ppb, 75 ppb and 150 ppb treatments in comparison to the CF control (0 ppb treatment). No statistical significance was observed for PAN 1666, while LS 6164 had a statistical difference between the CF control (0 ppb treatment) and the 150 ppb treatment.

The 150 ppb SO_2 treatment of the PAN 1666 plants did not vary from the CF control (0 ppb treatment) in respect to the A_{360} parameter (Fig. 4.2.2.2.d). The 25 ppb and the 75 ppb treatments had decreases of 19.5% and 6.7% respectively in relation to the CF control (0 ppb treatment). The A_{360} of LS 6164 25 ppb, 75 ppb and 150 ppb treatments experienced decreases of 30.5%, 44.4% and 67.44% respectively. The only statistical differences observed for either cultivar were between the 150 ppb treatment and the CF control (0 ppb treatment) of the LS 6164 cultivar.

In the circumstance where Ci is artificially 360 $\mu mol.mol^{-1}$, the stomatal resistance to CO_2 diffusion into the leaf, has been cancelled out (Küppers & Häder, 1999). Ci_{360} of PAN 1666 plants decreased for 75 ppb by 4.8%, and 0.8% for the150 ppb treatment. An increase of 9.8% for the 25 ppb treatment in comparison to the CF control (0 ppb treatment) were observed (Fig. 4.2.2.2.e). An increase in Ci may point towards an

increase in limitations within the mesophyll (mesophyll constraints) and a decreasing assimilation rate (Smit, *et al*., 2009:627). The Ci_{360} of only the 75 ppb treatment of LS 6164 decreases by 17.5%. LS 6164 treatments for the 25 ppb and the 150 ppb treatments, increases of 30.8% and 13.9% were observed in comparison to the CF control (0 ppb treatment). Neither of the cultivars had any significant difference in comparison to the CF control (0 ppb treatment) for any treatment.

The J_{max} is determined by Ribulose 1, 5-biphosphate carboxylase/oxygenase (Rubisco) activity, PEP regeneration capacity or photosynthetic electron transport rate (Smit, *et al*., 2009:627). The J_{max} is an indicator of RuBP regeneration capacity (Von Caemmerer & Farquhar, 1984:322). J_{max} decreased by 15.3%, 5.9% and 1.3% respectively for the 25 ppb, 75 ppb and 150 ppb treatments in comparison to the CF control (0 ppb treatment) plants (Fig. 4.2.2.2.f). The J_{max} parameter of LS 6164 plants decreased by 9.6% and 15.6% respectively for the 25 ppb and 150 ppb treatments respectively in comparison to the CF control (0 ppb treatment). The 75 ppb treatment however increases with 10.4% in contrast to the CF control (0 ppb treatment). None of the treatments had a statistical difference in respect to the CF control (0 ppb treatment).

Stomatal limitation decreased for all of the PAN 1666 treatments when compared to the CF control (0 ppb treatment), the percentage difference were 64.3% 21.8% and 5.2% for the 25 ppb, 75 ppb and 150 ppb treatments respectively (Fig. 4.2.2.2.g). The percentage stomatal limitation of photosynthesis in LS 6164 plants increased by 141.2% and 80.0% respectively for the 75 ppb and the 150 ppb treatments in comparison to the CF control (0 ppb treatment). The 25 ppb treatments indicated a decrease of 132.1%. No statistical differences to CF control (0 ppb treatment) were observed for any of either cultivar's treatments.

Evapo-transpiration (EVAP, where Ca = 360 $\mu mol.mol^{-1}$) increased by 13.9% for the 75 ppb and 9.8% for the 150 ppb, but however decreased by 3.9% for the 25 ppb treatments in comparison to the CF control (0 ppb treatment) treatment for PAN 1666 plants. The 75 ppb and the 150 ppb SO_2 treatments had decreases in EVAP by 57.1% and 50.6% respectively, meanwhile the 25 ppb treatments had an increase of 42.7% in comparison to the CF control (0 ppb treatment) (Fig. 4.2.2.2.h). No statistical

differences were observed in respect to the CF control (0 ppb treatment) for either cultivar.

Previous studies attributed the loss of photosynthetic capability to effects in the mesophyll, where they observed the inactivation of ribulosebisphosphate carboxylase and a decrease in levels of the substrate ribulosebisphosphate (Darrall, 1989:3). An increasing SO_2 concentration decreased the rate of photosynthesis (Heyneke, *et al*., 2012b:12). Exposure to 138 ppb SO_2 resulted in a decrease in J_{max} of 40 % (Rao & De Kok, 1994:284). The inhibition of J_{max} were expected to be observed but contradicting to Smit, *et al*., (2009:624) and their study done on trifluoroacetate on *Phaseolus vulgaris* and *Zea mays*, the expected concentration dependant inhibition, was observed in J_{max} only for the LS 6164 cultivar. The highest decrease, even though insignificant was observed for the 150 ppb treatment of the LS 6164 cultivar. The study on increasing SO_2 concentration and drought stress on soybeans by Heyneke, *et al*., (2012b:14) confirmed the findings of Smit, *et al*., (2009:624) that a decrease in J_{max} is expected in plants with increasing stress. The initial slope of the demand function (apparent carboxylation efficiency) were expected to decrease with increasing SO_2 concentrations as with previous results of stress studies observed by Smit, *et al*., (2009:624) as well as Heyneke, *et al*., (2012b:14). A significant decrease for the 150 ppb treatment of the LS 6164 cultivar correlated with the findings of the two previous mentioned studies.

An increase in the intercellular CO_2 concentration is expected when limitation within the mesophyll dominates (Farquhar & Sharkey, 1982:333). During induced salinity-alkalinity stress intercellular CO_2 concentration was observed to decrease (Zhang & Deng, 2012:882). The increases found for the 150 ppb treatment of LS 69164 in the CO_2 compensation point (□) and intercellular CO_2 concentration correlates with results of Heyneke, *et al*., (2012b:14), where it was observed as an indication of increasing limitation of the mesophyll processes. The operational point (A_{360}) as well as the assimilation rate was observed to have a significant decrease for the 150 ppb treatment of LS 6164. The percentage stomatal limitation did not have any significant increase which indicates that the limitations within the mesophyll is the dominant cause of reduction in the CO_2 assimilation rate of the 150 ppb treatment of LS6164 (Heyneke, *et al*., 2012b:14)

The decrease in $PI_{ABS,total}$ (Fig. 4.2.3.9.a and Fig. 4.2.3.9.b) corresponds with a decrease in CO_2 assimilation rate for both cultivars (Van Heerden, *et al*., 2004:247). A decrease in stomatal conductance will explain a decrease in the leaf water transpiration rate (Morgan, *et al*., 2003:1320). The decreases in evapo-transpiration was however insignificant in this study. It has been observed that in soybeans exposed to O_3 the changes in the photosynthetic capability are due to a decrease in activity and amount of Rubisco (Morgan, *et al*., 2003:1324).

4.2.3 Chlorophyll a fluorescence

Photosynthesis is the driving force behind biomass production therefore it is a valuable tool in defining SO_2 tolerance (Winner & Mooney, 1980:290). Different species varies in sensitivity of photosynthesis to air pollution (Darrall, 1989:11). The effects of elevated SO_2 on photosynthesis were evaluated using the JIP-test based on the "Theory of Energy Fluxes in Biomembranes" by Strasser (1981) (as cited by Strasser, *et al*., 2004:327). This theory is based on the facts that the primary quinone acceptor of PSII (Q_A) is reduced to Q_A^-, the reaction centre is closed and the chlorophyll fluorescence of the antenna is high, whereas when Q_A is in the oxidized state, the reaction centres are open and the fluorescence of the antenna is quenched (Yusuf, *et al*., 2010:1429). The JIP test allows the non-invasive efficient, rapid and sensitive obtainment of information of the structural and functional parameters that quantify the performance of the photosynthetic apparatus and therefore widely used in stress studies (Yusuf, *et al*., 2010:1429).

PSII is highly sensitive to stress conditions and to analyze these effects, the JIP test was done at the dark-adapted state (at least for 1 hour) to disclose any effects on different sites of the photosynthetic primary processes. The energy supply for metabolism and crop yield can therefore be quantified.

All of the transients displayed a typical OJIP transient after 3 weeks of SO_2 fumigation (Fig. 4.2.3.1). The OJIP transient started from the initial F_0 intensity and increased to a maximum fluorescence yield (P or F_M). The initial point, F_0, indicates the fluorescence signal immediately reached at the onset of illumination and is indicated by step O. It is

assumed that, after dark adaption, the primary quinone acceptor Q_A is completely oxidized and all of the reaction centres are open (and therefore able to receive electrons). After illumination the induction curve rises rapidly to a peak P, which represents the maximum (*P* or F_M) value where all Q_A is reduced and the reaction centres are closed. The rise in fluorescence is interpreted as the accumulation of the reduced form of acceptor Q_A. This is the result of the progressive closure of the reaction centres, which is the result of Q_A reduction due to the activity of PSII and Q_A^- reoxidation due to PSI activity.

Plotting the transients on a logarithmic scale, the rising transient from F_0 to F_M, is characterized by polyphasic behaviour (Fig. 4.2.3.1). The different steps of the polyphasic behaviour are labelled as O, J, I, P. There were not many differences between the treatments at F_0, but as the transient reaches F_M, more differences can be observed between the treatments. Analysis of the graphs after SO_2 fumigation revealed two intermediate steps F_J (about 2 ms) and F_I (about 30 ms) in between F_0 and F_M (Fig. 4.2.3.1). To visualize the comparative effect of SO_2 on each step, the curves are plotted as relative variable fluorescence, $V_t = (F_t - F_0)/(F_M - F_0)$ between steps O and P (Fig. 4.2.3.2).

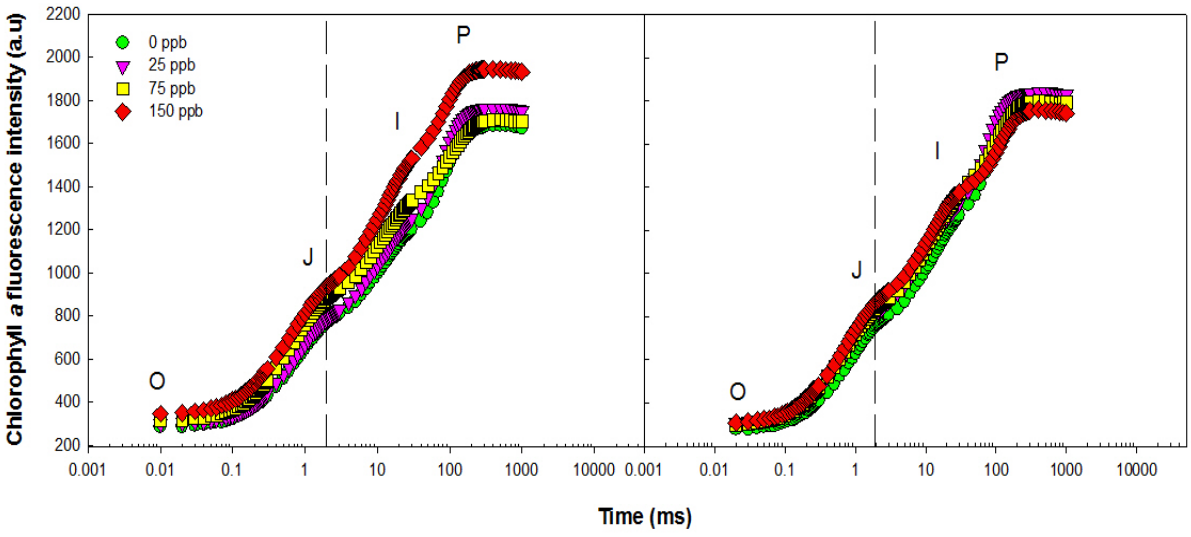

Figure 4.2.3.1: O-J-I-P curves: Changes of the fluorescence kinetics, O-J-I-P, plotted on a logarithmic scale from 50 µs to 1 s of intact dark-adapted leaves of *Glycine max*, cultivars (a) PAN 1666 and (b) LS 6164, after 3 weeks of SO_2 fumigation. (n = 24)

In figure 4.2.3.1 the average fast fluorescence transients are presented on a logarithmic scale and the typical OJIP shaped curve (polyphasic chlorophyll *a* fluorescence) can be observed for all treatments, which demonstrate that all samples were photosynthetically active. The O (50 µs) to J (2 ms) part of the transient curve refers to the single turnover range (i.e. Q_A is only reduced once) and therefore reflects mainly photochemical reactions resulting to the reduction of the electron acceptor Q_A. Multiple turnover events of PSII function is indicated in the transition J (2 ms) to P (300 ms). The OJIP fast fluorescence transients in Fig. 4.2.3.1. indicates that the SO_2 induced effects mainly occurred in the multiple turn-over events of PSII function, which correlates with the results of Heyneke, *et al*., (2012b:14). The JI phase is thought to indicate the reduction of intersystem electron carriers and the IP phase on the reduction of PSI electron acceptors (Yusuf, *et al*., 2010:1436).

The LS 6164 plants had a concentration dependent decrease in the multiple turnover phase of PSII, however a reversed effect was observed in the case of PAN 1666. The decrease in maximal fluorescence intensity will result in lower variable fluorescence levels (Panda & Sarkar, 2012:103). Panda & Sarkar, (2012:103) observed a decrease in maximal fluorescence, in rice when submerged, they attributed this phenomenon to some difference in the composition and organization of the PSII antenna and reaction centre, and is therefore a reflection on the changes in the PSII grouping. This phenomenon can be due to induced stomatal closure, probably caused by SO_2 exposure. A number of parameters can be derived from the polyphasic chlorophyll *a* fluorescence rise, OJIP that can provide information about photosynthetic fluxes, which make this test such a valuable tool in monitoring plant vitality (Oukarroum, *et al*., 2007:440). In the OJIP-curve the 150 ppb treatments of both cultivars have a higher F_0, this correlated with results of Pan, *et al*., (2010:985), they stated that this occurrence indicates photo inhibition and that it will correlate to damaged reaction centre (RC) at the acceptor side of PSII.

Subtracting the CF control (0 ppb treatment) relative variable fluorescence ($V_t = (F_t - F_0) / (F_M - F_0)$) from the fumigated plants enables us to investigate the effect of SO_2 fumigation on the O-J-I-P transient with greater accuracy (Fig. 4.2.3.2). The difference in kinetics reveals bands that are hidden between the steps O, J, I and P (Yusuf, *et al*.,

2010:1435). The average values were used to calculate the differences in kinetics. The fluorescence rise up to the J-step provides information concerning the single turnover events of the primary reaction of photochemistry in particularly Q_A reduction (Oukarroum, *et al*., 2007:439). This subtraction is referred to as the difference in relative variable fluorescence ($\Delta V_t = V_{treatment} - V_{control}$). From the difference in relative variable fluorescence graphs, we can recognize differences among different SO_2 treatments through the revealing of hidden bands between the different steps of normalised transients. These bands reveal more information than the original O-J-I-P and the relative variable fluorescence.

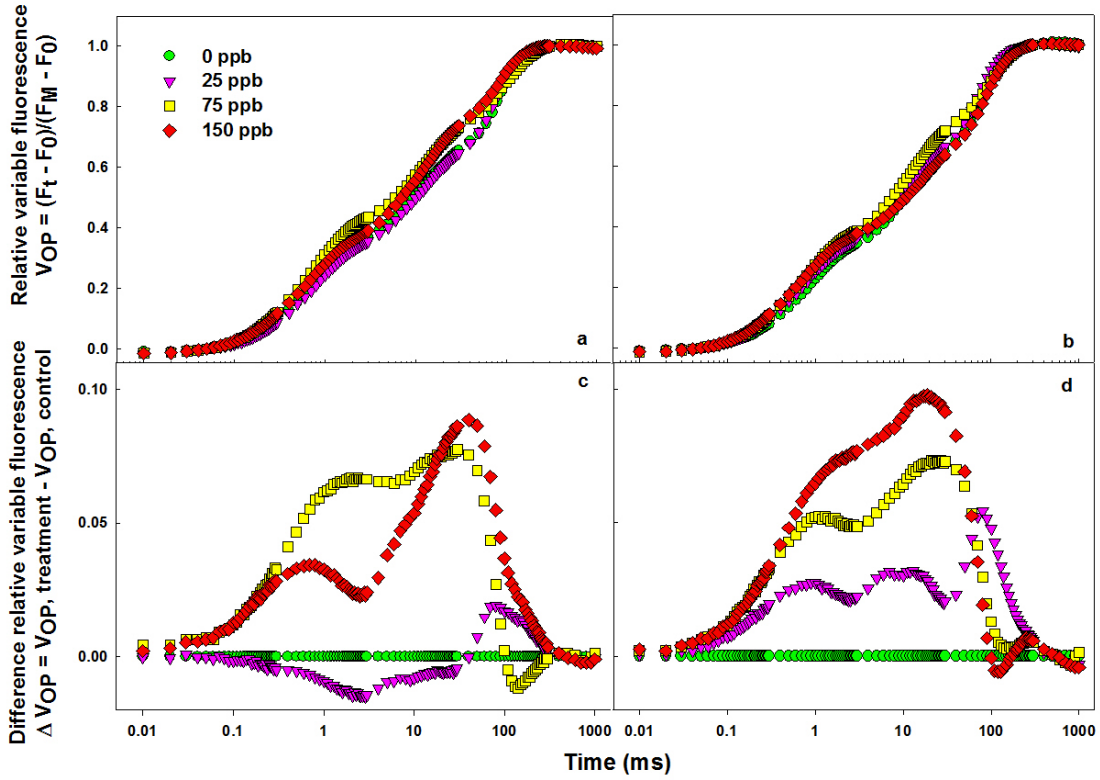

Figure 4.2.3.2: Relative variable chlorophyll *a* fluorescence transients normalised between F_0 and F_p: Relative variable chlorophyll *a* fluorescence transients normalised between F_0 (0.05µs) and F_p(300 ms) for (a) PAN 1666 and (b) LS 6164 *Glycine max* plants after three weeks of SO_2 fumigation. The difference in relative variable fluorescence ($\Delta V_{OP} = V_{OP, treatment} - V_{OP, control}$) for (c) PAN 1666 and (d) LS 6164 relative to the CF control (0 ppb treatment) plants exposed to CF air. The average fluorescence transients were normalised between the fluorescence extremes F_0 (50 µs) and F_P (300 ms). (n = 24)

The OJ phase reflects a single turnover photochemical event (Yusuf, *et al*., 2010:1431). Information on the energetic connectivity between the PSII units, can be deduced from the shape OJ phase where an exponential shape indicates no connectivity and a sigmoidal shape indicates connectivity (Yusuf, *et al*., 2010:1430). Double normalization between F_0 and F_J amplifies the K-band, which can be observed around 0.3ms, the effect of the acceptor side of PSII is excluded by this normalization (Panda & Sarkar, 2012:103) (Fig. 4.2.3.3). A positive K-band indicates the inactivation of the oxygen evolving complex (OEC) and/or it can indicate an increase in functional antenna size (Yusuf, *et al*., 2010:1436). Panda & Sarkar, (2012:103) stated that in addition to the inactivation of the OEC a positive K-band can be attributed to a loss of connectivity among PSII and a decrease of grouping probability. The processes from excitation trapping to PQ reduction are therefore affected. The negative ∆K-bands were amplified in PAN 1666 plants by 1.2% lower than the CF control (0 ppb treatment) for the 25 ppb treatments, while positive ∆K-band were 8.1% and 17.2% greater for 75 ppb and 150 ppb treatments respectively in respect to the CF control (0 ppb treatment). LS 6164 plants however indicated positive ∆K-band of 12.2%, 17.7% and 11.0% greater for the 25 ppb, 75 ppb and 150 ppb treatments respectively.

The PAN 1666 cultivar revealed a SO_2-concentration dependent ΔK-band, hidden in the fluorescence kinetics. The most inhibition of the OEC was observed at the 75 ppb treatment of the LS 6164 cultivar. The oxidation products of SO_2 are known to cause structural changes in proteins and through these changes the uncoupling of the OEC is probable due to conformational changes in the involved proteins. This increasing ΔK-band indicates increasing inhibition of the OEC and shows that compared to the CF control (0 ppb treatment) the SO_2 treatments have a lower activity at the oxygen evolving side. In a study with salinity-alkalinity stress the J-P step became a straight line and the researchers attributed this to the electron transport chain to be broken after Q_A and that the OEC failed to provide electrons for PSII to reduce the quinone acceptors (Deng, *et al*., 2010:53). The SO_2 concentration dependant ΔK-band correlates with results of Smit, *et al*., (2009:628); Oukarroum, *et al*., (2007:442) as well as Yusuf, *et al*., (2010: 1436) where they also observed positive ΔK-bands when plants are exposed to abiotic stress. The emergence of a positive ΔK-band can be contributed to the inhibition of root growth observed and the resulting inhibition of

nutrient uptake (Smit, *et al*., 2009:628). An imbalance within PSII between the electrons leaving the reaction centres at the acceptor side and the electrons donated by the donor side can explain the positive ΔK-band observed (Oukarroum, *et al*., 2007:445).

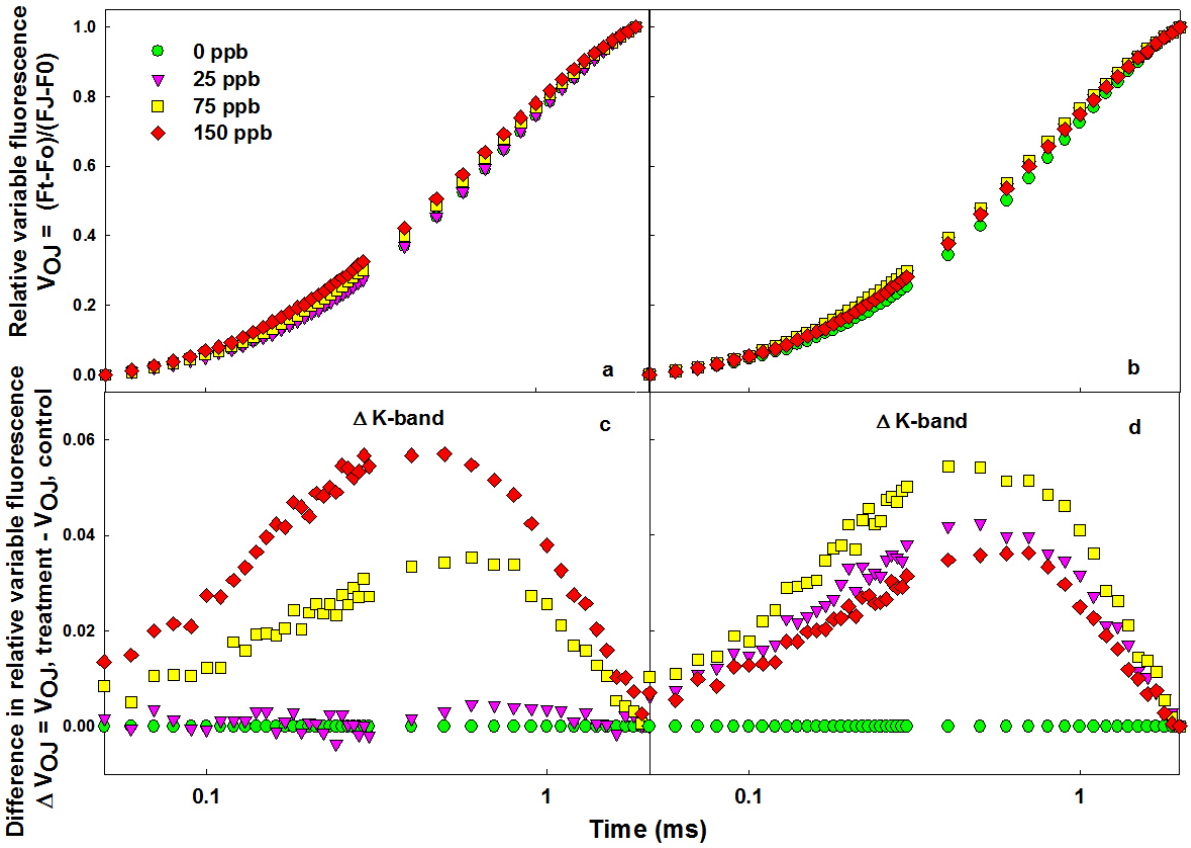

Figure 4.2.3.3: Relative variable fluorescence normalised between F_0 and F_J: Relative variable fluorescence ($\Delta V_{OJ} = V_{OJ,\ treatment} - V_{OJ,\ control}$) for (a) PAN 1666 and (b) LS 6164 as well as difference in relative variable fluorescence for (c) PAN 1666 and (d) LS 6164 exposed to different SO_2 concentrations for three weeks relative to the CF control (0 ppb treatment) plants exposed to CF air. The average fluorescence transients were normalised between the fluorescence extremes F_0 (50 µs) and F_J (2 ms). (n = 24)

The intensification of the ΔI-band visible at 30ms in Fig. 4.2.3.4 indicates the inhibition of reduction of end electron acceptors (decrease in production of reducing equivalents) (Smit, *et al*., 2009:628). The effect on electron flow from PQH_2 to PSII end electron acceptors can be observed. At the 25 ppb a positive ΔI-band of 0.6% for PAN 1666 and 1.9% for LS 6164 greater than the CF control (0 ppb treatment)s was revealed,

whereas for 75 ppb the positive ΔI-band had a higher difference relative to the CF control (0 ppb treatment) treatment of 15.5% (PAN 1666) and 14.7% (LS 6164). As expected the plants exposed to 150 ppb SO_2 were predominantly influenced with positive ΔI-bands of respectively 24.1% and 17.2% higher for PAN 1666 and LS 6164 higher in comparison with respect to their CF control (0 ppb treatment) plants.

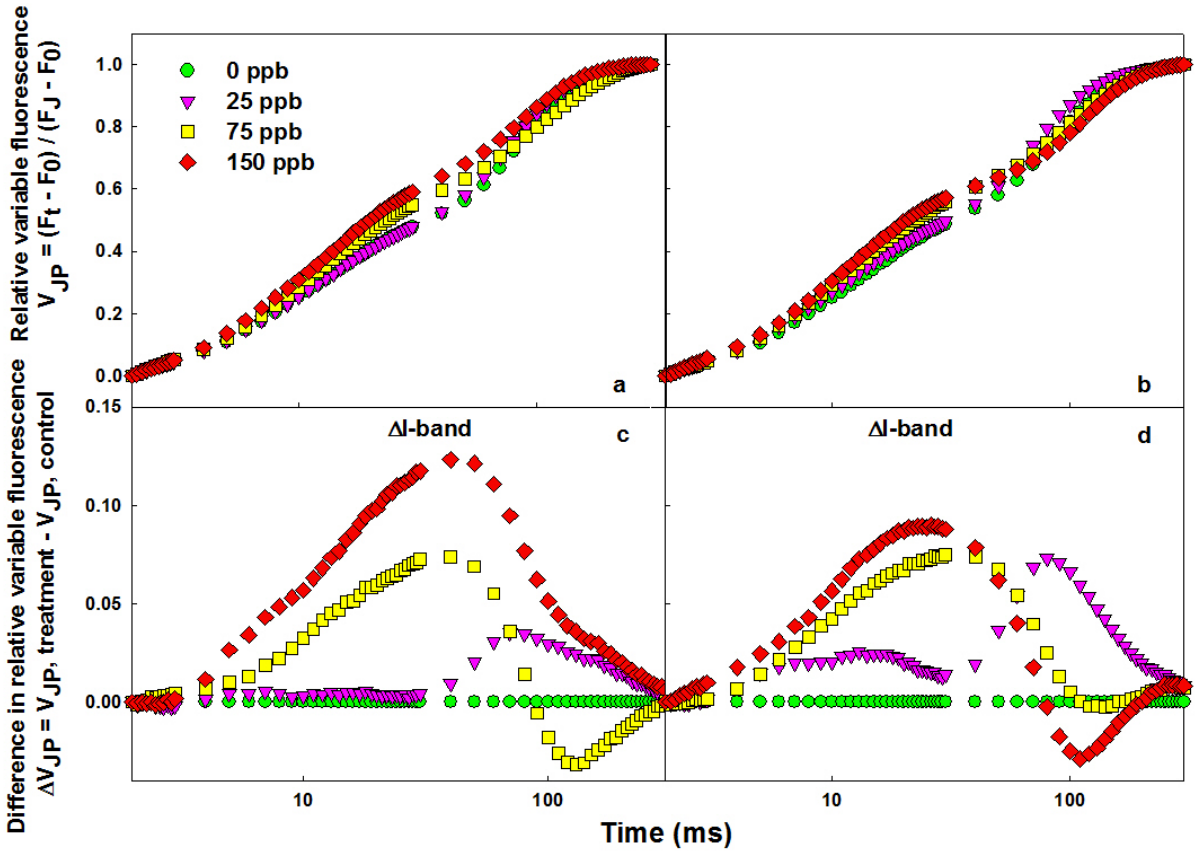

Figure 4.2.3.4: Relative variable fluorescence normalised between F_J and F_P: Relative variable fluorescence ($\Delta V_{JP}=V_{JP\ treatment} - V_{JP\ control}$) (a) PAN 1666 as well as (b) LS 6164 and difference in relative variable fluorescence of (c) PAN 1666 and (d) LS 6164 exposed to different SO_2 concentrations for three weeks relative to the CF control (0 ppb treatment) plants exposed to CF air. The average fluorescence transients were normalised between the fluorescence extremes F_J (2 ms) and F_P (300 ms). (n = 24)

The inhibition of the reduction of end e-receptors (represented by the ΔI-band) were SO_2 concentration dependant in both cultivars, even though PAN 1666 had more inhibition at 150 ppb SO_2 compared to LS 6164. The electron flow further down from

the reduced intersystem electron acceptors, of the electron transport chain, to the PSI and electron acceptors was therefore inhibited. This indicates that reducing equivalents are decreased and a lower extent of energetic connectivity exits among PSII units. An SO_2 concentration-dependant ΔI-band was observed by Heyneke, *et al*., (2012b:16) and was interpreted as an indicator of inhibition of the final reduction of end electron acceptors and observed to corroborate a decrease in J_{max}.

The OK phase of the curve shed light on the energetic connectivity (grouping) of PSII units where a higher connectivity will result in more effective utilization of excitation energy (Yusuf, *et al*., 2010:1436). In Fig. 4.2.3.5 the ΔL-band, which is visible at 0.15 ms, is amplified and this reflects an influence on the overall grouping probability within the PSII antenna (P_{2G}). The appearance of the positive ΔL-bands at 0.15 ms indicated that all treatments resulted in a decrease of energetic connectivity. The consequence of this is a lower stability of the PSII system. A positive ΔL-band indicates the transformation of a sigmoidal fluorescence rise and exponential rise (Oukarroum, *et al*., 2007:442). A negative ΔL-band indicates more of hyperbolic initial fluorescence rise, which is a reflection of an increase of the energetic connectivity (Oukarroum, *et al*., 2007:444). PAN 1666 indicated a greater ΔL-band of 2.7%; 3.4% and 8.8% correspondingly to 25 ppb; 75 ppb and 150 ppb SO_2 treatments in comparison to the CF control (0 ppb treatment). The LS 6164 plants indicated a positive ΔL-band greater by 4.2%; 4.0% and 3.3% respective to 25 ppb; 75 ppb and 150 ppb when compared to the CF control (0 ppb treatment).

PAN 1666 had an increasing ΔL-band with increasing SO_2 concentrations and therefore an inhibition on the overall grouping probability within the PSII antenna, while LS 6164 did not have mentionable variation in energetic connectivity between different treatments. The positive ΔL-bands pointed towards a decrease of the energetic connectivity (Yusuf, *et al*., 2010:1436). The results of a positive ΔL-band are similar to results of Oukarroum, *et al*., (2007:442) of drought stress and re-watering on barley cultivars, as well as results observed by Yusuf, *et al*., (2010:1436) due to abiotic stress. A higher energetic connectivity between PSII units increases the utilization of excitation energy and is a factor of stability of the photosynthetic system (Tsimilli-Michael, *et al*., 2008:77).

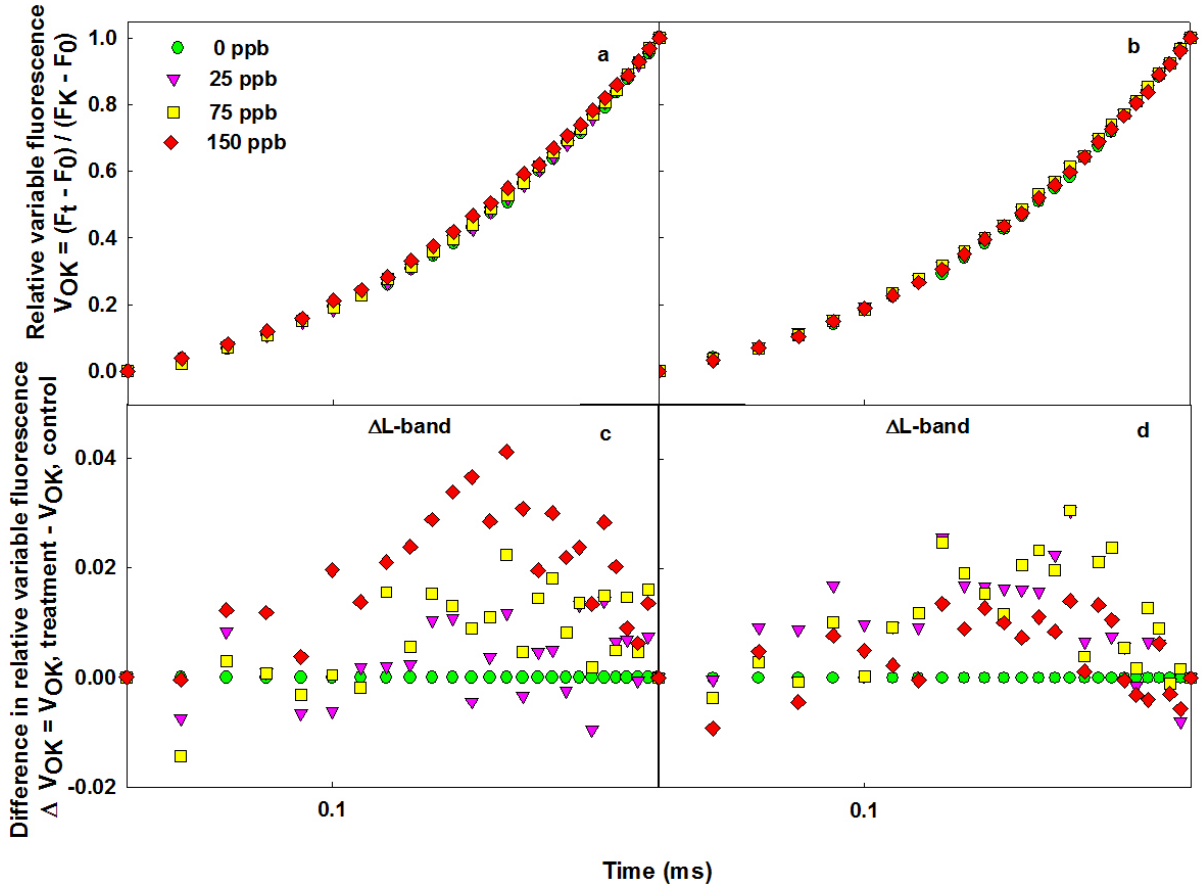

Figure 4.2.3.5: Relative variable fluorescence normalised between F_0 and F_K: Changes in relative variable fluorescence ($\Delta V_{OK} = V_{OK\ treatment} - V_{OK\ control}$) for (a) PAN 1666 as well as (b) LS 6164 and difference in relative variable fluorescence of (c) PAN 1666 and (d) LS 6164 exposed to different SO_2 concentrations for three weeks relative to the CF control (0 ppb treatment) plants exposed to CF air. The average fluorescence transients were normalised between the fluorescence extremes F_0 (50 µs) and F_K (0.3 ms). (n = 24).

Double normalisation between 0.3 ms and 30 ms intensify the J-band, which appears around 2ms (Fig. 4.2.3.6). An increase in the J-band is attributed to fast accumulation of Q_A, which can be interpreted as evidence for an interruption of electron flow beyond Q_A. The presence of this peak is also associated with the inhibition of Q_A^- re-oxidation (Smit, *et al.*, 2009:628). Positive ΔJ-band was observed for PAN 1666 for 75 ppb (5.7%) greater than the CF control (0 ppb treatment), on the other hand 25 ppb and 150 ppb indicated negative ΔJ-bands of 3.9% and 11.1% respectively lower compared to the CF control (0 ppb treatment). LS 6164 plants only revealed positive ΔJ-bands,

which were 1.0% (25 ppb); 0.2% (75 ppb) and 6.6% (150 ppb) higher at 2 ms compared to the CF control (0 ppb treatment) plants.

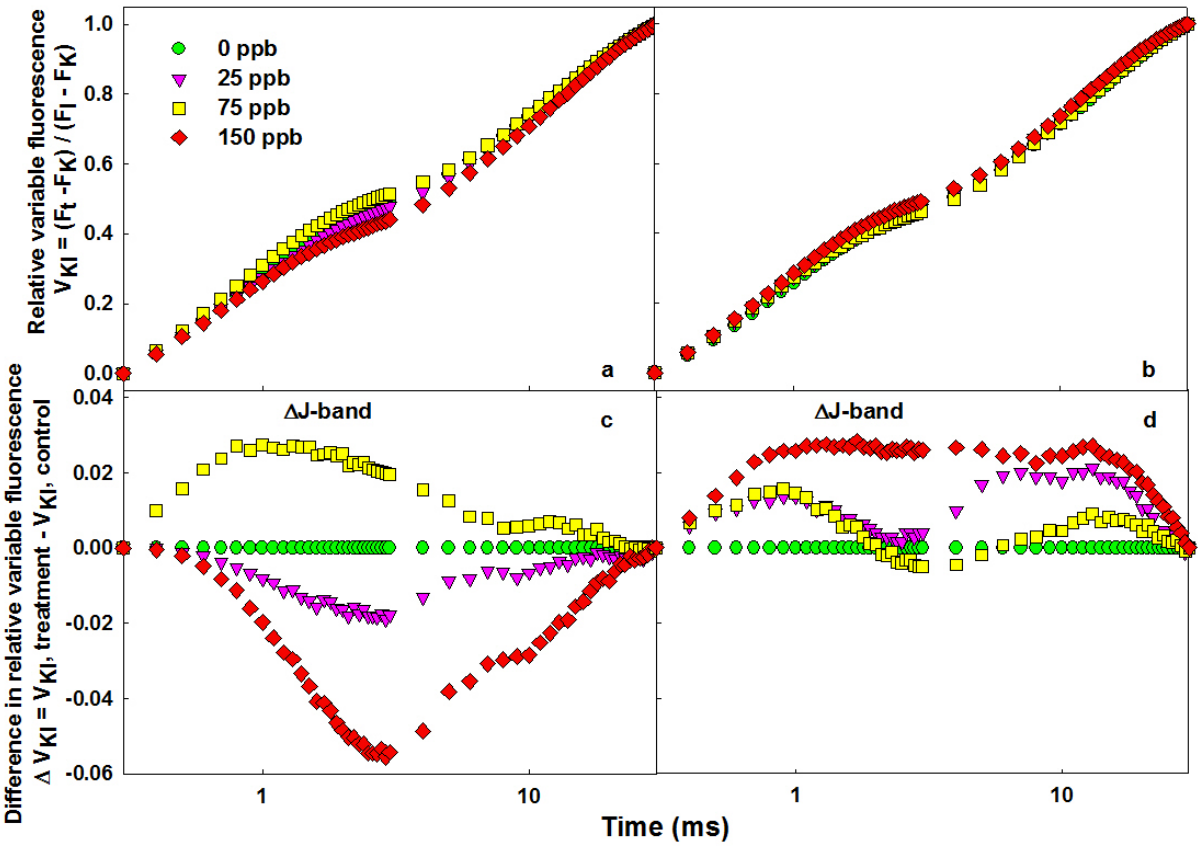

Figure 4.2.3.6: Relative variable fluorescence normalised between F_K and F_I: Changes in relative variable fluorescence ($\Delta V_{KI} = V_{KI\ treatment} - V_{KI\ control}$) for (a) PAN 1666 and (b) LS 6164 as well as difference in relative variable fluorescence for (c) PAN 1666 and (d) LS 6164 exposed to different SO_2 concentrations for three weeks relative to the CF control (0 ppb treatment) plants exposed to CF air. The average fluorescence transients were normalised between the fluorescence extremes F_K (0.3 ms) and F_I (30 ms). (n = 24)

Positive ΔJ-bands were observed in results of Smit, *et al.*, (2009:628), that reflect the inhibition of the reoxidation of Q_A^-. Panda & Sarkar, (2012:103) established an increase in the fluorescence at the 2 ms (J-band), and interpreted it as an indication of an accumulation of the reduced Q_A^- pool due to the decrease of electron transport beyond Q_A^- or acceptor side of PSII.

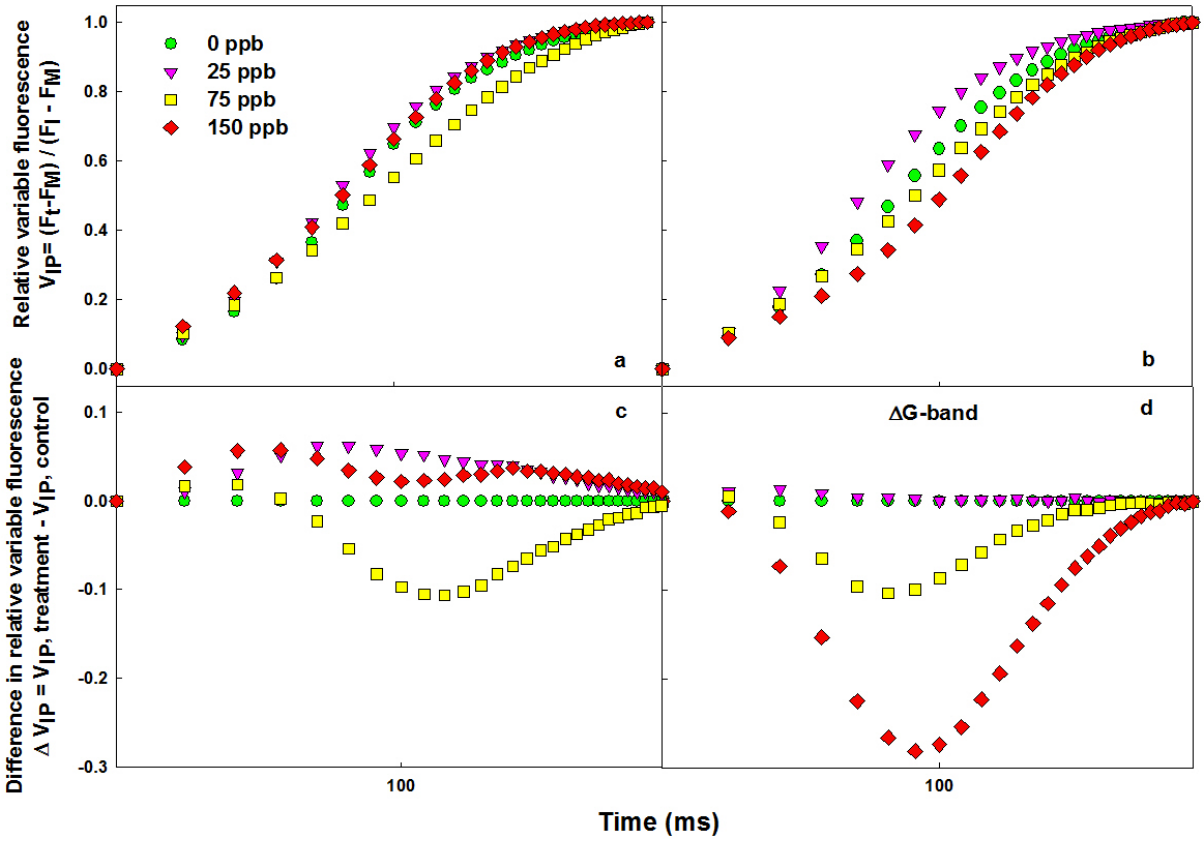

Figure 4.2.3.7: Relative variable fluorescence normalised between F_I and F_P: Changes in relative variable fluorescence ($\Delta V_{IP} = V_{IP\ treatment} - V_{IP\ control}$) for (a) PAN 1666 and (b) LS 6164 as well as difference in relative variable fluorescence for (c) PAN 1666 and (d) LS 6164 exposed to different SO_2 concentrations for three weeks relative to the CF control (0 ppb treatment) plants exposed to CF air. The average fluorescence transients were normalised between the fluorescence extremes F_I (30ms) and F_P (300ms). (n = 24)

Normalization between I (30 ms) and P (300ms) ease a comparison of the reduction rates of the end electron acceptors pool with different SO_2 treatments (Yusuf, *et al*., 2010:1436). An intensified G-band provides information about the protonated secondary quinone acceptor (Q_BH_2). Positive ΔG-band for PAN 1666 for the 25 ppb and 150 ppb treatments of 7.1% and 2.4% higher relative to the CF control (0 ppb treatment) (Fig. 4.2.3.7). ΔG-bands were observed for the 75 ppb treatment that was 14.6% lower than the CF control (0 ppb treatment). LS 6164 only had one positive ΔG-peak for the 25 ppb treatment which were 14.9 % higher than the 0 ppb. The 75 and 150 ppb treatments displayed negative ΔG-peaks of 9.6% and 23.0% lower than 0 ppb.

The LS 6164 cultivar had a stimulation of the protonated secondary quinone acceptor for increasing SO_2 treatments as indicated by the G-band, however for the PAN 1666 cultivar stimulation was observed for the 75 ppb treatment while slight stimulation was observed for the 150 ppb treatment.

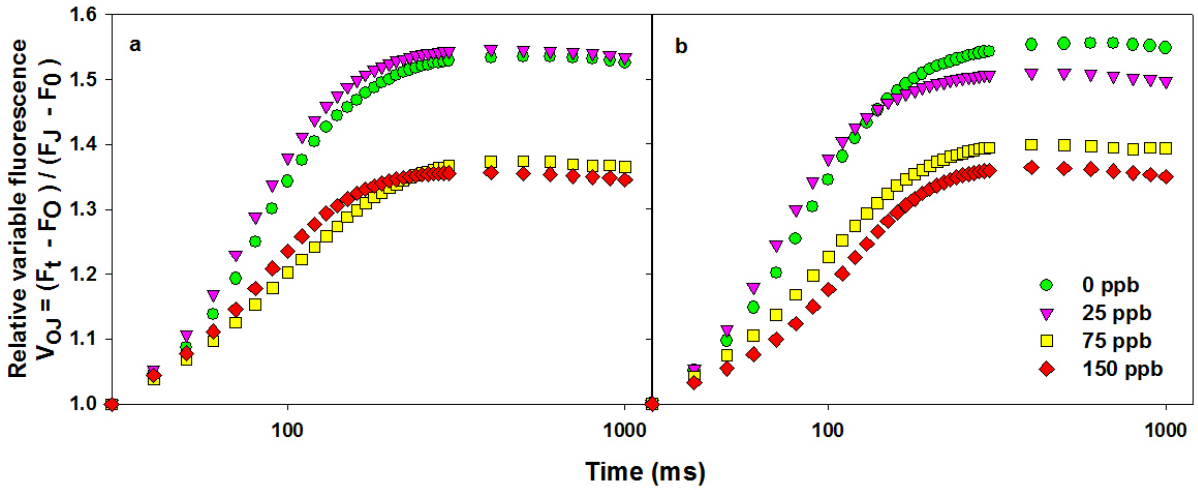

Figure 4.2.3.8: Relative variable fluorescence normalised between F_0 and F_J: Double normalization at the O-J phase, $V_{OJ} = (F_t - F_0)/(F_J - F_0)$ giving forth relative variable fluorescence for (a) PAN 1666 and (b) LS 6164 *Glycine max* plants after three weeks of SO_2 fumigation. Only transients above 1.0 ($V_{OI} \geq 1.0$) are illustrated since it reflects the reduction of electron acceptors at PSI. (n = 24)

The maximal amplitude of the fluorescence rise reflects the size of the pool of the end electron acceptors at the PSI acceptor side (Yusuf, *et al*., 2010:1437). The 75 ppb and the 150 ppb had a decrease in the pool size of end electron acceptors for both cultivars, compared to the CF control (0 ppb treatment)s (Fig. 4.2.3.8). The 25 ppb treatment gave a slightly higher result of 0.9 % whereas for 75 ppb and 150 ppb indicated a decrease of 10.6% and 11.4% respectively. All LS 6164 plants had a decrease in pool size as the SO_2 concentration increased. Decreases were observed of 2.4 %, 9.6% and 11.9% respectively for 25 ppb; 75 ppb and 150 ppb when compared to the CF control (0 ppb treatment). An increase in SO_2 exposure therefore leads to a decrease in pool size of end electron acceptors at the PSI acceptor side.

Concentration dependant inhibitions were observed in pool size of end electron acceptors for both cultivars. A decrease in pool size of end electron acceptors at the PSII acceptor side correlates with a decrease observed due to abiotic stress by Yusuf, *et al*., (2010:1436).

The $PI_{ABS,total}$ of intact dark-adapted leaves was the most sensitive parameter of chlorophyll a fluorescence and is a representation for the overall potential for energy conservation from excitation to the reduction of PSI end acceptors (Fig. 4.2.3.9). The average $PI_{ABS,total}$ values were calculated with the JIP-test from the direct fluorescence transients without normalization. This is a multi-parametric expression considering four independent fluorescence parameters namely efficiency of light energy absorption [RC/ABS or γ_{RC} / (1-γ_{RC})]; trapping of excitation energy [φ_{PO} / (1- φ_{PO})]; conversion of trapped excitation energy to electron transport [Ψ_O/ (1- Ψ_O)] and reduction of end electron transport [δ_{Ro} / (1- δ_{Ro})]. A clearly visible concentration dependant effect was observed with increasing SO_2 concentration over a period of time, for both *Glycine max* cultivars. Before SO_2 fumigation started (week 0) the $PI_{ABS,total}$ of all treatments of both cultivars were more or less the same however from week 2 onward there were noteworthy differences between treatments.

A significant ($p \leq 0.05$) decrease in the $PI_{ABS,total}$ of PAN 1666 and LS 6164 cultivars were observed over time when the plants were fumigated with 75 ppb and 150 ppb SO_2, when compared to the CF control (0 ppb treatment) (0 ppb). The 150 ppb treatment resulted in the lowest $PI_{ABS,total}$ of both cultivars followed by the 75 ppb treatment. After 1, 2, 3 and 4 weeks of fumigation the 150 ppb treatment were 48.5 % ($p \leq 0.05$), 69.8% ($p \leq 0.05$), 64.3% ($p \leq 0.05$) and 65.5% ($p \leq 0.05$) lower, while the 75 ppb treatments were 20.3% ($p \leq 0.05$), 35.4% ($p > 0.05$), 54.3% ($p \leq 0.05$) and 60.5% ($p \leq 0.05$) lower compared to the CF control (0 ppb treatment) of PAN 1666 (Fig. 4.2.3.9.a). The 25 ppb treatment of PAN 1666 was only significant higher after 1 (15.7%, $p \leq 0.05$) and 2 weeks (0.9%, $p \leq 0.05$), of fumigation and from then on there were no significant differences between the 25 ppb treatment and the CF control (0 ppb treatment). A significant decline in the $PI_{ABS,total}$ for both the 75 ppb and the 150 ppb treatments were observed from 2 weeks onward. A decline of 43.7% ($p \leq 0.05$), 56.9% ($p \leq 0.05$) and 72.5% ($p \leq 0.05$) were observed for the 150 ppb treatment, while the 75 ppb were 25.8 ($p \leq 0.05$), 46.1% ($p \leq 0.05$) and 68.5% ($p \leq 0.05$) compared to

the CF control (0 ppb treatment) (Fig. 4.2.3.9.b). The $PI_{ABS,total}$ of the 25 ppb treatment of LS 6164 was significantly higher after 1, 2, and 3 weeks of fumigation 33.7% ($p \leq 0.05$), 22.8% ($p \leq 0.05$) and 23.6% ($p \leq 0.05$) but declined significantly after 4 weeks of fumigation with 18.8% ($p \leq 0.05$) compared to the CF control (0 ppb treatment). The 75 ppb and 150 ppb treatments differed significantly compared to each other for the PAN 1666 plants only after 1 and 2 weeks of fumigation, while these treatments of LS 6164 plants differed significantly from each other after 2 and 3 weeks of fumigation.

The influence of SO_2 fumigation on PSII, measured with the JIP test gave an insight in the capability of the photosynthesis apparatus for energy supply, metabolism and eventually the yield delivered by the plants. The multi-parametric nature of $PI_{ABS,total}$ is an excellent indicator of the overall capability of the photosynthesis apparatus. The largest decrease in fluorescence data was observed in the $PI_{ABS,total}$, this is due to the fact that it expresses the overall potential for energy conservation that depends on all the efficiencies for the sequential energy transduction (Yusuf, *et al*., 2010:1437).

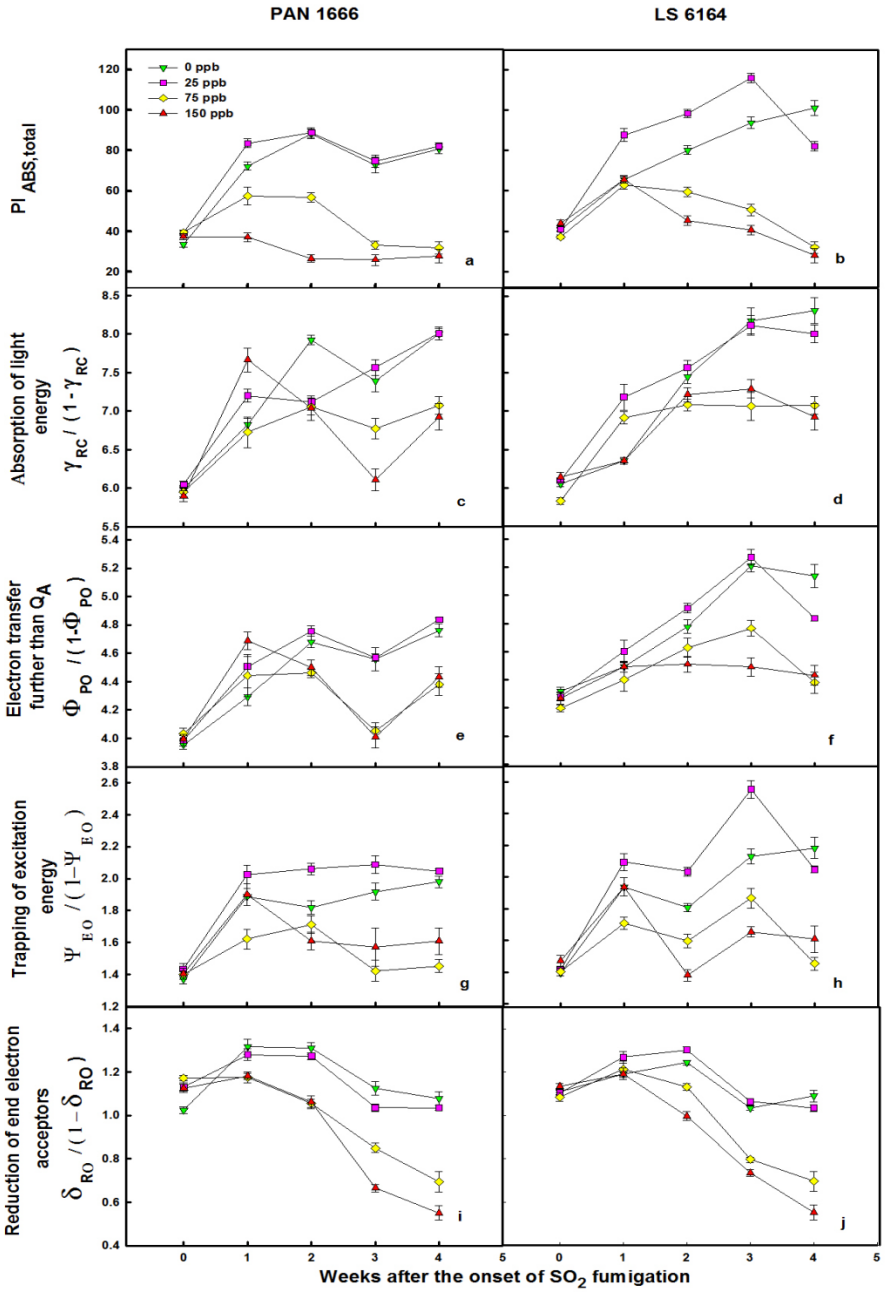

Figure 4.2.3.9: $PI_{ABS,total}$ and partial components: $PI_{ABS,total}$ of (a) PAN 1666 and (b) LS 6164 of *Glycine max* plants over a period of 4 weeks exposure to SO_2. The four partial processes reflected by the $PI_{ABS,total}$ namely: absorption of light energy (γ_{RC} / (1-γ_{RC}) also known as RC/ABS) for (c) PAN 1666 and (d) LS 6164; trapping of excitation energy (φ_{PO} /(1-φ_{PO}) for (e) PAN 1666 and (f) LS 6164; the conversion of trapped excitation energy to electron transport further than Q_A^- [ψ_{EO} / (1-ψ_{PO})] for (g) PAN 1666 and (h) LS 6164 (h) as well as the reduction of end electron acceptors [δ_{RO} / (1-δ_{RO})] for (i) PAN 1666 and for (j) LS 6164. (n = 24)

The increased inhibition of $PI_{ABS,total}$ due to increasing SO_2 concentration on soybean was established in a previous study by Heyneke, *et al*., (2012b:16), which support the results observed. The decrease in $PI_{ABS,total}$, as well as the decrease of the individual component parameters of $PI_{ABS,total}$ when plants are exposed to abiotic stress are also confirmed with results of Smit, *et al*., (2009:626) and Yusuf, *et al*., (2010:1437). $PI_{ABS,total}$ decreased significantly overtime for both cultivars for increasing SO_2 treatments. The two parameters that contributed most to the decrease in the $PI_{ABS,total}$ is the conversion of trapped excitation energy to electron transport as well as the reduction of end electron acceptors. Concentration dependant decreases in the all four partial processes of $PI_{ABS,total}$ were observed. The decrease of $PI_{ABS,total}$ may possibly be due to a decrease of stomatal conductance at the corresponding SO_2 levels. The reduced stomatal conductance will also lead to a reduction in SO_2 conductance and ultimately influence the rate of electron transport. $PI_{ABS,total}$ demonstrated the largest difference (negative), since it expresses the overall potential for energy conservation that depends on all the efficiencies for the sequential energy transduction. A decrease in $PI_{ABS,total}$ expresses a decrease in plants ability for energy conservation, there is a strong correlation to the final outcome of a plant's physiological parameters for example growth (Yusuf, *et al*., 2010:1437).

Significant differences in the absorption of light energy [Y_{RC} / (1- Y_{RC})] parameter had significant differences within PAN 1666 treatments before increasing SO_2 fumigation (Fig. 4.2.3.9.c). The absorption of light energy of the 150 ppb treatment after 1 week of fumigation had an absorption of 12.3% ($p \leq 0.05$) higher than the CF control (0 ppb treatment), where after 2, 3 and 4 weeks of fumigation the absorption were 11.1% ($p \leq 0.05$), 17.3% ($p \leq 0.05$) and 13.5% ($p \leq 0.05$) lower compared to the CF control (0 ppb treatment). The 75 ppb treatment had significant decreases of 10.9% ($p \leq 0.05$), 8.4% ($p \leq 0.05$) and 11.6% ($p \leq 0.05$) compared to the CF control (0 ppb treatment). After 2 weeks the 25 ppb treatment had significant change in absorption of 10.1% ($p \leq 0.05$) where after no significant change in comparison to the CF control (0 ppb treatment) were observed for the PAN 1666. The LS 6164 plants had significant differences observed from 2 weeks of SO_2 fumigation (Fig. 4.2.3.9.d). The absorption at 2, 3 and 4 weeks of fumigation the 150 ppb treatment was 3.1% ($p > 0.05$), 10.8% ($p \leq 0.05$) and 16.7% ($p \leq 0.05$) lower, while the 75 ppb treatment were 4.9% ($p \leq 0.05$), 13.6% ($p \leq 0.05$) and 14.9% ($p \leq 0.05$) lower compared to the CF control (0 ppb treatment). The

75 ppb and 150 ppb treatment had no significant differences ($p > 0.05$) in respect to each other only at week 2 and 3. The $PI_{ABS,total}$ of the 25 ppb treatment were insignificant higher for week 1, 2 and 3 weeks of fumigation, but declined insignificantly ($p > 0.05$) after 4 weeks when compared to the CF control (0 ppb treatment).

The ability of the plant to photosynthesize and to produce carbon for its metabolism finds its origin where light is absorbed by the PSII antenna pigments and the cascade ends at the reduction of end electron acceptors at PSI electron acceptor side (Yusuf, *et al*., 2010:1430). The absorbance of light energy is an indicator for the apparent antenna size (total absorption / reaction centre) and can therefore be influenced either by inactivation of a part of the antenna or by a decrease in the size of the antenna. A decrease in antenna size indicates also a photo-protective regulation (Tsimilli-Michael, *et al*., 2008:78).

The ability of PSII to create a charge separation, also referred to as trapping [φ_{PO} / (1-φ_{PO})] decreases significantly for PAN 1666 and LS 6164 when fumigated with 150 ppb and 75 ppb SO_2 in comparison to the CF control (0 ppb treatment). After one week of fumigation the 150 ppb treatment was 9.2% ($p \leq 0.05$) higher, however at 2, 3 and 4 weeks of fumigation trapping was 3.9% ($p \leq 0.05$), 12.0 ($p \leq 0.05$) and 6.9% ($p \leq 0.05$) lower (Fig. 4.2.3.9.e). The 75 ppb treatment was 3.5% higher at 1 week of fumigation, while after 2, 3 and 4 weeks the significant decreases to the CF control (0 ppb treatment) were 4.7% ($p \leq 0.05$), 11.1% ($p \leq 0.05$) and 9.0% ($p \leq 0.05$). The 75 ppb and 150 ppb treatments had no significant differences in respect to each other at any of the time intervals. The trapping of the 25 ppb treatment was higher at all weeks than the CF control (0 ppb treatment), but none of these differences were significant. For LS 6164 significant differences were observed from week 2, the 150 ppb treatment decreased by 5.6% ($p \leq 0.05$), 13.8% ($p \leq 0.05$) and 13.7% ($p \leq 0.05$) respectively at weeks 2, 3 and 4 respectively in comparison to the CF control (0 ppb treatment) (Fig. 4.2.3.9.f). The 75 ppb treatment decreased with 3.1% ($p > 0.05$), 8.6% ($p > 0.05$) and 14.8% ($p > 0.05$) at weeks 2, 3 and 4 of fumigation when compared to the CF control (0 ppb treatment), while a significant difference between the 75 ppb and 150 ppb treatments were observed at 3 weeks of fumigation. After 1, 2 and 3 weeks of fumigation the 25 ppb treatment had a slight insignificant increase in respect to the CF control (0 ppb treatment), however decreased significantly after 4 weeks of fumigation.

Trapping and absorption decreased proportionally to each other for both cultivars and are the least influenced of the parameters of $PI_{ABS,total}$. Trapping is an intermediate energy flux that leads to the reduction of electron acceptors of PSII, pheophytin-pheo and Q_A (Yusuf, *et al*., 2010:1430).

Electron transport [ψ_{EO} / (1-ψ_{EO})] had no statistical significance neither for PAN 1666 plants between the SO_2 treatments and the CF control (0 ppb treatment) at week 0 and 1 week of SO_2 fumigation (Fig. 4.2.3.9.g). The 75 ppb and 150 ppb treatments decreased significantly in electron transport when compared to the CF control (0 ppb treatment). After 2, 3 and 4 weeks of fumigation the electron transport for the 150 ppb were 11.5% ($p \leq 0.05$), 18.1% ($p > 0.05$) and 18.8% ($p > 0.05$) lower, while the 75 ppb treatment was 5.9% ($p > 0.05$), 25.8% ($p \leq 0.05$) and 26.6% ($p \leq 0.05$) lower than the CF control (0 ppb treatment). In the 25 ppb treatment after 2 weeks of fumigation the only significant difference to the CF control (0 ppb treatment) was observed where the 25 ppb was 13.2% ($p \leq 0.05$) higher. The 75 ppb and 150 ppb treatments did not differ significantly after 2, 3 and 4 weeks of fumigation. This is the parameter that decreased the most for the 75 ppb after 3 weeks of fumigation. For LS 6164 at week 1, 2, 3 and 4 the 75 ppb treatment were 11.9% ($p \leq 0.05$), 11.7% ($p \leq 0.05$), 12.4% ($p \leq 0.05$) and 33.5% ($p \leq 0.05$) lower, while the 150 ppb treatment were 23.7% ($p \leq 0.05$), 22.4% ($p \leq 0.05$) and 26.4% ($p \leq 0.05$) significantly lower than the CF control (0 ppb treatment) after 2, 3 and 4 weeks of fumigation (Fig. 4.2.3.9.h). The 25 ppb treatment was significantly ($p \leq 0.05$) higher after 2 and 3 weeks of fumigation, while after 4 weeks of fumigation it was significantly lower than the CF control (0 ppb treatment). Significant differences were observed between the 75 ppb and 150 ppb treatments at 2 and 3 weeks of fumigation. Electron transport was the largest contributor to the decrease in $PI_{ABS,total}$ for the 75 ppb treatment after 1 week of fumigation as well as the 75 ppb and 150 ppb treatments after 2 weeks of fumigation.

In drought stress studies of Oukarroum, *et al*., (2007:444) photosynthetic electron transport was the largest contributor to a decrease in PI_{ABS}. PI_{ABS} is similar to $PI_{ABS,total}$ but does not take the reduction of end electron acceptor into account.

A significant difference ($p \leq 0.05$) in the reduction of end electron acceptors [δ_{RO} / (1-δ_{RO})] of both PAN 1666 and LS 6164 were observed with fumigation with 75 ppb and

150 ppb SO_2 when compared to the CF control (0 ppb treatment). After 1, 2, 3 and 4 weeks of fumigation the 150 ppb treatment was 10.4% ($p \leq 0.05$), 19.0% ($p \leq 0.05$), 40.9% ($p \leq 0.05$) and 48.8% ($p \leq 0.05$) lower, while the 75 ppb treatment were 10.8% ($p \leq 0.05$), 19.3% ($p \leq 0.05$), 24.5% ($p \leq 0.05$) and 35.4% ($p \leq 0.05$) lower than the CF control (0 ppb treatment) (Fig. 4.2.3.9.i). No significant differences were observed at any of the time intervals for the PAN 1666 25 ppb treatment in respect to the CF control (0 ppb treatment). Significant difference was observed between the 75 ppb and 150 ppb treatments only after 3 weeks of fumigation. This parameter had the largest influence on the $PI_{ABS,total}$ for the 150 ppb treatment after 1 week of fumigation; 75 ppb and 150 ppb treatments after 2 weeks of fumigation; the 150 ppb treatment after 3 weeks of fumigation as well as for the 75 ppb and 150 ppb treatment after 4 weeks of fumigation. For LS 6164 significant differences were observed from 2 weeks of fumigation in respect to the CF control (0 ppb treatment). The 150 ppb treatment at 2, 3 and 4 weeks of SO_2 exposure were 19.9% ($p \leq 0.05$), 29.1% ($p \leq 0.05$) and 49.4% ($p \leq 0.05$) lower, while the 75 ppb were 9.1% ($p \leq 0.05$), 23.1% ($p \leq 0.05$) and 36.2% ($p \leq 0.05$) lower than the CF control (0 ppb treatment) (Fig. 4.2.3.9.j). No significant differences were observed between the CF control (0 ppb treatment) and the 25 ppb treatment. Significant differences were observed between the 75 ppb and 150 ppb treatments at 2 and 4 weeks of fumigation. Both the 75 ppb and the 150 ppb treatments experienced the largest difference in this parameter after 3 as well as 4 weeks of fumigation.

Çíçek, *et al*., (2012:535) observed a decrease in the efficiency of electron movement from the reduced intersystem electron acceptors (The electron acceptors refer to the electron transport further than Q_A^-) to the PSI end electron acceptors when examining the effect of textile azo dyes on maize.

The $PI_{ABS,total}$ is in essence an indicator of sample vitality, whereas the driving force (DF) is an indicator which describes the forces of redox reactions and generally movements of Gibbs free energy in biological systems. The total driving force for photosynthetic activity ($DF_{ABS,total}$) was calculated by the summation of the logarithm of the partial driving forces for efficiency of absorption of light energy, trapping of excitation energy, the conversion of excitation energy to electron transport and the formation of reducing equivalents (Fig. 4.2.3.10). Both cultivars indicate clear

concentration dependence for all treatments which is even more evident over longer exposure to SO_2.

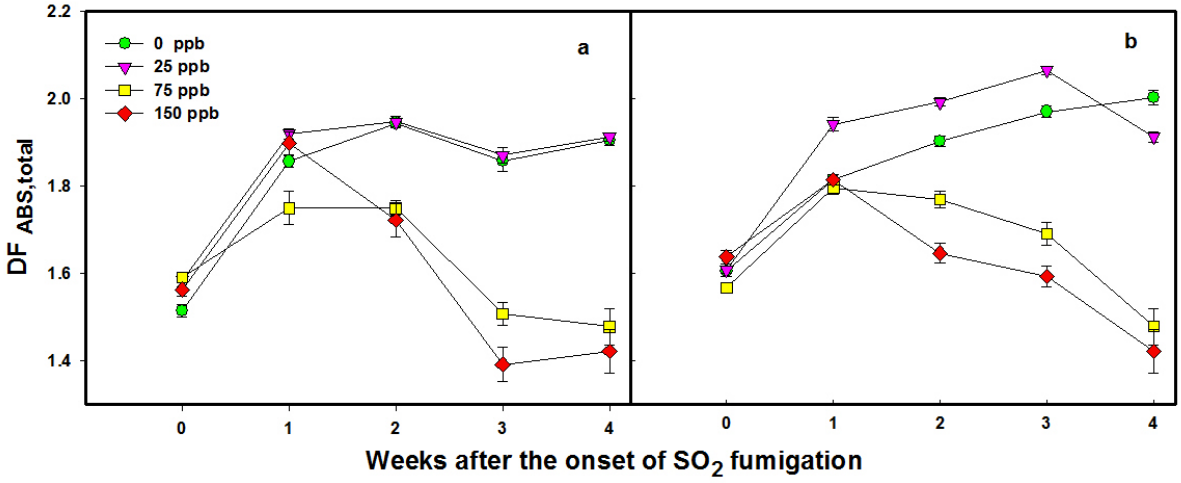

Figure 4.2.3.10: Driving force total: Driving force total over a time period of 4 weeks for (a) PAN 1666 and (b) LS 6164. (n = 24)

A significant ($p \leq 0.05$) decrease in the $DF_{ABS,total}$ of both PAN 1666 and LS 6164 cultivars were observed with fumigation with 75 ppb and 150 ppb SO_2. The 150 ppb SO_2 treatment resulted in the lowest $DF_{ABS,total}$ of both cultivars followed by the 75 ppb treatment. After 2, 3 and 4 weeks of SO_2 fumigation the $DF_{ABS,total}$ of the150 ppb treatment were 11.4% ($p \leq 0.05$), 25.0% ($p \leq 0.05$) and 25.3% ($p \leq 0.05$) lower, while the 75 ppb were 10.0% ($p \leq 0.05$), 18.8% ($p \leq 0.05$) and 22.4% ($p \leq 0.05$) lower compared to the CF control (0 ppb treatment) (Fig. 4.2.3.10.a). The 25 ppb treatment had no significant difference ($p > 0.05$) for any of the time intervals in respect to the CF control (0 ppb treatment). A significant difference between the 75 ppb and 150 ppb treatment were observed only after 3 weeks of fumigation. For LS 6164 at 2, 3 and 4 weeks of SO_2 fumigation the 150 ppb treatment were 13.5% ($p \leq 0.05$), 19.1% ($p \leq 0.05$) and 29.0% ($p \leq 0.05$) lower, while the 75 ppb treatment were 7.0% ($p \leq 0.05$), 14.2% ($p \leq 0.05$) and 26.2% ($p \leq 0.05$) lower compared to the CF control (0 ppb treatment) (Fig. 4.2.3.10.b). The 75 ppb and 150 ppb treatments had significant differences between them after 2 and 3 weeks of SO_2 fumigation. The only significant difference observed between the 25 ppb and the CF control (0 ppb treatment) were an increase of 4.7% ($p \leq 0.05$) after 2 weeks fumigation.

The driving force of photosynthesis is the logarithm of the performance index ($PI_{ABS,total}$). Even though the decreases are not as dramatic as in the case of $PI_{ABS,total}$ an obvious concentration-dependant relationship with SO_2 in both cultivars is evident.

A multi-parametric description of the structure and function of PSII for all of the treatments of both cultivars is presented to compare the changes of phenomenological energy fluxes. Energy fluxes started to experience mentionable differences from week 2 onwards (Fig. 4.2.3.11). The reaction centre of PSII is the most sensitive protein complex of the photosynthetic electron transfer chain, damage to this complex will result in a loss of photosynthetic capacity and the degradation of the reaction centre proteins (Kim, *et al*., 2007:912). Energy fluxes per reaction centre (RC) are functional parameters (Yusuf, *et al*., 2010:1436).

Significant increases ($p \leq 0.05$) in the amount of light energy absorbed (ABS/RC) were observed in both the PAN 1666 and LS 6164 cultivars. After 1, 2, 3 and 4 weeks of SO_2 fumigation, the 150 ppb treatment was 10.5% ($p \leq 0.05$) lower, 13.6% ($p \leq 0.05$), 21.3% ($p \leq 0.05$) and 15.9% ($p \leq 0.05$) higher, while the 75 ppb treatment was 1.8% ($p > 0.05$), 12.6% ($p \leq 0.05$), 9.6% ($p \leq 0.05$) and 13.3% ($p \leq 0.05$) higher when compared with the CF control (0 ppb treatment) of the PAN 1666 cultivar. Significant differences between the 75 ppb and 150 ppb treatments were observed after 1 and 3 weeks of SO_2 fumigation. The only significant increase between the 25 ppb treatment and the CF control (0 ppb treatment) were 11.4% ($p \leq 0.05$) after 2 weeks of SO_2 fumigation. For LS 6164 at 2, 3 and 4 weeks of SO_2 exposure, the 150 ppb treatment was 3.4% ($p > 0.05$), 12.3% ($p \leq 0.05$) and 20.0% ($p \leq 0.05$) higher, while the 75 ppb treatment was 5.2% ($p \leq 0.05$), 16.7% ($p \leq 0.05$) and 17.3% ($p \leq 0.05$) higher than the CF control (0 ppb treatment).

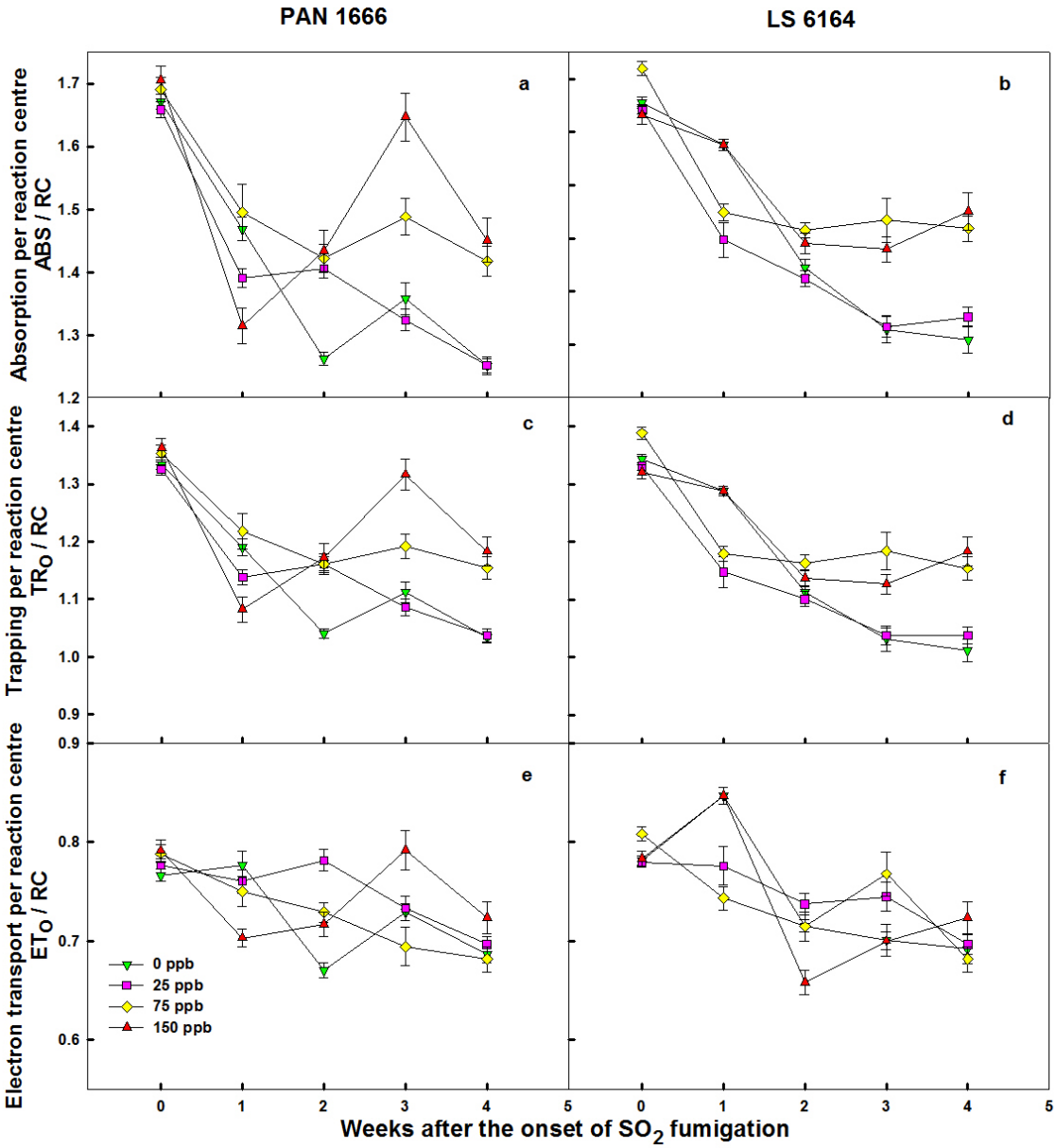

Figure 4.2.3.11: Specific energy fluxes: Specific energy fluxes per reaction centre (RC) through PSII in soybean over a period of four weeks of SO_2 fumigation. Light absorption per active reaction centre (ABS/RC) for (a) PAN 1666 and (b) LS 6164; trapping of excitation energy per active reaction centre (TR_0/RC) for (c) PAN 1666 and (d) LS 6164; electron transport per active reaction centre (ET_0/RC) for (e) PAN 1666.and (f) LS 6164. (n = 24)

The increase in ABS/RC corresponds with the results observed by Yusuf, *et al*., (2010:1437). ABS/RC is regarded as a measure of the size of the antenna leading to light absorption per reaction section. Increases in this parameter will be a result of an

effort to compensate for inactivation of reaction centres. An increase in the ABS/RC may indicate that a fraction of the RC is inactivated and therefore no longer reduces Q_A or a decrease in antenna size (which was previously mentioned) (Yusuf, *et al*., 2010:1437). An increase in antenna size can be used to compensate for a decrease in the density of the reaction centres (RC/CS_0) this will however only explain LS 6164 data at 75 ppb SO_2 exposure after three weeks. An increase of the antenna size (ABS/RC) concurs with the results of Smit, *et al*., (2009:626). Increases in ABS/RC and TR_0/RC were also observed in a study of Pan & Li (2012:739) in the case of chilling and freezing temperatures. Maize exposed to mine drainage also had an increase in ABS/RC as well, Pan, *et al*., (2010:986) stated that this increase indicates that the plants were unable to regulate the light-harvesting capacity in order to adapt.

An increase in both the ABS/RC as well as theTR_0/RC were observed which indicates that both the fraction of RCs transformed to non-Q_A^- reducing centres and functional antenna size were influenced (Yusuf, *et al*., 2010:1437). Even though both fluxes increased they had slightly different increases because TR_0/RC did not increase as much as ABS/RC. Functional antennas are still absorbing chlorophylls that did not feed the active RC but dissipated their excitation energy as heat (Yusuf, *et al*., 2010:1437). This still keep us in the dark as to whether the transformation at the reaction centres is due to structural changes to heat sinks or due to the inactivation of the oxygen evolving complex (OEC) or even a combination of the two.

Significant increases in the trapping of light energy per reaction centre (TR_0/RC) of both cultivars were observed with SO_2 fumigation of 75 ppb and 150 ppb when compared to the CF control (0 ppb treatment) (Fig 4.2.3.11c and d). After 2, 3 and 4 weeks fumigation, the PAN 1666 150 ppb treatment were 12.7% ($p \leq 0.05$), 18.3% ($p \leq 0.05$) and 14.4% ($p \leq 0.05$) higher, while the 75 ppb treatment were 11.6% ($p > 0.05$), 7.1% ($p \leq 0.05$) and 11.9% ($p \leq 0.05$) higher than the CF control (0 ppb treatment). A significant difference was observed between the 75 ppb and 150 ppb treatments after 3 weeks of fumigation. The 25 ppb treatment had no significant differences in respect to the CF control (0 ppb treatment) after any of the time intervals. For LS 6164 at 2, 3 and 4 weeks of SO_2 fumigation the 150 ppb treatment were 2.3% ($p \leq 0.05$), 9.3% ($p \leq 0.05$) and 17.0% ($p \leq 0.05$) higher, while the 75 ppb treatment were 4.6% ($p \leq 0.05$), 14.9% ($p \leq 0.05$) and 14.1% higher than the CF control (0 ppb treatment). The 25 ppb

treatment of the LS 6164 had no significant difference ($p > 0.05$) in comparison to the CF control (0 ppb treatment).

The increase in the specific trapping flux (TR_0/RC) will limit the change on the quantum yield of primary photochemistry (φ_{PO}). An increase in TR_0/RC correlates with results observed by Smit, *et al*., (2009:626) and Yusuf, *et al*., (2010:1437) in abiotic stress studies. The increase in TR_0/RC compensates for the inactivation of reaction centres (Smit, *et al*., 2009:626).

Only a few significant differences ($p \leq 0.05$) were observed for the ET_0/RC flux. The 150 ppb treatment of PAN 1666 after 1 week of SO_2 fumigation was the only ET_0/RC flux where a significant difference to the CF control (0 ppb treatment) was observed for 1 week of fumigation, this was a decrease of 9.5% ($p \leq 0.05$). After 2 weeks of SO_2 fumigation, the 25 ppb, 75 ppb and 150 ppb treatments increased significantly with 16.5%, 8.7% and 6.9% respectively. For LS 6164 the 75 ppb decrease insignificantly with 0.1% ($p > 0.05$) after 2 weeks of fumigation, while an increase of 9.5% ($p \leq 0.05$) was observed after 3 weeks of fumigation. Pan & Li (2012:741) observed a decrease in ET_0/RC of due to chilling and freezing. Pan, *et al*., (2010:986) observed a decrease in ET_0/RC (which they states relates to the re-oxidation of reduced Q_A via electron transport in active RC) of maize due to mine drainage.

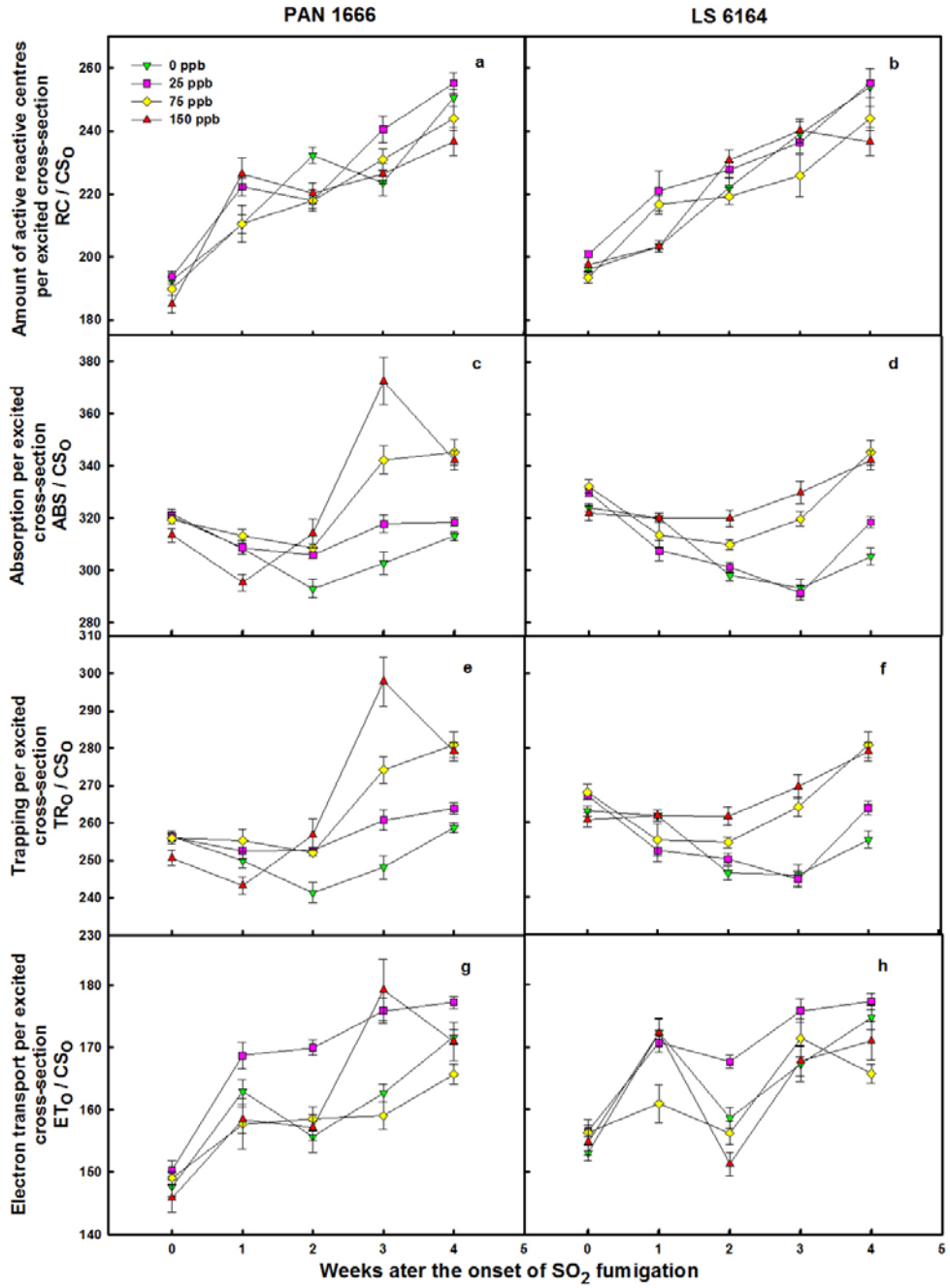

Figure 4.2.3.12: Phenomenological energy fluxes: Phenomenological energy fluxes per cross section (CS) through PSII in *Glycine max* plants over a period of 4 weeks of SO_2 fumigation. Density of active reaction centres per cross-section (RC/CS_0) for (a) PAN 1666 and (b) LS 6164, absorption per cross section ABS/CS_0 for (c) PAN 1666 and (d) LS 6164, trapping of excitation energy per cross-section of leaf (TR_0/CS_0) for (e) PAN 1666 and (f) LS 6164, electron transport per cross-section (ET_0/CS_0) for (g) PAN 1666 and (h) LS 6164. (n = 24)

LS 6164 had no significant differences for the amount of active reactive centres per excited cross-section (RC/CS_0) flux for any of the SO_2 treatments in comparison with the CF control (0 ppb treatment) at any of the time intervals. The significant differences observed for PAN 1666 were increases in the 25 ppb treatment at 2 and 3 weeks of fumigation of 4.4% ($p \leq 0.05$) and 5.0% respectively. The 150 ppb treatment decreased with 4.1% ($p \leq 0.05$) after 1 week of fumigation. The 75 ppb treatment had a significant increase after 2 weeks of SO_2 fumigation of 5.4% ($p \leq 0.05$). The active reactive centres per excited cross-section (RC/CS_0) phenomenological flux characterize the active PSII reaction centre per exited cross-section (Panda & Sarkar, 2012:104). Smit, *et al*., (2009:626) observed a decrease in RC/CS_0 when plants were exposed to trifluoroacetate.

The absorption per excited cross-section (ABS/CS_0) flux increased significantly ($p \leq 0.05$) for the PAN 1666 as well as the LS 6164 cultivars with 75 ppb and 150 ppb SO_2 fumigation in comparison to the CF control (0 ppb treatment). After 2, 3 and 4 weeks of fumigation the 150 ppb treatment of PAN 1666 were 7.2% ($p \leq 0.05$), 23.1% ($p \leq 0.05$) and 9.3% ($p \leq 0.05$) higher, while the 75 ppb treatment were 5.4% ($p \leq 0.05$), 13.1% ($p \leq 0.05$) and 10.3% ($p \leq 0.05$) higher than the CF control (0 ppb treatment). No statistical differences ($p > 0.05$) were observed for the 25 ppb treatment for either the PAN 1666 or the LS 6164 cultivar. A significant difference ($p \leq 0.05$) was observed between the 75 ppb and 150 ppb treatment after 3 weeks of SO_2 fumigation. For LS 6164 the 150 ppb treatment after 2, 3 and 4 weeks were 7.3% ($p \leq 0.05$), 12.6% ($p \leq 0.05$) and 12.1% ($p \leq 0.05$) higher, while the 75 ppb were 3.9% ($p \leq 0.05$), 9.1% ($p \leq 0.05$) and 13.1% ($p \leq 0.05$) higher than the CF control (0 ppb treatment). The only significant difference ($p \leq 0.05$) between the 75 ppb and 150 ppb were observed after 2 weeks of SO_2 fumigation.

A significant ($p \leq 0.05$) increase in the trapping per excited cross-section (TR_0/CS_0) of both the PAN 1666 and LS 6164 cultivars were observed with fumigation with 75 ppb and 150 ppb SO_2 in respect to the CF control (0 ppb treatment). These significant differences were observable from 2 weeks of fumigation. After 2, 3 and 4 weeks of fumigation the 150 ppb were 6.4% ($p \leq 0.05$), 20.0% ($p \leq 0.05$) and 7.9% ($p \leq 0.05$) higher, while the 75 ppb were 4.5% ($p > 0.05$), 10.5% ($p \leq 0.05$) and 8.6% ($p \leq 0.05$) higher than the CF control (0 ppb treatment) of PAN 1666 (Fig. 4.2.3.12.e). After 3

weeks of SO_2 fumigation a significant difference between the 150 ppb and 75 ppb treatments were observed. The 25 ppb treatment had no significant differences in respect to the CF control (0 ppb treatment). For LS 6164 at 2, 3 and 4 weeks of SO_2 fumigation the 150 ppb treatment were 6.2% ($p \leq 0.05$), 9.6% ($p \leq 0.05$) and 9.3% ($p \leq 0.05$) higher, while the 75 ppb treatment were 3.3% ($p \leq 0.05$), 7.4% ($p \leq 0.05$) and 10.0% ($p \leq 0.05$) higher when compared to the CF control (0 ppb treatment). A significant difference between the 75 ppb and 150 ppb treatment were observed after 2 weeks of SO_2 fumigation. The 25 ppb treatment had no significant difference compared to the CF control (0 ppb treatment) (Fig. 4.2.3.12.f).

Not many significant differences were observed for the electron transport per excited cross-section (ET_0/CS_0) flux, for neither the PAN 1666 nor the LS 6164 cultivar. The 25 ppb treatment of PAN 1666 had significant increases of 9.2% ($p \leq 0.05$) and 5.1% ($p \leq 0.05$) after 2 and 3 weeks of fumigation in comparison to the CF control (0 ppb treatment). The 75 ppb treatment had a significant decrease of 3.5% ($p \leq 0.05$) after 4 weeks of fumigation, while the 150 ppb treatment had a significant increase of 10.1% ($p \leq 0.05$) after 3 weeks of fumigation compared to the CF control (0 ppb treatment). (Fig. 4.2.3.12.g). For LS 6164 the statistical differences observed were for the 75 ppb, a decrease of 6.6% after 1 week of fumigation and an increase of 10.0% ($p \leq 0.05$) after 4 weeks of fumigation. The 25 ppb treatment had a increase of 5.6% ($p \leq 0.05$) after 2 weeks of SO_2 fumigation. For the 150 ppb the only observed statistical difference was a decrease of 4.6% ($p \leq 0.05$) after 2 weeks of fumigation (Fig. 4.2.3.12.h).

The increases observed in the electron transport per cross-section (ET_0/CS_0), differs from the decrease observed by Smit, *et al*., (2009:626) during a study of trifluoroacetate on *Zea mays* and *Phaseolus vulgaris*. ET_0/CS_0 indicates the re-oxidation of reduced Q_A through the electron transport over a cross-section of active and inactive reaction centre (Panda & Sarkar, 2012:104). Panda & Sarkar, (2012:104) stated that an increase in ET_0/CS_0 as well as RC/CS_0 will lead better structural and functional integrity of PSII.

The specific trapping flux (TR_0/RC) and trapping per excited cross-section (TR_0/CS_0) increased with SO_2 exposure. After 3 weeks of SO_2 exposure all phenomenological

fluxes increased. A decrease of RC/CS_0 is expected to lead to corresponding decreases in electron transport per cross section however the highest increases are observed in respect to ET_0/RC with these levels of SO_2. The ET_0/CS_0 and J_{max} were expected to have corresponding decreases (Smit, *et al*., 2009:626).

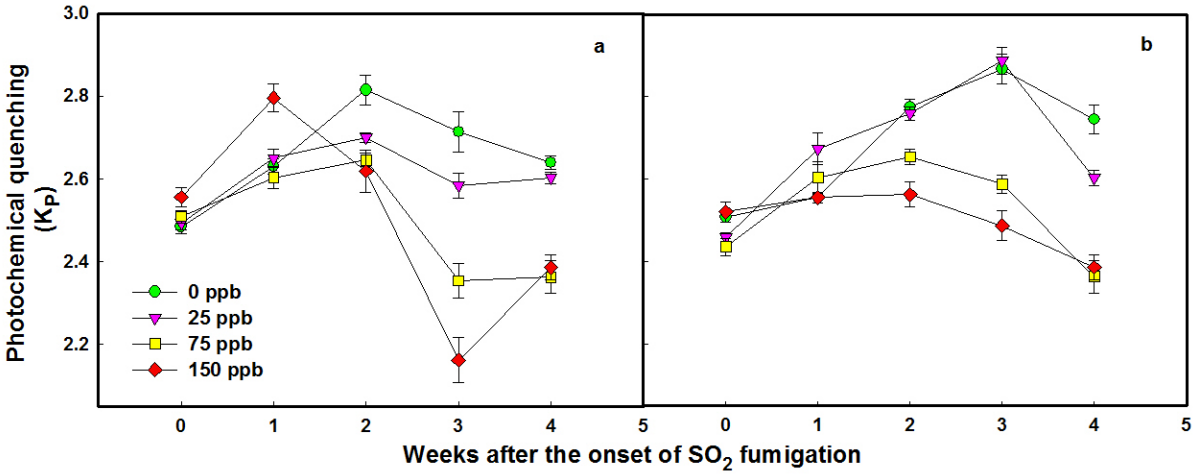

Figure 4.2.3.13: Photochemical quenching: Photochemical quenching (k_P) over a period of four weeks of SO_2 fumigation at different SO_2 concentrations for (a) PAN 1666 and (b) LS 6164. (n = 24)

When a chlorophyll molecule absorbs light energy it gets excited from its ground state, lowest energy state, to its first singlet excited state. In the excited state, the energy can be passed to another chlorophyll molecule through inductive resonance, or it can return to the ground state by emitting energy as heat (called non-photochemical quenching , K_N) or it can return to the ground state by emitting photons (photochemical quenching, K_P)

The effect of SO_2 exposure over a period of four weeks on the photochemical quenching revealed a significant concentration dependant effect (Fig. 4.2.3.13). After 1, 2, 3 and 4 weeks of SO_2 fumigation, the 150 ppb treatment were 6.3% ($p \leq 0.05$), 7.0% ($p \leq 0.05$), 20.3% ($p \leq 0.05$) and 9.6% ($p \leq 0.05$) lower, while the 75 ppb treatment were 1.1% ($p > 0.05$), 6.0% ($p \leq 0.05$), 13.3% ($p \leq 0.05$) and 10.5% ($p \leq 0.05$) lower than the CF control (0 ppb treatment) of PAN 1666. Only after 3 weeks of fumigation, a significant difference was observed between the 75 ppb and 150 ppb treatment. The 25 ppb had no significant difference to the CF control (0 ppb

treatment) at any of the time intervals. For LS 6164 the statistical decreases that were observed after 2, 3 and 4 weeks of fumigation with 150 ppb SO_2 were 7.7% ($p \leq 0.05$), 13.3% ($p \leq 0.05$) and 13.1% ($p \leq 0.05$), while the 75 ppb were 4.4% ($p \leq 0.05$), 9.7% ($p \leq 0.05$) and 13.9% ($p \leq 0.05$). There were a significant difference between the 75 ppb and 150 ppb treatments after 2 weeks of fumigation. The only significant influence of 25 ppb SO_2 on the photochemical quenching was after 4 weeks of fumigation where a decrease of 5.2% ($p \leq 0.05$) was observed.

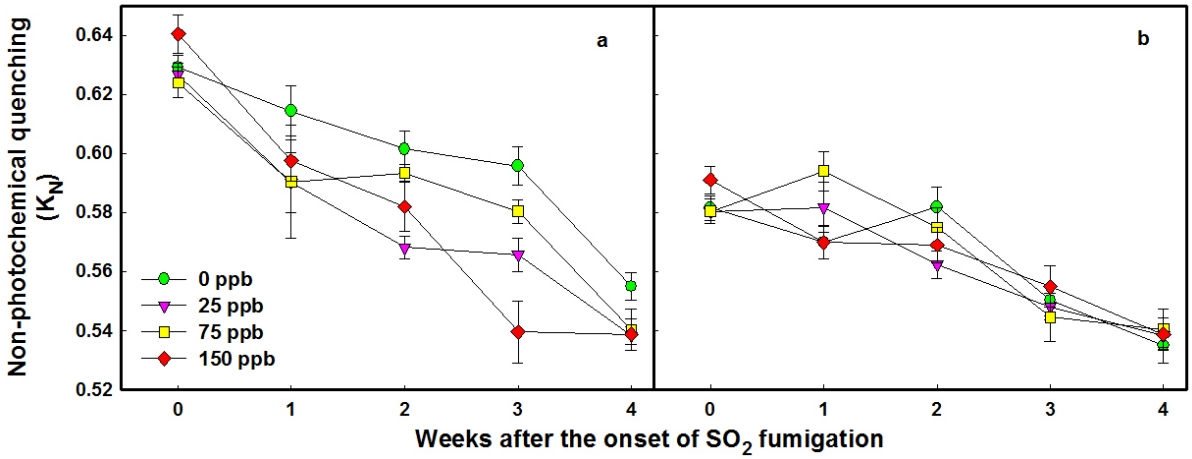

Figure 4.2.3.14: Non-photochemical quenching: Non-photochemical quenching (k_N) over a period of four weeks of SO_2 fumigation at different concentrations for (a) PAN 1666 and (b) LS 1666. (n = 24)

Non-photochemical quenching is a mechanism plants have to protect themselves from the adverse effects of high light intensity. During non-photochemical quenching, singlet excited state chlorophyll is quenched through enhanced internal conversion to the ground state (non-radiative decay). Excess excitation energy is therefore harmlessly dissipated as heat energy through molecular vibration.

The influence of SO_2 fumigation on the non-photochemical quenching over 4 weeks of exposure can be seen in Fig. 4.2.3.14. LS 6164 had no significant differences at any of the weeks between the 25 ppb, 75 ppb or the 150 ppb in comparison the CF control (0 ppb treatment). However, PAN 1666 treatment only had significant ($p \leq 0.05$) decreases in the non-photochemical quenching of 5.5%, 5.1% and 9.4% respectively

for the 25 ppb treatment after 2 weeks of fumigation as well as the 25 ppb treatment and the 150 ppb treatment after 3 weeks of fumigation.

The PSII results indicated that the 150 ppb treatment of the LS 6164 cultivar had the largest decrease in $PI_{ABS,total}$, trapping, electron transport, reduction of end electron acceptors, RC/CS_0 and $DF_{ABS,total}$ after 4 weeks of fumigation. Biomass, yield as well as seed quality was measured after 4 weeks of fumigation and the correlation between these parameters and photosynthesis is evident. It was observed that even though the PAN 1666 were more affected up until 3 weeks of SO_2 fumigation, the significant decrease in PSII efficiency after 4 weeks of fumigation led to a larger effect on the LS 6164 cultivar at harvest time.

Sulphur dioxide fumigations have been observed to result in the progressive decrease in photosynthesis (Winner & Mooney, 1980:293). A decrease in photosynthetic activity ($F_v/F_m=\Phi_{PO}$) which represented the maximal yield of the photochemical reaction on PSII following air pollutant exposure have been observed in previous studies done on sweet potato (Kim, *et al*., 2007:909). The damage to PSII is probably caused by the occurrence of excess ROS as a result of SO_2 exposure and therefore it is necessary to examine the mechanisms of defence against oxidative stress to confirm this suspicion. The loss in chlorophyll content will have a negative effect on the photosynthetic abilities of plant exposed to high concentrations of SO_2.

The lowest SO_2 concentration previously observed to inhibit photosynthesis in long-term fumigations is 60 ppb (Darrall, 1989:4). Photosynthetic changes due to air pollutants occur even before inhibition of growth as well as visible injury as we observed from 2 weeks of SO_2 exposure, this will be due to the fact the growth and foliar injury take time to become visible (Darrall, 1989:26). An overall strong concentration dependant inhibition was observed for LS 6164 and PAN 1666 for *Glycine max* plants exposed to SO_2 fumigation. Previous studies observed a strong correlation between $PI_{ABS,total}$ and physiological parameters for example growth and survival in the case of stress (Yusuf, *et al*., 2010:1437). This is confirmed with earlier data mentioned for example the decrease in plant height measured. The presence of excess ROS has been suggested to cause the destruction of PSII (Ali & Alqurainy,

2006:188). A loss in photosynthetic capacity in leaves of wheat has been observed when exposed to O_3 (Morgan, *et al*., 2003:1324).

4.2.4 Stomatal conductance

The Stomatal conductance decreased as the SO_2 concentration increased. The 150 ppb SO_2 concentration resulted in a significant ($p \leq 0.05$) decrease in the stomatal conductance of LS 6164 after 7 and 11 days when compared to the CF control (0 ppb treatment). For PAN 1666 a significant decrease of 79.6% ($p \leq 0.05$) was observed for the 150 ppb treatment. After 11 days of fumigation the significant decreases in stomatal conductance for the 25 ppb, 75 ppb and 150 ppb treatment were 35.5% ($p \leq 0.05$), 50.8% ($p \leq 0.05$) and 65.2% ($p \leq 0.05$) in the PAN 1666 cultivar in respect to the CF control (0 ppb treatment). The decrease in stomatal conductance can indicate partial stomatal closure.

The changes in stomatal function can be directly caused by the oxidation products of SO_2, ROS or the accumulation of CO_2 in intercellular spaces which will inevitably affect stomatal movement. An accumulation of CO_2 in the intercellular spaces will be confirmed by an increase in C_i values and may result in stomatal closure which will in turn reduce photosynthetic rate. Stomatal conductance was overall higher in LS 6164 plants compared to PAN 1666. The reduction in stomatal conductance will cause a decrease in photosynthetic activity due to a decrease in CO_2 availability. Concentration dependant reduction was however evident in both cultivars for both after 7 days and 11 days of fumigation. The decrease in stomatal conductance is evidence for mechanical transformations in the stomata.

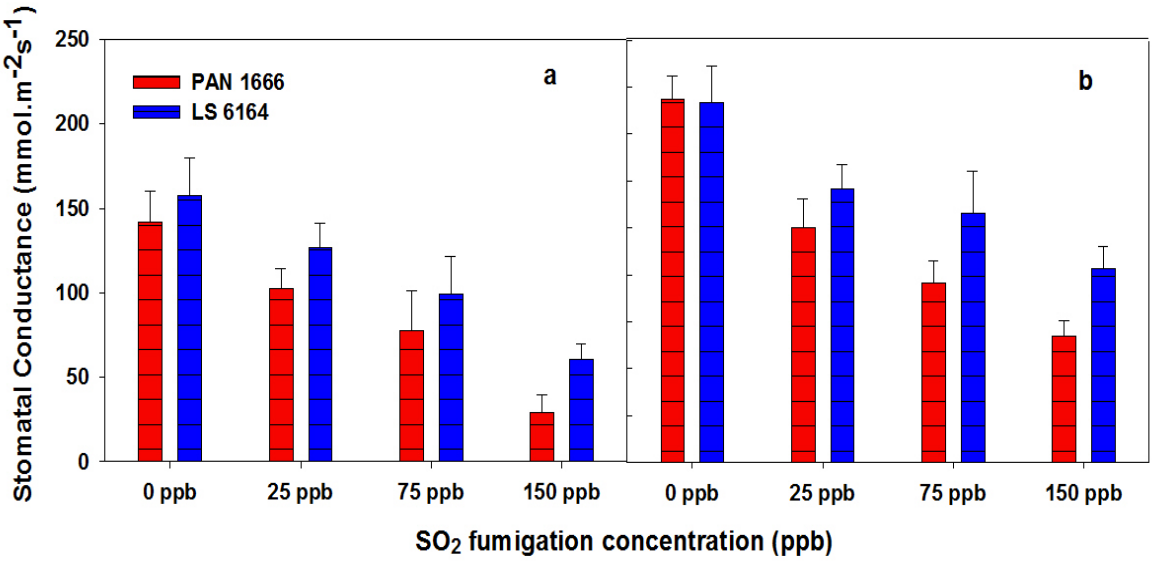

Figure 4.2.4.1: Stomatal conductance: The effect of SO_2 fumigation on the stomatal conductance of PAN 1666 and LS 6164 *Glycine max* measured after respectively (a) 7 days; (b) 11 days of SO_2 fumigation. (n = 8)

Several previous studies have been conducted to examine the effect of air pollutants on stomatal conduction. It was observed that plants exposed to medium to high salinity-alkalinity had a decrease in stomatal conductance (Zhang & Deng, 2012:882)
A decrease in stomatal conductance often observed in correlation with the inhibition of net photosynthesis (Darrall, 1989:18). Stomatal opening or closure will have an effect on the effective dose of the air pollutant and also the sensitivity of the plant to SO_2 (Darrall, 1989:18). Stomatal closure and a reduction in photosynthetic activity have been observed in the case of SO_2 fumigation of several species (Ranieri, *et al*., 1999:926; Winner & Mooney, 1980:293). However not all species react to SO_2 exposure with stomatal closure, some species like *Zea mays* react by opening stomata (Winner & Mooney, 1980:295). The state of the stomata will directly influence the amount of SO_2 absorbed, we can therefore conclude that the response of the stomata contributes to variations in SO_2 sensitivity between plants (Winner & Mooney, 1980:293). Species with a higher stomatal conductance were observed to be less tolerant to air pollutants (Darrall, 1989:12). An increase in carbon dioxide concentration has been observed in leaves due to the inhibition of photosynthesis and consequently a decrease in stomatal conductance. A decrease in stomatal conductance will cause a limitation on the amount of air pollutant that enters the plant (Morgan, *et al*., 2003:1324). The decrease in stomatal conductance suggests that the

decrease in photosynthetic activity may be influenced by stomatal closure. Stomatal closure as a response causes a decrease in CO_2 availability in the carboxylation site and therefore the leaf CO_2 uptake. A decrease in stomatal conduction was also observed during SO_2 fumigation by Winner & Mooney (1980:294).

4.2.5 Sulphur content

The sulphur content of the dried leaves of LS 6164 and PAN 1666 *Glycine max* plants increased significantly as the SO_2 concentration increased (Fig. 4.2.5.1). The 150 ppb treatment of both cultivars resulted in the largest percentage sulphur content followed by the 75 ppb treatments. Neither of the cultivars had a significant increase for the 25 ppb treatments. The percentage sulphur content for 25 ppb, 75 ppb and 150ppb for the PAN 1666 cultivar increased with 26.0% ($p > 0.05$), 160.5% ($p \leq 0.05$) and 295.4% ($p \leq 0.05$), while for the LS 6164 cultivar sulphur content was 34.6% ($p > 0.05$), 200.8% ($p \leq 0.05$) and 362.8% ($p \leq 0.05$) higher compared to the CF control (0 ppb treatment). The sulphur content was similar for the cultivars at the CF control (0 ppb treatment) as well as at the 25 ppb treatments, however the LS 6164 had a higher sulphur content for the 75 ppb and 150 ppb treatments in comparison to the PAN 1666 cultivar.

Sulphur is a structural part of amino acids, proteins, vitamins and chlorophyll and SO_2 may be reduced and incorporated into organic S compounds and utilized as sulphur nutrient (Li & Yi, 2012:46; Tauz, *et al*., 2003:212). SO_2 exposure is expected to lead to an increase in total sulphur and sulphate (Tauz, *et al*., 2003:212). The increase in total sulphur content correlated with results of Tauz, *et al*., (2003: 214), where the uptake and distribution of sulphate due to SO_2 and H_2S was examined, as well as Yang, *et al*., (2006:238), where the effects of SO_2 on Chinese cabbage were investigated. The increase in total sulphur content can be ascribed to an increase of SO_2 oxidized into sulphate and metabolized into organic sulphur content (Yang, *et al*., 2006:238). Sulphite is oxidized to sulphate, if the sulphate is in excess it is most likely transferred to the vacuoles where it may not be accessible for remobilization (Tauz, *et al*., 2003:216; Yang, *et al*., 2006:237).

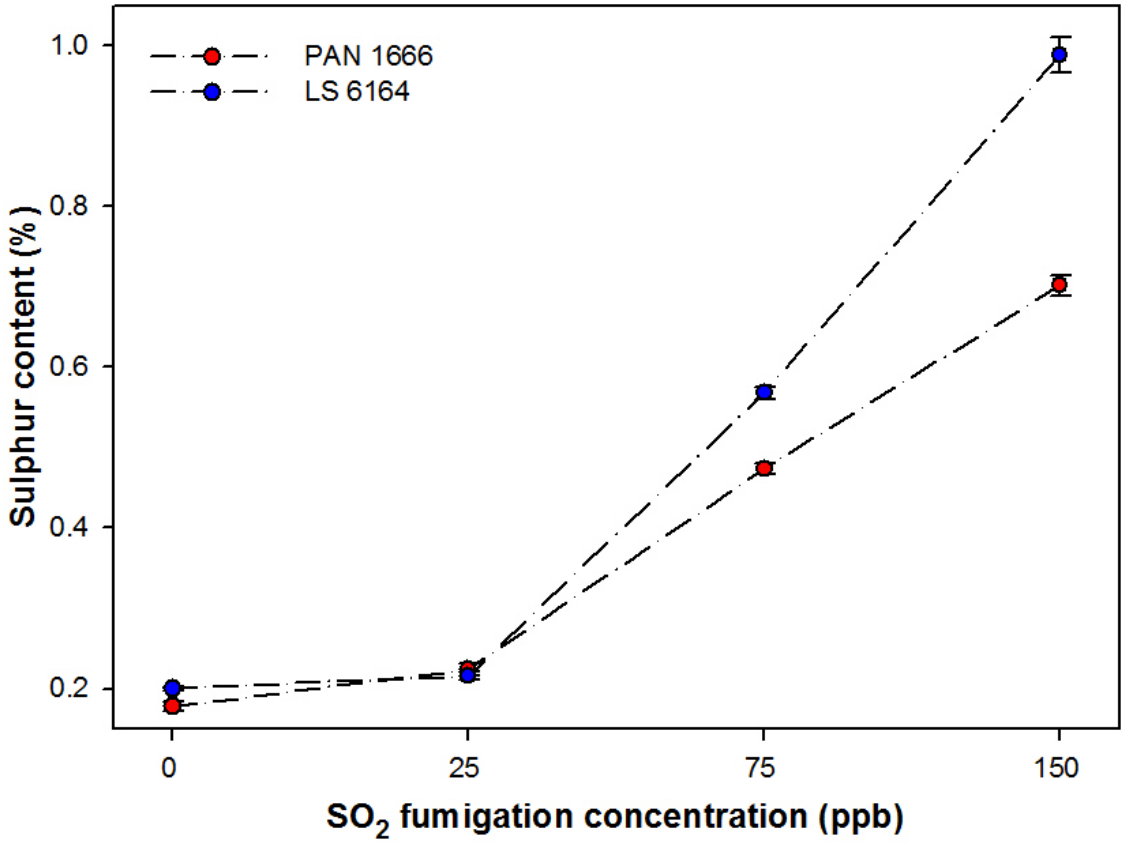

Figure 4.2.5.1: Sulphur content: The effect of different levels of SO_2 on the sulphur content in PAN 1666 and LS 6164 at harvest. (n = 8)

4.3 Enzyme activity

4.3.1 Specific lipoxygenase (LOX) activity

The specific LOX activity measured on the fourth trifoliate leaves harvested after 3 weeks of fumigation as well as in the sixth trifoliate leaf harvested after 6 weeks of fumigation increased significantly when fumigated with 150 ppb SO_2. After 3 weeks of fumigation with 25 ppb, 75 ppb and 150 ppb SO_2 the increases in specific LOX activity in PAN 1666 plants were 43.3% ($p > 0.05$), 61.5% ($p > 0.05$) and 124.1% ($p \leq 0.05$), while in LS 6164 increases were 83.0% ($p \leq 0.05$), 85.2 ($p \leq 0.05$) and 106.7% ($p \leq 0.05$) when compared to the CF control (0 ppb treatment) (Fig. 4.3.1.1.a). However after 6 weeks of fumigation significant increases were observed in the sixth trifoliate leaves for all treatments of both cultivars. The specific LOX activity of the 25 ppb, 75 ppb and 150 ppb treatments for PAN 1666 were induced by 186.0% ($p \leq 0.05$), 191.2% ($p \leq 0.05$) and 233.2% ($p \leq 0.05$) higher, while the LS 6164 were 16.3% ($p \leq$

0.05), 18.1% ($p \leq 0.05$) and 16.0% ($p \leq 0.05$) higher compared to the CF control (0 ppb treatment) (Fig. 4.3.1.1.b). The LS 6164 cultivar had a lower specific LOX activity in comparison to the PAN 1666 cultivar.

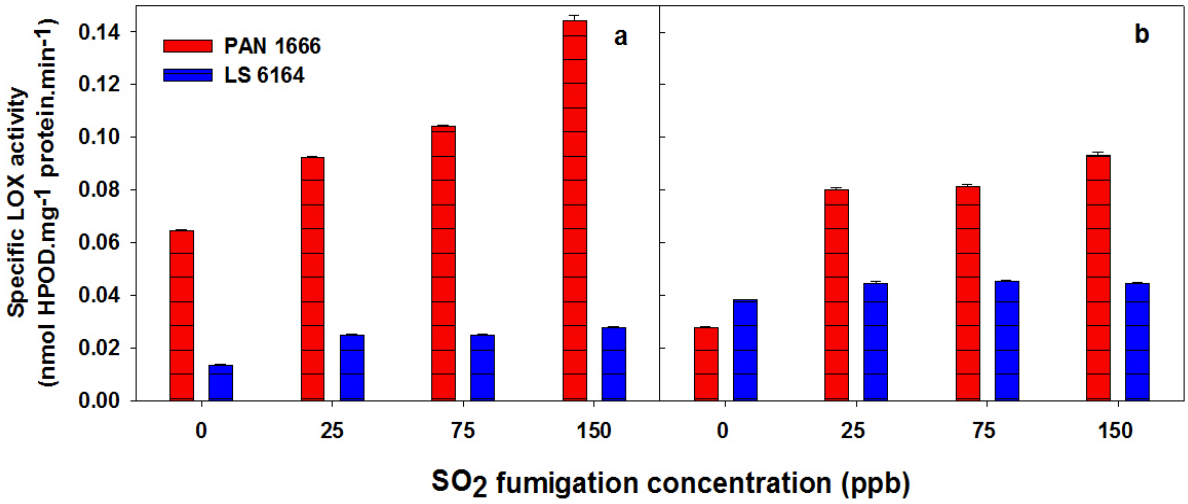

Figure 4.3.1.1: Specific LOX activity: Specific lipoxygenase (LOX) activity measured of leaves harvested at (a) 3 weeks (4th trifoliate leaf) and (b) 6 weeks (6th trifoliate leaf) after SO_2 fumigation. (n = 8)

Four evergreen plant species in the area of a refinery, with high levels of air pollution, had higher lipid peroxidation in leaves (Deniz & Duzenli, 2006:74), this study confirm results observed with the increasing SO_2 concentration in this study. Specific LOX activity increases with a correlation to the duration of anoxia have been observed in potato cells (Ali & Alqurainy, 2006:210). Ali, *et al.*, (2005:218) observed plants to have an increased LOX activity in plants exposed to temperature stress, they emphasized that the increase in LOX activity will cause enhanced lipid peroxidation and result in ROS production. The generation of ROS by LOX might contribute to hypersensitive cell death (Blée, 2002:317). Increased LOX activity was also implicated in potassium leakage and the loss of membrane integrity (Ali, *et al.*, 2005:218). ROS are small and able to move over short distances and they are ideal to act as signalling molecules that cells use to activate and control various genetic stress response reaction (Karuppanapandian, *et al.*, 2011:721).

4.3.2 Specific Peroxidase (POD) activity

The specific peroxidase (POD) activity in the fourth trifoliate leaf harvested after 3 weeks of fumigation, had significant increases for 150 ppb treatments of both the PAN 1666 and LS 6164 cultivars when compared to the CF control (0 ppb treatment) (Fig. 4.3.1.2.a). The specific POD activity for the 25 ppb, 75 ppb and 150 ppb treatment of the PAN 1666 were 2.8% ($p > 0.05$), 38.2% ($p > 0.05$) and 172.6% ($p \leq 0.05$) higher, while the LS 6164 were only significant higher for the 150 ppb treatment with 96.4% ($p \leq 0.05$) in comparison to the CF control (0 ppb treatment). The 25 ppb and 75 ppb treatments of the LS 6164 cultivar were insignificantly lower compared to the CF control (0 ppb treatment). The POD activity of the sixth trifoliate leaf, harvested after 6 weeks of fumigation, had insignificant differences from the CF control (0 ppb treatment) (Fig 4.3.1.2.b). The specific POD activity of the 25 ppb, 75 ppb and 150 ppb treatments of the PAN 1666 cultivar were 132.6% ($p > 0.05$), 240.8 ($p \leq 0.05$) and 455.1% ($p \leq 0.05$) higher compared to the CF control (0 ppb treatment). The PAN 1666 cultivar had a higher specific POD activity in comparison to the LS 6164 cultivar.

Peroxidases are one of the primary H_2O_2 scavenging enzymes in plant cells (Li & Yi, 2012:48). Increase in POD activity has been observed in several plant species grown near air pollution sources (Tripathi & Gautam, 2007:129). A significant increase in POD activity has been observed in sweet potato when exposed to SO_2 and O_3 due to its involvement in overcoming the oxidative stresses derived from air pollutants and UV radiation (Kim, *et al*., 2007:909).

A concentration dependant increase in POD activity suggests that POD plays a role in protection against oxidative stress due to the accumulation of ROS (Kim, *et al*., 2007:911). Significant increases in POD activity have been observed when *Arabidopsis* shoots were exposed to SO_2 (Li & Yi, 2012:48). POD can initiate cell-wall toughening events which will strengthen tissue against further damage (Li & Yi, 2012:48). Leaves of plants resistant to air pollution might have a higher POD activity (Tripathi & Gautam, 2007:131). It was observed that water stress causes a significant increase in POD activity, the stress could increase the accumulation of peroxidase substrates which in turn are scavenger of ROS (Ali & Alqurainy, 2006:201). It was proposed that peroxidases oxidise sulphite to sulphate as a side-reaction (Tauz, *et al*.,

2003:216). The increase in specific POD activity indicates the presence of an antioxidant defence mechanism where SO_2 most probably induced ROS formation.

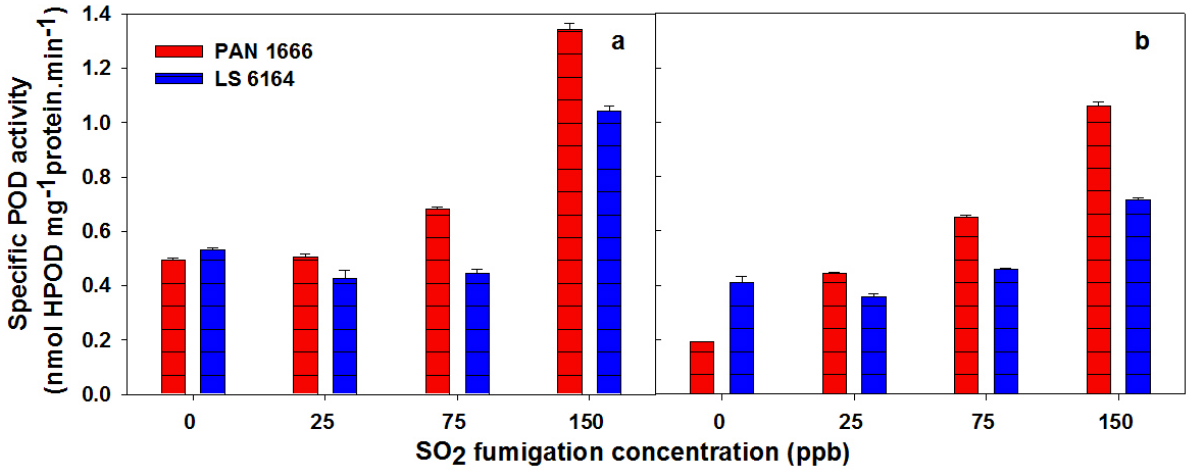

Figure 4.3.1.2: Specific POD activity: Specific POD activity for leaves of LS 6164 and PAN 1666 *Glycine max* plants harvested after (a) 3 weeks (4th trifoliate leaf) and (b) 6 weeks (6th trifoliate leaf) of SO_2 fumigation. (n = 8)

Chapter 5
Conclusion

Throughout this study it was evident that SO_2 can cause an overall concentration dependant inhibition on morphological and physiological parameters of both PAN 1666 and LS 6164 of *Glycine max* plants.

Sulphur dioxide damage to LS 6164 and PAN 1666 had the predicted acute and chronic foliar injury (Fig. 4.1.2), which correlated with the decrease in chlorophyll content (Fig. 4.2.1.1). Though there were visible reductions in plant growth (Fig. 4.1.3.2. & Fig. 4.1.3.3), there were no statistical differences in the shoot length of both cultivars (Fig. 4.1.3.1). The 75 ppb and the 150 ppb SO_2 treatments did significantly reduce the shoot and root biomass of both cultivars (Fig. 4.1.4.1). From the results it appeared that the reduction in root biomass was more affected than the shoot biomass. There were no statistical differences between the effects of the 75 ppb and the 150 ppb SO_2 treatments on biomass production. The 75 ppb and the 150 ppb SO_2 treatments caused a large percentage wise decrease in the nodule dry mass (Fig. 4.1.4.3), average number of pods (Fig. 4.1.5.1) and the average mass of thirty seeds of both cultivars (Fig. 4.1.5.2). However, these decreases were not significant because of the large variation in data sets. Sulphur dioxide fumigation, even at 25 ppb, resulted in a general increase in oil percentage and a decrease in the protein percentage of both cultivars (Fig. 4.1.5.3).

The gas exchange results indicated a significant decrease in the carboxylation efficiency for the 150 ppb treatment of LS 6164 where the 150 ppb treatment of LS 6164 was the treatment with the largest reduction in root biomass. It therefore could relate to the theory that a decrease in root biomass causes a nutrient shortage and a decrease in the amount and/or activity of photosynthetic enzymes. The ability of Rubisco to regenerate depends on the capability of the electron transport chain to produce ATP and NADPH, the LS 6164 was observed to be less proficient in doing so than PAN 1666. The percentage of stomatal limitation did not decrease significantly and therefore the significant decrease in CO_2 assimilation rate of LS 6164 at 150 ppb SO_2 can be attributed to dominant limitations in the mesophyll.

Long term exposure of SO_2 resulted in a decrease in the total chlorophyll content as the SO_2 concentration increased from 25 ppb to 150 ppb (Fig. 4.2.1.1). The ability of both cultivars to absorb light energy was reduced especially by the 75 ppb and the 150 ppb SO_2 levels. The dramatic effect that SO_2 has on photosynthesis is evident from the chlorophyll *a* fluorescence kinetics analyses. The photosynthetic vitality index indicated that LS 6164 was more sensitive to SO_2 from 25 ppb SO_2 and higher, whereas PAN 1666 became sensitive to SO_2 from 75 ppb SO_2. A decrease in the ability to absorb light energy, the trapping of excitation energy, to transfer electrons beyond Q_A^-, and the reduction of end electron acceptors all contributed to the decline in the vitality index (Fig. 4.2.3.9).

The difference in relative fluorescence analyses also indicates that LS 6164 is more sensitive to SO_2 compared to PAN 1666. A slight stimulation effect on photosynthesis was noticeable with the 25 ppb SO_2 treatment on the PAN 1666 plants. Sulphur dioxide concentrations of 75 ppb and higher, reduced the photosynthetic capabilities of both cultivars significantly. The detection of a positive ΔK-band indicates that SO_2 can either enlarge the functional antenna size or it can inactivate the oxygen evolving complex of PSII (Fig. 4.2.3.3). Further evidence that indicates that LS 6164 is more sensitive to SO_2 is the detection of a positive ΔJ-band, which indicates the accumulation of Q_A^- (Fig. 4.2.3.6). PAN 1666 was more successful in reducing Q_A^- compared to LS 1666. The ability of both cultivars to reduce the end electron acceptors was influenced by 75 ppb and 150 ppb SO_2. With LS 6164, the 25 ppb treatment was able to negatively influence the reduction of end electron acceptors (Fig. 4.2.3.8). Photosynthetic inhibition in LS 6164 could already be observed at 25 ppb, even though damage was not visible yet, whereas with PAN 1666, photosynthetic inhibition started at 75 ppb SO_2.

The process of photosynthesis can be linked to growth via the process of carbon allocation. The carbon can either be allocated to the roots or the shoots. Sulphur dioxide stress favoured a higher shoot-to-root ratio (Fig. 4.1.4.2). This implies that the root biomass was more affected by increasing SO_2 concentration than the shoot. The potential for SO_2 to alter photosynthesis and growth of both roots and shoots increases the value of photosynthesis as a biomarker of plants to air pollution stress.

Plants that experience stress conditions usually express higher levels of peroxidase activity than are normally detected of healthy plants. This higher expression in peroxidase activity has been used as a biochemical marker of stress tolerance or resistance in various studies. Treatments of 25 and 75 ppb fumigation in LS 6164 were not able to induce increased levels of peroxidase activity. Only a fumigation of 150 ppb SO_2 was able to induce significantly higher levels of peroxidise activity. In PAN 1666 fumigation levels as low as 25 ppb SO_2 were able to induce significantly high levels of peroxidise activity (Fig. 4.3.2.1). The expression of peroxidase activity under stress conditions is important since one of the functions of peroxidase activity is the detoxification of reactive oxygen species. The ability of PAN 1666 to induce peroxidase activity to higher levels and to do it at relatively low SO_2 concentrations, enables PAN 1666 to minimize damage due to reactive oxygen species.

Lipoxygenase activity is usually associated with biotic stress conditions. Its function during biotic stress conditions varies from the biosynthesis of defence compounds to the synthesis of signalling molecules. In this study we have demonstrated that air pollution stress, in the form of SO_2, is able to induce lipoxygenase activity to significantly higher levels in PAN 1666, but not in LS 6164 (Fig. 4.3.1.1). The function of higher lipoxygenase activity during high levels of SO_2 is not yet clear at this point in time. A most likely function might be that it can lead to the production of signalling molecules that can assist the plant to tolerate high levels of SO_2.

Sulphur dioxide gain entry into the leaves via the same diffusion pathway as CO_2 through the stomata of the plant. Stomatal conductance is therefore an important factor that determines the amount of SO_2 taken up by the plant. The higher the stomatal conductance the more CO_2 and consequently the more SO_2 is taken up. The stomatal conductance of both PAN 1666 and LS 6164 cultivars decreases with an increase in SO_2 concentration. PAN 1666 had a lower level of stomatal conductance compared to LS 6164 (Fig. 4.2.4.1). This means that LS 6164 have the potential to take up more SO_2 than PAN 1666, which is confirmed by the total sulphur content of the leaf material (Fig. 4.2.5.1). LS 6164 had a higher sulphur content compared to PAN 1666. The formation of HSO_3^- (hydrogen sulphite ion) and SO_3^- (sulphite ion) due to SO_2 absorption resulted in the inhibition of photosynthesis. The inhibition of photosynthesis and the detoxification of these sulphite ions, leads to the formation of

reactive oxygen species. The higher peroxidase activity of PAN 1666 (Fig. 4.3.2.1) enabled this cultivar to detoxify these reactive oxygen species with more success than LS 6164.

Although the objectives of this study were adequately reached, the following aspects need further investigation:

- It was observed in this study that increasing SO_2 levels influence the composition of *Glycine max* seeds, more knowledge of the extend of these changes will be valuable.
- The in-*vitro* determination of Rubisco activity of both cultivars, to confirm the variation between the cultivars.
- The determination of ATP and NADPH levels in isolated chloroplasts to confirm the inhibition of their formation by SO_2.
- The evaluation of the effect of SO_2 on symbiotic nitrogen fixation.
- The determination of the contribution of the sulphate-reduction pathway by the determination of the glutathione levels as well as the activity of other known enzymes involved in antioxidant scavenging for example superoxide dismutase.

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APPENDIX

Table 7.1: Percentage difference to CF control (0 ppb treatment) for all data: Data calculated as percentage difference to CF control (0 ppb treatment). A – indicates a decrease and + an increase in comparison to the respective CF control (0 ppb treatment). Statistical significance ($p \leq 0.05$) and insignificance ($p > 0.05$) can also be seen where applicable

	PAN 1666			.LS 6164		
	25 ppb	**75 ppb**	**150 ppb**	**25 ppb**	**75 ppb**	**150 ppb**
Shoot length	+ 2.2% ($p > 0.05$)	– 4.1% ($p > 0.05$)	– 14.2% ($p > 0.05$)	+ 11.2% ($p > 0.05$)	– 6.1% ($p > 0.05$)	– 9.7% ($p > 0.05$)
Shoot biomass	- 0.2% ($p > 0.05$)	– 24.8% ($p \leq 0.05$)	– 24.4% ($p \leq 0.05$)	– 9.6% ($p > 0.05$)	– 31.4% ($p \leq 0.05$)	– 26.3% ($p \leq 0.05$)
Root biomass	+ 2.2% ($p > 0.05$)	–47.2% ($p \leq 0.05$)	47.1% ($p \leq 0.05$)	– 23.9% ($p > 0.05$)	– 58.1% ($p \leq 0.05$)	– 61.2% ($p \leq 0.05$)
Shoot-to-root ratio	+ 8.4% ($p > 0.05$)	+ 45.6% ($p > 0.05$)	+ 45.0% ($p > 0.05$)	+ 15.7% ($p > 0.05$)	+ 72.3% ($p \leq 0.05$)	+ 85.7% ($p \leq 0.05$)
Nodule biomass	+ 18.2% ($p > 0.05$)	– 19.4% ($p > 0.05$)	– 51.0% ($p > 0.05$)	– 2.6% ($p > 0.05$)	– 43.7% ($p > 0.05$)	– 43.0% ($p > 0.05$)
Number of pods	+ 0.6% ($p > 0.05$)	– 4.5% ($p > 0.05$)	– 16.0% ($p > 0.05$)	– 10.4% ($p > 0.05$)	– 18.4% ($p > 0.05$)	– 21.2% ($p > 0.05$)
Average seed weight	– 3.0%	– 48.2%	– 56.4%	– 3.9%	– 38.9%	43.3%
Seed oil content	– 2.5%	+ 10.5%	+ 10.0%	+ 6.3%	+ 7.2%	10.8%
Seed protein content	+ 2.4%	+ 0.2%	– 1.9%	– 6.1%	– 3.3%	– 4.9%
Oil-to-protein ratio	– 4.8%	+ 10.3%	+ 12.1%	+ 13.2%	+ 10.8%	+16.6%
Chlorophyll content after 15 days	+ 17.1% ($p > 0.05$)	– 9.4% ($p > 0.05$)	– 14.0% ($p \leq 0.05$)	+11.1% ($p > 0.05$)	– 6.3% ($p > 0.05$)	– 11.0% ($p > 0.05$)
Chlorophyll content after 29 days	- 5.5% ($p > 0.05$)	– 10.4% ($p \leq 0.05$)	– 32.1% ($p \leq 0.05$)	- 6.2% ($p > 0.05$)	– 17.7% ($p \leq 0.05$)	– 26.7% ($p \leq 0.05$)
Chlorophyll content after 37 days	- 16.4% ($p > 0.05$)	– 16.7% ($p \leq 0.05$)	– 38.7% ($p \leq 0.05$)	- 8.8% ($p \leq 0.05$)	– 10.5% ($p \leq 0.05$)	– 23.3% ($p \leq 0.05$)

Table 1 continues:

	PAN 1666			LS 6164		
	25 ppb	**75 ppb**	**150 ppb**	**25 ppb**	**75 ppb**	**150 ppb**
Γ	+ 23.3% (p > 0.05)	+ 4.1% (p > 0.05)	– 4.9% (p > 0.05)	+ 52.7% (p > 0.05)	+ 2.7% (p > 0.05)	+ 45.0% (p > 0.05)
A_0	– 27.5% (p > 0.05)	– 10.8% (p > 0.05)	– 0.2% (p > 0.05)	– 48.0% (p ≤ 0.05)	– 13.6% (p > 0.05)	– 57.9 (p ≤ 0.05)
CE	+ 65.2% (p > 0.05)	+ 19.5% (p > 0.05)	– 3.3% (p > 0.05)	- 45.1% (p > 0.05)	- 9.6% (p > 0.05)	- 58.0% (p ≤ 0.05)
A_{360}	- 19.5% (p > 0.05)	- 6.7% (p > 0.05)	0% (p > 0.05)	- 30.5% (p > 0.05)	- 44.4 (p > 0.05)	- 67.4% (p ≤ 0.05)
Ci_{360}	+ 9.8% (p > 0.05)	- 4.8% (p > 0.05)	- 0.8% (p > 0.05)	+ 30.8% (p > 0.05)	- 17.5% (p > 0.05)	+13.9% (p > 0.05)
J_{max}	- 15.3% (p > 0.05)	- 5.9% (p > 0.05)	- 1.3% (p > 0.05)	- 9.6% (p > 0.05)	+ 10.4% (p > 0.05)	- 15.6% (p > 0.05)
ℓ	- 64.3% (p > 0.05)	- 21.8% (p > 0.05)	- 5.2% (p > 0.05)	- 132.1% (p > 0.05)	+ 141.2% (p > 0.05)	+ 80.0% (p > 0.05)
Evap	- 3.9% (p > 0.05)	+ 13.9% (p > 0.05)	+ 9.8% (p > 0.05)	+ 42.7% (p > 0.05)	- 57.1% (p > 0.05)	- 50.6% (p > 0.05)
K-band	- 1.2%	+ 8.1%	+ 17.2%	+ 12.2%	+17.7%	11.0%
I-band	+ 0.6%	+ 15.5%	+ 24.1%	+ 1.9%	+ 14.7%	+17.2%
L-band	+ 2.7%	+ 3.4%	+ 8.8%	+ 4.2%	+ 4.0%	+ 3.3%
J-band	- 3.9%	+ 5.7%	- 11.1%	+ 1.0%	+ 0.2%	+ 6.6%
G-band	+ 7.1%	- 14.6%	+ 2.4%	+14.9%	- 9.6%	- 23.0%
Pool size	+ 0.9%	- 10.6%	- 11.4%	- 2.4%	- 9.6%	- 11.9%

Table 1 continues

	PAN 1666			LS 6164		
	25 ppb	**75 ppb**	**150 ppb**	**25 ppb**	**75 ppb**	**150 ppb**
$PI_{ABS,total}$ **2 weeks**	+ 0.9% (p ≤ 0.05)	- 35.4% (p > 0.05)	- 69.7% (p ≤ 0.05)	+ 22.8% (p ≤ 0.05)	- 25.8% (p ≤ 0.05)	- 43.7% (p ≤ 0.05)
$PI_{ABS,total}$ **3 weeks**	+ 2.8% (p > 0.05)	- 54.3% (p ≤ 0.05)	- 64.3% (p ≤ 0.05)	+ 23.6% (p ≤ 0.05)	- 46.1% (p ≤ 0.05)	- 56.9% (p ≤ 0.05)
$PI_{ABS,total}$ **4 weeks**	+ 1.9% (p > 0.05)	- 60.5% (p ≤ 0.05)	- 65.5% (p ≤ 0.05)	- 18.8% (p ≤ 0.05)	- 68.5% (p ≤ 0.05)	- 72.5% (p ≤ 0.05)
Absorption 2 Weeks	- 10.1% (p ≤ 0.05)	-10.9% (p ≤ 0.05)	- 11.1% (p ≤ 0.05)	+ 1.6% (p > 0.05)	- 4.9% (p ≤ 0.05)	- 3.1% (p > 0.05)
Absorption 3 Weeks	+ 0.4% (p > 0.05)	- 8.4% (p ≤ 0.05)	-17.3% (p ≤ 0.05)	- 0.7% (p > 0.05)	- 13.6% (p ≤ 0.05)	- 10.8% (p ≤ 0.05)
Absorption 4 Weeks	+ 0.1% (p > 0.05)	- 11.6% (p ≤ 0.05)	- 13.5% (p ≤ 0.05)	- 3.7% (p > 0.05)	- 14.9% (p ≤ 0.05)	- 16.7% (p ≤ 0.05)
Trapping 2 weeks	+ 1.6% (p > 0.05)	- 4.7% (p ≤ 0.05)	- 3.9% (p ≤ 0.05)	+ 2.8% (p > 0.05)	- 3.1% (p > 0.05)	- 5.6% (p ≤ 0.05)
Trapping 3 weeks	+ 0.3% (p > 0.05)	-11.1% (p > 0.05)	- 12.0% (p ≤ 0.05)	+ 1.1% (p > 0.05)	- 8.6% (p ≤ 0.05)	- 13.8% (p ≤ 0.05)
Trapping 4 weeks	+ 1.6% (p > 0.05)	- 8.0% (p ≤ 0.05)	- 6.9% (p ≤ 0.05)	- 5.9% (p ≤ 0.05)	- 14.8% (p ≤ 0.05)	- 13.7% (p ≤ 0.05)
Electron transport 2 weeks	+ 13.2% (p ≤ 0.05)	- 5.9% (p > 0.05)	- 11.5% (p ≤ 0.05)	+ 12.5% (p ≤ 0.05)	- 11.7% (p ≤ 0.05)	- 23.7% (p ≤ 0.05)
Electron transport 3 weeks	+ 8.8% (p > 0.05)	- 25.8% (p ≤ 0.05)	- 18.1% (p ≤ 0.05)	+ 19.8% (p ≤ 0.05)	- 12.4% (p ≤ 0.05)	- 22.4% (p ≤ 0.05)
Electron transport 4 weeks	+ 3.4% (p > 0.05)	- 26.6% (p ≤ 0.05)	- 18.8% (p ≤ 0.05)	- 6.3% (p > 0.05)	- 33.5% (p ≤ 0.05)	- 26.4% (p ≤ 0.05)

Table 1 continues

	PAN 1666			LS 6164		
	25 ppb	**75 ppb**	**150 ppb**	**25 ppb**	**75 ppb**	**150 ppb**
Reduction of end electron acceptors 2 weeks	- 2.8% (p > 0.05)	- 19.3% (p ≤ 0.05)	- 19.0% (p ≤ 0.05)	+ 4.6% (p > 0.05)	- 9.1% (p ≤ 0.05)	- 19.9% (p ≤ 0.05)
Reduction of end electron acceptors 3 weeks	- 8.0% (p > 0.05)	- 24.5% (p ≤ 0.05)	- 40.9% (p ≤ 0.05)	+ 2.9% (p > 0.05)	- 23.1% (p ≤ 0.05)	- 29.1% (p ≤ 0.05)
Reduction of end electron acceptors 4 weeks	- 3.8% (p > 0.05)	- 35.4% (p ≤ 0.05)	- 48.8% (p ≤ 0.05)	- 5.0% (p > 0.05)	- 36.2% (p ≤ 0.05)	-49.4% (p ≤ 0.05)
DF_{total} 2 weeks	+ 0.1% (p > 0.05)	- 10.0% (p ≤ 0.05)	- 11.4% (p ≤ 0.05)	+ 4.7% (p ≤ 0.05)	- 7.0% (p ≤ 0.05)	- 13.5% (p ≤ 0.05)
DF_{total} 3 weeks	+ 0.8% (p > 0.05)	- 18.8% (p ≤ 0.05)	-25.0% (p ≤ 0.05)	+ 4.8% (p > 0.05)	- 14.2% (p ≤ 0.05)	- 19.1% (p ≤ 0.05)
DF_{total} 4 weeks	+ 0.4% (p > 0.05)	- 22.4% (p ≤ 0.05)	- 25.3% (p ≤ 0.05)	- 4.4% (p > 0.05)	- 26.2% (p ≤ 0.05)	- 29.0% (p ≤ 0.05)
ABS/RC 2 weeks	+ 11.4% (p ≤ 0.05)	+ 12.6% (p ≤ 0.05)	+ 13.6% (p ≤ 0.05)	- 1.6% (p > 0.05)	+ 5.2% (p ≤ 0.05)	+ 3.4% (p > 0.05)
ABS/RC 3 weeks	- 2.5% (p > 0.05)	+ 9.6% (p ≤ 0.05)	+ 21.3% (p ≤ 0.05)	+ 0.4% (p > 0.05)	+ 16.7% (p ≤ 0.05)	+ 12.3% (p ≤ 0.05)
ABS/RC 4 weeks	+ 0.04% (p > 0.05)	+ 13.3% (p ≤ 0.05)	+ 15.9% (p ≤ 0.05)	+ 3.5% (p > 0.05)	+ 17.3% (p ≤ 0.05)	+ 20.0% (p ≤ 0.05)
TR_0/RC 2 weeks	+ 11.7% (p > 0.05)	+ 11.6% (p > 0.05)	+ 12.7% (p ≤ 0.05)	- 1.1% (p > 0.05)	+ 4.6% (p ≤ 0.05)	+ 2.3% (p ≤ 0.05)
TR_0/RC 3 weeks	- 2.4% (p > 0.05)	+ 7.1% (p ≤ 0.05)	18.3% (p ≤ 0.05)	+ 0.6% (p > 0.05)	+ 14.9% (p ≤ 0.05)	+ 9.3% (p ≤ 0.05)
TR_0/RC 4 weeks	+ 0.3% (p > 0.05)	+ 11.9% (p ≤ 0.05)	+ 14.4% (p ≤ 0.05)	+2.6% (p > 0.05)	+ 14.1% (p ≤ 0.05)	+ 17.0% (p ≤ 0.05)

Table 1 continues

	PAN 1666			LS 6164		
	25 ppb	**75 ppb**	**150 ppb**	**25 ppb**	**75 ppb**	**150 ppb**
ET_0/RC 2 weeks	+ 16.5% (p ≤ 0.05)	+ 8.7% (p ≤ 0.05)	+ 6.9% (p ≤ 0.05)	+ 3.1% (p > 0.05)	- 0.1% (p > 0.05)	- 8.0% (p ≤ 0.05)
ET_0/RC 3 weeks	+ 0.5% (p > 0.05)	- 4.8% (p > 0.05)	+ 8.5% (p > 0.05)	+ 6.3% (p > 0.05)	+ 9.5% (p ≤ 0.05)	- 0.1% (p > 0.05)
ET_0/RC 4 weeks	+ 1.5% (p > 0.05)	- 0.7% (p > 0.05)	+ 5.5% (p > 0.05)	+ 0.7% (p > 0.05)	- 1.5% (p > 0.05)	+ 4.6% (p > 0.05)
RC/CS_0 2 weeks	- 6.1% (p ≤ 0.05)	- 6.1% (p ≤ 0.05)	- 5.2% (p > 0.05)	+ 2.6% (p > 0.05)	- 1.2% (p > 0.05)	+ 3.9% (p > 0.05)
RC/CS_0 3 weeks	+ 7.6% (p ≤ 0.05)	+ 3.3% (p > 0.05)	+ 1.3% (p > 0.05)	- 1.2% (p > 0.05)	- 5.6% (p > 0.05)	+ 0.4% (p > 0.05)
RC/CS_0 4 weeks	+ 1.9% (p > 0.05)	- 2.6% (p > 0.05)	- 5.6% (p > 0.05)	+ 0.5% (p > 0.05)	- 3.9% (p > 0.05)	- 6.8% (p > 0.05)
ABS/CS_0 2 weeks	+ 4.4% (p > 0.05)	+ 5.4% (p ≤ 0.05)	+ 7.2% (p ≤ 0.05)	+ 1.0% (p > 0.05)	+ 3.9% (p ≤ 0.05)	+ 7.3% (p ≤ 0.05)
ABS/CS_0 3 weeks	+ 5.0% (p > 0.05)	+ 13.1% (p ≤ 0.05)	+ 23.0% (p ≤ 0.05)	- 0.6% (p > 0.05)	+9.1% (p ≤ 0.05)	+ 12.6% (p ≤ 0.05)
ABS/CS_0 4 weeks	+ 1.7% (p > 0.05)	+ 10.3% (p ≤ 0.05)	+ 9.3% (p ≤ 0.05)	+ 4.3% (p > 0.05)	+ 13.1% (p ≤ 0.05)	12.1% (p ≤ 0.05)
TR0/CS_0 2 weeks	+ 4.7% (p > 0.05)	+ 4.5% (p > 0.05)	+ 6.4% (p ≤ 0.05)	+ 1.5% (p > 0.05)	+ 3.3% (p ≤ 0.05)	+ 6.2% (p ≤ 0.05)
TR0/CS_0 3 weeks	+ 5.1% (p > 0.05)	+ 10.5% (p ≤ 0.05)	+ 20.0% (p ≤ 0.05)	- 0.4% (p > 0.05)	+ 7.4% (p ≤ 0.05)	+ 9.6% (p ≤ 0.05)
TR0/CS_0 4 weeks	+ 2.0% (p > 0.05)	+ 8.6% (p ≤ 0.05)	+ 7.9% (p ≤ 0.05)	+3.3% (p > 0.05)	+10.0% (p ≤ 0.05)	+ 9.3% (p ≤ 0.05)
ET0/CS_0 2 Weeks	+ 9.2% (p ≤ 0.05)	+ 1.9% (p > 0.05)	+ 1.0% (p > 0.05)	+ 5.7% (p ≤ 0.05)	-1.5% (p > 0.05)	- 4.6% (p ≤ 0.05)
ET0/CS_0 3 Weeks	+ 8.1% (p ≤ 0.05)	- 2.2% (p > 0.05)	+ 10.1 (p ≤ 0.05)	+ 5.1% (p > 0.05)	+ 2.5% (p > 0.05)	+ 0.3% (p > 0.05)
ET0/CS_0 4 Weeks	+ 3.3% (p > 0.05)	- 3.5% (p ≤ 0.05)	- 0.4% (p > 0.05)	+ 1.5% (p > 0.05)	- 5.1% (p ≤ 0.05)	- 2.1% (p > 0.05)

Table 1 continues

	PAN 1666			LS 6164		
	25 ppb	**75 ppb**	**150 ppb**	**25 ppb**	**75 ppb**	**150 ppb**
Quantum yield of K_p 2 weeks	- 4.1% (p > 0.05)	- 6.0% (p ≤ 0.05)	-7.0% (p ≤ 0.05)	- 0.6% (p > 0.05)	- 4.4% (p ≤ 0.05)	- 7.7% (p ≤ 0.05)
Quantum yield of K_p 3 weeks	- 4.8% (p > 0.05)	- 13.3% (p ≤ 0.05)	- 20.3% (p ≤ 0.05)	+ 0.7% (p > 0.05)	- 9.7% (p ≤ 0.05)	- 13.3% (p ≤ 0.05)
Quantum yield of K_p 4 weeks	- 1.4% (p > 0.05)	- 10.5% (p ≤ 0.05)	-9.6% (p ≤ 0.05)	- 5.2% (p ≤ 0.05)	- 13.9% (p ≤ 0.05)	- 13.1% (p ≤ 0.05)
Quantum yield of K_N 2 weeks	- 5.5% (p ≤ 0.05)	- 1.4% (p > 0.05)	- 3.2% (p > 0.05)	- 3.3% (p > 0.05)	- 1.2% (p > 0.05)	- 2.2% (p > 0.05)
Quantum yield of K_N 3 weeks	- 5.1% (p ≤ 0.05)	- 2.6% (p ≤ 0.05)	- 9.4% (p > 0.05)	- 0.4% (p > 0.05)	- 1.0% (p > 0.05)	+ 0.8% (p > 0.05)
Quantum yield of K_N 4 weeks	- 3% (p > 0.05)	- 2.6% (p > 0.05)	- 2.9% (p > 0.05)	+ 0.6% (p > 0.05)	+ 1.0% (p > 0.05)	+ 0.7% (p > 0.05)
Stomatal conductance after 7 days	- 27.7% (p > 0.05)	- 45.4% (p > 0.05)	– 79.6% (p ≤ 0.05)	- 19.7% (p > 0.05)	- 37.0% (p > 0.05)	– 61.8% (p ≤ 0.05)
Stomatal conductance after 11 days	– 35.5% (p ≤ 0.05)	– 50.8% (p ≤ 0.05)	– 65.2% (p ≤ 0.05)	- 23.8% (p > 0.05)	- 30.8% (p > 0.05)	– 46.0% (p ≤ 0.05)
Sulphur content	+ 26.0% (p > 0.05)	+ 160.5% (p ≤ 0.05)	+ 295.4% (p ≤ 0.05)	+ 34.6% (p > 0.05)	+ 200.8% (p ≤ 0.05)	+ 362.*% (p ≤ 0.05)

Table 1 continues

	PAN 1666			LS 6164		
	25 ppb	**75 ppb**	**150 ppb**	**25 ppb**	**75 ppb**	**150 ppb**
Specific LOX activity after 3 weeks	+ 43.3% ($p > 0.05$)	+ 61.5% ($p > 0.05$)	+ 124.1% ($p \leq 0.05$)	+ 83.1% ($p \leq 0.05$)	+ 85.2% ($p \leq 0.05$)	+ 106.7% ($p \leq 0.05$)
Specific LOX activity after 6 weeks	+ 186.0% ($p \leq 0.05$)	+ 191.2% ($p \leq 0.05$)	+ 233.2% ($p \leq 0.05$)	+ 16.3% ($p \leq 0.05$)	+ 18.1% ($p \leq 0.05$)	+ 16.0% ($p \leq 0.05$)
Specific POD activity after 3 weeks	+ 2.8% ($p > 0.05$)	+ 38.2% ($p > 0.05$)	+ 172.6% ($p \leq 0.05$)	– 19.3% ($p > 0.05$)	– 16.1% ($p > 0.05$)	+ 96.4% ($p \leq 0.05$)
Specific POD activity after 6 weeks	+ 132.6% ($p > 0.05$)	+ 240.8% ($p \leq 0.05$)	+ 455.1% ($p \leq 0.05$)	- 13.1% ($p > 0.05$)	+ 11.7% ($p > 0.05$)	+ 73.3% ($p > 0.05$)