PHYSIOLOGICAL RESPONSE OF THE SUCCULENT *Augea capensis* (ZYGOPHYLLACEAE) OF THE SOUTHERN NAMIB DESERT TO SO_2 AND DROUGHT STRESS

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	CO_2 assimilation rate at ambient CO_2 concentration (350 µmol mol^{-1})
A_0	CO_2 assimilation rate at an intercellular CO_2 concentration of 350 µmol mol^{-1} or above where no stomatal limitation is present
ABA	Abscisic acid
ABS/CS_M	The phenomenological energy flux (per excited cross section of leaf) for light absorption
ABS/RC	The specific energy flux (per PSII reaction centre) for light absorption
APS	adenosine 5'- phosphosulphate
ATP	Adenosine tri-phosphate
γEC	Dipeptide γ-glutamylcysteine
c_a	Atmospheric CO_2 concentration
CAM	Crassulacean acid metabolism
CE	Carboxylation efficiency
c_i	Intercellular CO_2 concentration
CS	Excited cross section of leaf
CS-SH	Protein-sulfide complex
$CS\text{-}SO_3^{2-}$	Protein bound sulfite-complex
DI_0/RC	Dissipation at the level of the antenna chlorophylls
ET	Electron transport
ET_0/CS_M	The phenomenological energy flux (per excited cross section of leaf) for electron transport
ET_0/RC	The specific energy flux (per PSII reaction centre) for electron transport
FBPase	Fructose-1,6-bisphosphatase

F_V/F_M	Quantum yield of primary photochemistry
g_s	Stomatal conductance
GSH	Glutathione
HSO_3^-	Hydrogen sulfite
J_{max}	Maximum CO_2 assimilation rate at saturating CO_2 concentration
l	Relative stomatal limitation of photosynthesis
NADPH	β-Nicotinamide adenine dinucleotide
OEC	Oxygen evolving complex
PCR cycle	Photosynthetic carbon reduction cycle
PEA	Plant efficiency analyser
Pi	Inorganic phosphate
PI_{ABS}	Performance index expressed on absorption basis
PLC	Photosynthetic leaf chamber
ppm	Parts per million
ppb	Parts per billion
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
φ_{EO}	Quantum yield of electron transport
ψ_0	Efficiency with which a trapped exciton can move an electron further than Q_A^- into the electron transport chain
Ψ_L	Leaf water potential
Q_A	Primary bound plastoquinone
Q_A^-	Primary bound plastoquinone in reduced state
Q_B	Secondary bound plastoquinone
Q_B^-	Secondary bound plastoquinone in reduced state
RC	Photosystem II reaction centre

RC/ABS	The density of active PSII reaction centres on a chlorophyll basis
RC/CS_M	The density of active PSII reaction centres per excited cross section
Γ	CO_2 compensation concentration
RuBP	Ribulose-1,5- bisphosphate
Rubisco	Ribulose-1,5- bisphosphate carboxylase/oxygenase
RWC	Relative water content
SBPase	Sedoheptulose-1,7-bisphosphatase
S.E.	Standard error
SO_4^{2-}	Sulfate
SO_3^{2-}	Sulfite
TR	Trapping of excitation energy
TR_0/CS_M	The phenomenological energy flux (per excited cross section of leaf) for trapping
TR_0/RC	The specific energy flux (per PSII reaction centre) for trapping
WUE	Water use efficiency

PREFACE

The Skorpion Zinc Mine (in operation since 2003) is located in the southwestern corner of Namibia. It is situated inside diamond area no. 1, also known as the *Sperrgebiet*, an area that has virtually been undisturbed for 80 years because of highly restricted access. The southern part of the Namib Desert in Namibia falls within the northern boundaries of the Succulent Karoo biome (Cowling *et al.*, 1999). The succulent vegetation in this area is regarded as highly sensitive and the specialized habitat supports a unique diversity of fauna and flora, some of which are endemic to Namibia.

During 2002, Mr Norman Green (former manager, Skorpion Zinc Project) and Ms Michele Kilbourn-Louw (former environmental consultant, Skorpion Zinc Project) consulted Prof GHJ Krüger and Dr PDR van Heerden (Section Botany, North-West University, Potchefstroom Campus, South Africa) about the feasibility of monitoring the effects of possible SO_2 pollution on the unique succulent vegetation in the vicinity of the mine. Sulphuric acid, used during the zinc refinery process, is produced in an acid plant at the mine. During acid production, low levels of SO_2 gas are emitted to the atmosphere. A decision was taken to initiate a vegetation-monitoring program to determine whether SO_2 emissions from the fume stack of the acid plant had any negative effects on the vegetation. In addition to the field visits, it was decided to initiate a series of laboratory experiments to determine the precise effects that water deprivation and SO_2 pollution had on *Augea capensis* Thunb., a plant species with a C_3 photosynthetic pathway occuring in the vicinity of the mine. Water availability is very often the dominant stress factor in desert environments. Characterization of the specific response to water deprivation was therefore deemed vital for successful distinction between symptoms of water deprivation and SO_2 pollution.

The importance of the study lies in the fact that the sensitive succulent vegetation in the vicinity of the mine is unique and has a high conservation status. The experiments described in this thesis is a first step towards long-term monitoring of possible pollution effects at Skorpion Zinc mine. This should provide valuable data to the mine management in order for them to determine their environmental management strategies. If any detrimental pollution effects are noticed, mine management can immediately take action and set strategies in place to minimize the

effects of pollution in the area. This study can therefore be seen as a management tool for the mine, but also contributing to the field of science as very little research has been done on the effects of SO_2 pollution on succulents in desert environments.

The work embodied in this thesis was carried out at Skorpion Zinc mine (Namibia) and the School of Environmental Sciences and Development: Section Botany, North-West University, Potchefstroom, South Africa.

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I hereby declare that this thesis presented for the degree *Master Scientiae*, 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.

ABSTRACT

PHYSIOLOGICAL RESPONSE OF THE SUCCULENT *Augea capensis* (ZYGOPHYLLACEAE) OF THE SOUTHERN NAMIB DESERT TO SO_2 AND WATER AVAILABILITY

The main aim of this study was to investigate the effects of water availability and SO_2 pollution, imposed separately or simultaneously, on the photosynthetic metabolism of *Augea capensis* Thunb., a succulent of the Namib Desert in the region of Skorpion Zinc mine, Namibia. The main driver for this investigation was the need to distinguish between the effects of water availability on plants native to a desert environment, where water availability dominates plant response, but where the possibility of anthropogenic SO_2 pollution poses a new threat to the unique succulent vegetation.

Fifteen measuring sites were selected in the vicinity of the mine to determine how rainfall influenced the physiological status of the vegetation. Chlorophyll *a* fluorescence measurements, and analysis of recorded OJIP fluorescence transients with the JIP-test, were used for this purpose. A series of laboratory experiments were also conducted on *A. capensis* to determine the precise physiological response that water deprivation and SO_2 pollution had under controlled growth conditions. Potted plants were exposed to water deprivation or SO_2 fumigation in the light or dark. Besides chlorophyll *a* fluorescence, photosynthetic gas exchange and Rubisco activity were also measured.

Changes in fast fluorescence rise kinetics observed under field conditions suggest considerable modulation of photosystem II function by rainfall with concomitant involvement of a heat stress component as well. In both the field and laboratory experiments, one of the JIP-test parameters, the so-called performance index (PI_{ABS}), was identified as a very sensitive indicator of the physiological status of the test plants. Moreover, under laboratory conditions, a good correlation existed between the water deprivation-induced decline in CO_2 assimilation rates and the decline in PI_{ABS} values. The JIP-test in general, and the PI_{ABS} in particular, shows considerable potential for application in the investigation of water availability influences on desert ecosystems. In the laboratory experiments, water deprivation caused stomatal closure but also a slight elevation in intercellular CO_2 concentration and inhibition of Rubisco activity, suggesting that mesophyll limitation was the dominant factor

contributing to the decrease in CO_2 assimilation rates. Following re-watering, *A. capensis* showed remarkable recovery capacity.

Fumigation of *A. capensis* with 1.2 ppm SO_2 in the dark or light revealed relatively small effects on CO_2 assimilation. The inhibitory effects on photosynthesis were also fully reversible, indicating no permanent metabolic/structural damage. The effects on photosynthesis were more pronounced when fumigation occurred in the dark. This phenomenon might be related to diurnal differences in cellular capacity for SO_2 detoxification. When long-term moderate water deprivation was combined with simultaneous SO_2 fumigation, there was no additional inhibitory effect on photosynthesis. These findings suggest that water deprivation do not increase sensitivity towards SO_2 pollution in *A. capensis*. Fumigation with SO_2, singly or in combination with water deprivation also had no major effect on chloroplast ultrastructure. It appears that *A. capensis* is remarkably resistant to SO_2 pollution even in the presence of low water availability, which is a common phenomenon in desert ecosystems.

Since *A. capensis* seems to be highly tolerant to SO_2, its suitability as an indicator species for the detection of SO_2 pollution effects at Skorpion Zinc mine is questionable. Because water availability dominates the physiological/biochemical response in this species, subtle SO_2 pollution effects might be difficult to detect against this dominant background. The high water content of *A. capensis* and similar succulents might act as a substantial sink for SO_2 and could convey considerable tolerance against this form of air pollution.

Keywords:

Augea capensis Thunb., chlorophyll *a* fluorescence, CO_2 assimilation, water availability/deprivation, photosynthesis, SO_2 pollution, leaf ultrastructure, Namib Desert, succulents, *Zygophyllum prismatocarpum* E. Meyer ex Sond.

OPSOMMING

FISIOLOGIESE REAKSIE VAN *Augea capensis* (ZYGOPHYLLACEAE), 'N SUKKULENT VAN DIE SUIDELIKE-NAMIBWOESTYN, OP SO_2 EN WATERBESKIKBAARHEID

Die hoofdoel van hierdie studie was om die effek van gesamentlike- of afsonderlike toediening van waterbeskikbaarheid en SO_2 besoedeling op fotosintese in *Augea capensis* Thunb., 'n sukkulent vanuit die Namibwoestyn in die omgewing van Skorpion Zinc myn (Namibië), te ondersoek. The hoof dryfveer vir hierdie ondersoek was die behoefte om onderskeid te kan tref tussen die effekte van hierdie stremmingsfaktore op plante endemies tot 'n *woestyngebied*, waar waterbeskikbaarheid plantrespons oorheers, maar waar die moontlikheid van SO_2 besoedeling van menslike oorsprong 'n nuwe bedreiging inhou vir die unieke sukkulente plantegroei.

Vyftien studielokaliteite is in die omgewing van die myn geselekteer sodat vasgestel kon word in welke mate reënval die fisiologiese status van die plantegroei beïnvloed. Chlorofil *a* fluoressensie-metings, en analise van OJIP fluoressensie-krommes met behulp van die JIP-toets, is vir hierdie doel aangewend. 'n Aantal laboratoriumproewe is ook uitgevoer om vas te stel hoe waterweerhouding en SO_2 besoedeling die fisiologiese reaksie van *A. capensis* onder gekontroleerde toestande beïnvloed het. Potplante is blootgestel aan waterweerhouding of SO_2 besoedeling in die lig of donker. Buiten chlorofil *a* fluoressensie, is fotosintetiese gaswisseling sowel as Rubisco aktiwiteit ook bepaal.

Veranderinge in *vinnige-fase fluoressensie-kinetika*, wat onder veldtoestande waargeneem is, suggereer aansienlike modifikasie van fotosisteem II funksie deur reënval, met die addisionele betrokkenheid van 'n hitte-stremmingskomponent. In beide die veld- en laboratoriumproewe is een van die JIP-toets parameters, die sogenaamde vitaliteitsindeks (PI_{ABS}), geïdentifiseer as 'n besonder sensitiewe indikator van die fisiologiese status van proefplante. 'n Goeie korrelasie tussen waterweerhouding-geïnduseerde afname in die CO_2 assimileringstempo en 'n afname in PI_{ABS} waardes is ook onder laboratoriumkondisies waargeneem. Die JIP-toets in die algemeen, en die PI_{ABS} in besonder, toon dus aansienlike potensiaal om gebruik te word om waterbeskikbaarheidseffekte in woestynekosisteme te bestudeer.

In die laboratoriumproewe het waterweerhouding stomasluiting tot gevolg gehad, maar ook 'n klein verhoging in intersellulêre CO_2 konsentrasie en die inhibisie van Rubisco aktiwiteit veroorsaak. Hierdie bevindinge dui op mesofilbeperking as die hoof beperkende faktor wat tot die afname in CO_2 assimileringstempos aanleiding gegee het. Na herbenatting, het *A. capensis* 'n merkwaardige herstelvermoë vertoon.

Klein veranderinge ten opsigte van CO_2 assimilering is waargeneem wanneer *A. capensis* met 1.2 dpm SO_2 in die lig of donker behandel is. Die inhiberende effek op fotosintese was ook ten volle omkeerbaar wat daarop dui dat geen permanente metaboliese/strukturele skade aangerig is nie. Die effek op fotosintese was groter wanneer SO_2 behandeling in die donker plaasgevind het. Hierdie verskynsel kan moontlik die gevolg wees van dag/nag verskille in sellulêre SO_2 detoksifiseringskapasiteit. Gelyktydige blootstelling aan langdurige matige waterweerhouding en SO_2 het geen bykomende inhiberende effek op fotosintese gehad nie. Hierdie bevinding suggereer dat waterweerhouding nie die sensitiwiteit van *A. capensis* teenoor SO_2 besoedeling verhoog het nie. Behandeling met SO_2, alleen of in kombinasie met waterweerhouding, het ook geen invloed op die ultrastruktuur van chloroplaste gehad nie. Dit blyk dus dat *A. capensis* hoogs weerstandbiedend is teenoor SO_2 besoedeling, selfs in die teenwoordigheid van lae waterbeskikbaarheid wat 'n algemene verskynsel in 'n woestynomgewing is.

As gevolg van die skynbare hoë weerstand teenoor SO_2 besoedeling, kan die bruikbaarheid van *A. capensis* as 'n indikatorspesie vir die monitering van SO_2 besoedelingseffekte, bevraagteken word. Omdat waterbeskikbaarheid die fisiologiese/biochemiese reaksie van hierdie spesie oorheers, mag dit moeilik wees om meer subtiele SO_2 besoedelingseffekte teen hierdie dominante agtergrond te bespeur. Die hoë waterinhoud van *A. capensis* en ander soortgelyke sukkulente mag moontlik as 'n effektiewe absorbeerder van SO_2 dien wat aansienlike weerstand teenoor hierdie vorm van besoedeling mag verleen.

Sleutelterme:

Augea capensis Thunb., chlorofil *a* fluoressensie, CO_2 assimilering, waterweerhouding/beskikbaarheid, fotosintese, SO_2 besoedeling, blaarultrastruktuur, Namibwoestyn, sukkulente, *Zygophyllum prismatocarpum* E. Meyer ex Sond.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

To understand how plants function in their natural environment, it is important to know how plants will react to different stressors. These stress responses will also explain why plants favor certain environmental gradients and why they are distributed along them (Osmond *et al.*, 1987).

Abiotic stressors seldom act independently and the stress environment may involve a complex of interacting stress factors. Plants may counteract these stressors by modifications on cellular, metabolic and genetic level.

Individual plants, by virtue of their stationary nature, cannot migrate like animals to avoid unfavorable conditions. According to Alscher & Cummings (1990), species survival during exposure to short-term stress depends on acclimatisation whereas physiological changes, which are based on continued growth under stress conditions, depend on adaptation.

Nowhere is the reality of plants facing daily stressful situations more evident than in a desert environment. A desert is an area of low rainfall (less than 200 mm precipitation annually), an environment where drought often prevails (Salisbury & Ross, 1991). The Namib Desert stretches along the west coast of southern Africa between 15° and 32° S latitude. It is a narrow land strip seldom exceeding 150 km in the east-west direction. Rainfall (ca. 70 mm per annum) in the southern Namib, where Skorpion Zinc mine is situated, occurs preferentially in winter from March to October. During winter the high-pressure system over Southern Africa moves to the north so that cold fronts can extent northwards as far as Lüderitz. It is an area of diversity, in its landscapes, as well as in its floristic composition (Von Willert *et al.*, 1992).

Abiotic stress in the form of water limitation is a natural and common feature in the Namib Desert. The release of SO_2 of anthropogenic origin, on the other hand, is not a characteristic of arid environments, which is usually far removed from human activities. In the sections below, the effects of drought and SO_2 pollution on plants are

reviewed. Because of the central role of photosynthesis in whole-plant metabolism, the literature review will concentrate mainly on the effects of these stress factors on this key process.

1.2 Inhibition of photosynthesis by drought stress

Plants are subjected to a wide range of environmental stresses that adversely affect growth, metabolism and yield (Reddy *et al.*, 2004). Drought stress develops in plants during periods when the water supply to the roots become limiting or when transpiration rates become very high.

Water availability is one of the most important limitations to photosynthesis and plant productivity (Boyer, 1982; Tezara *et al.*, 1999). Plants respond to drought stress through various strategies. These strategies may involve adaptive changes and/or deleterious effects as well as a mixture of stress avoidance and tolerance mechanisms (Chaves *et al.*, 2002). It is essential to understand how photosynthesis will be affected by drought stress because the nature and sensitivity of metabolic processes determine the response of plants to water deficits and the processes required to prevent damage. These protective mechanisms allow plants to function in terms of productivity, reproduction and ecological fitness in different environments and under varying water balances (Lawlor, 2002).

The debate as to whether drought mainly limits photosynthesis through stomatal closure (stomatal limitation) or through metabolic impairment (mesophyll limitation) is still ongoing (Tezara *et al.*, 1999; Lawlor, 2002; Reddy *et al.*, 2004). Chaves (1991) reported that when drought periods are lengthened and dehydration becomes more severe, metabolic changes might occur in plants. In cases of mild drought stress, stomatal limitation plays the most important role in the inhibition of photosynthesis. Thus, the severity of drought stress seems to be the determining factor as to whether the reduction of photosynthesis is due to stomatal and/or mesophyll limitation. The precise mechanisms and sequence of events leading to the inhibition of photosynthesis by drought stress still remains uncertain and varies between species.

1.2.1 Stomatal limitation of photosynthesis

Stomatal limitation is generally regarded to be the primary cause for reduced photosynthesis during drought stress (Sharkey, 1990; Chaves, 1991; Ort *et al.*, 1994;

Cornic, 2000). Under these conditions a concomitant decrease in CO_2 assimilation rate (A) and intercellular CO_2 concentration (c_i) is often observed.

It is clear that stomata close progressively as drought stress progresses, accompanied by a parallel decrease of net photosynthesis. Various experiments have shown that stomatal responses to drought stress are often more closely linked to soil moisture content than to leaf water status (Chaves *et al.*, 2002). This root-shoot signaling pathway involves abscisic acid (ABA), which is synthesized in the roots in response to soil drying (Davies & Zhang, 1991). Photosynthetic rates often begin to decline when cell turgor is reduced to zero (Boyer & Potter, 1973), while ABA synthesis is already initiated when cell turgor approaches zero (Pierce & Raschke, 1980).

Many studies have shown that drought stress-induced loss of O_2 evolution and net CO_2 assimilation capacity can be restored under high external CO_2 concentrations where stomatal limitation of photosynthesis is excluded (Frederik *et al.*, 1990; Cornic, 1994). These findings imply that stomata play a dominant role in decreased CO_2 assimilation during drought stress. It is often argued that any non-stomatal (mesophyll) effects can be attributed to the presence of non-homogeneous (patchy) stomatal closure, which is a potential artifact during gas exchange measurements in drought-stressed plants (Downtown *et al.*, 1988; Terashima *et al.*, 1988). However, Gunasekera & Berkowitz (1992) concluded that patchy stomatal closure is rarely encountered by plants growing under field conditions because this phenomenon apparently does not occur when drought stress is imposed at a relatively gradual rate. They also concluded that patchy stomatal closure is not a universal phenomenon in all plant species.

A high degree of co-regulation of stomatal conductance (g_s) and photosynthesis is usually found. It presents a more integrative basis for the assessment of drought stress effects than for example, leaf water potential (Ψ_L) or relative water content (RWC). Medrano *et al.* (2002) used g_s as an integrative parameter and found a decline in carboxylation efficiency (CE), c_i and quantum yield of primary photochemistry (F_v/F_m).

The importance of stomatal responses during drought stress in plants growing in arid environments was demonstrated in the crassulacean acid metabolism (CAM) species *Portulacaria afra* (Hanscom & Ting, 1978). Stomatal responses in this species

followed the typical night-daytime cycle of CAM species. Nocturnal stomatal opening has a major effect on the water economy of CAM plants. In non-CAM species, daytime stomatal opening, and the accompanying increase in the temperature-related vapor pressure gradient, results in high transpiration rates. Complete daytime stomatal closure in CAM plants is therefore an important survival mechanism in arid environments (Hanscom & Ting, 1978).

1.2.2 Mesophyll limitation of photosynthesis

The limitation of photosynthesis during drought stress through metabolic impairment is a more complex phenomenon than stomatal limitation. Sites at which photosynthetic metabolism may be impaired include: 1) ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity; 2) regeneration of ribulose-1,5-bisphosphate (RuBP) by the photosynthetic carbon reduction (PCR) cycle; 3) supply of reducing equivalents (ATP and NADPH) for the operation of the PCR cycle; 4) electron transport and generation of a proton motive force across the thylakoid membrane; and 5) starch and sucrose synthesis (Lawlor, 2002).

1.2.2.1 Rubisco activity

The rate of photosynthesis in higher plants depends on the activity of Rubisco as well as regeneration of RuBP (Tezara *et al.*, 1999; Parry *et al.*, 2002; Chaitanya *et al.*, 2003;). Loss of Rubisco activity has been reported in several plants during drought stress (Parry *et al.*, 2002). The amount of Rubisco in leaves is controlled by the rate of synthesis and degradation and even under conditions of drought stress the Rubisco holo-protein is relatively stable with a half-life of several days (Webber *et al.*, 1994). Drought stress in tomato (Bartholomew *et al.*, 1991), *Arabidopsis* (Williams *et al.*, 1994) and rice (Vu *et al.*, 1999) resulted in a rapid decline in the abundance of Rubisco transcripts. The loss of Rubisco protein under drought stress conditions has often been ascribed to enhanced rates of degradation of the enzyme (Mehta *et al.*, 1992; Ishibashi *et al.*, 1996). However, there is increasing evidence that the loss of Rubisco protein is a function of changes in gene expression.

Short-term responses of Rubisco to drought stress are not clear, as different studies have produced conflicting results. Gimenéz *et al.* (1992) and Gunasekera and Berkowitz (1993) found little effect of drought stress on Rubisco. Majumdar *et al.* (1991) observed rapid loss of Rubisco during drought stress in soybean. Increasing

severity and duration of drought stress, however, do decrease both Rubisco activity (Tezara & Lawlor, 1995) and protein content (Kicheva *et al.*, 1994) in sunflower and wheat respectively. Under Mediterranean conditions, Parry *et al.*, (1993) found that Rubisco activity of tobacco was decreased by the action of tight-binding inhibitors that block the catalytic sites of the enzyme. Parry *et al.*, (2002) also found that drought stress decreased the initial and total extractable activities of Rubisco. Decreased CE and CO_2 saturated rates of photosynthesis (J_{max}) with decreasing RWC also suggest loss of Rubisco activity. However, the recovery of J_{max} by subsequent rehydration suggests that Rubisco and other key enzymes are not impaired irreversibly during drought stress. A reduction in the amount of Rubisco during drought stress may be related to stimulation of leaf senescence, which is difficult to distinguish from a direct effect of low RWC (Majumbar *et al.*, 1991).

In the CAM-succulent *Sedum pulchellum*, Smith & Eickemeier, (1983) investigated the effect of drought stress on PEP carboxylase and Rubisco activity and found only small changes in the activities of both enzymes. Similar investigations in *Portulacaria afra* (L.), however, revealed a 50% decrease in the activity of both enzymes during a period of drought stress (Guralnick & Ting, 1987).

1.2.2.2 RuBP regeneration

The capacity for RuBP regeneration is a key factor in CO_2 assimilation and depends on the supply of ATP and NADPH and the function of PCR cycle enzymes, predominantly the stromal bisphosphatases, fructose-1,6-bisphosphatase (FBPase) and sedoheptulose-1,7-bisphosphatase (SBPase). A strong relationship between CO_2 assimilation and RuBP availability were demonstrated by Gimenéz *et al.* (1992) in drought-stressed sunflower leaves. High PGA/RuBP ratios suggested limitation in the RuBP regeneration part of the PCR cycle, either caused by enzyme limitation or inadequate reductant supply.

According to the model for photosynthetic gas exchange (Farquhar *et al.*, 1980) the reduced RuBP regeneration capacity in drought-stressed plants might be due to decreased photochemical activity. Recent advances in chlorophyll fluorescence techniques have shown that J_{max} is usually reduced to a much greater extent than electron transport under mild drought stress conditions, suggesting that the photochemical reactions are rather tolerant to drought stress. However, Tezara *et al.* (1999) have suggested that decreased ATP synthesis, through ATPsynthase

impairment, would lead to reduced RuBP regeneration capacity under more severe drought stress conditions.

1.2.2.3 Photosystem II (PSII) function

Photosystem II (PSII) is composed of a reaction centre (RC) complex, the inner antennae, the light-harvesting antenna system and the oxygen-evolving complex (Anderson & Styring, 1991; Green & Durnford, 1996). Upon moderate drought stress conditions, photosynthesis decreases mainly because of stomatal closure. As the drought stress progresses, biochemical constraints limit CO_2 assimilation more directly (Lawlor, 1995). As limitation of CO_2 assimilation by the PCR cycle frequently precedes inactivation of electron transfer reactions, an excess of reducing equivalents is generated in drought stressed plants. Balancing the supply of, and demand for, reducing equivalents requires the concerted regulation of photosynthetic electron transport and PCR cycle activity. Thus, under drought stress conditions, photosynthetic electron transport has to be down regulated to meet the lower demand for reducing equivalents because of reduced capacity for CO_2 assimilation. Strong evidence has accumulated indicating that PSII, which catalyses the oxidation of water into oxygen and initiates photosynthetic electron transport, is essential for this regulation (Golding & Johnson, 2003). There is however also evidence of direct damage to PSII by drought stress. Some studies demonstrated that drought stress results in damage to the oxygen-evolving complex (OEC) of PSII (Canaani *et al.*, 1986; Toivonen & Vidaver, 1988) and also to PSII reaction centres (Havaux *et al.*, 1986, 1987). Giardi *et al.* (1996) demonstrated that drought stress caused considerable degradation of the D1 reaction centre protein and enhanced phosphorylation of PSII core proteins. Similar effects were also observed by He *et al.* (1995).

Chlorophyll *a* fluorescence can be regarded as a bio-sensing technique for stress detection in plants (Kocheva *et al.*, 2004). Environmental stresses that affect PSII efficiency lead to a characteristic decrease in the F_v/F_m ratio. Kocheva *et al.* (2004) found no significant decrease in the F_v/F_m ratio of drought-stressed barley leaves, suggesting that the quantum yield of PSII was not lowered. Similar findings were made by Cornic & Briantais (1991); Epron *et al.* (1993); Liang *et al.* (1997) and Lima *et al.* (2002), supporting the idea that PSII is rather tolerant to drought stress.

Even when the F_V/F_M ratio show changes during drought stress, it only provides limited information about overall PSII function. For example, it gives no direct information on the heterogeneity (active *versus* deactivated) of the PSII reaction centres. In contrast, rapid fluorescence induction kinetics provides a multitude of information about the structure and function of PSII. Using direct (not modulated) fluorescence techniques, Strasser *et al.* (1995) demonstrated that when a dark-adapted leaf is illuminated with a saturated light pulse (3000 photons μmol m^{-2} s^{-1}), the fluorescence induction curve is polyphasic (see chapter 2 for an illustration and full explanation). The individual steps have been denoted as O, J, I and P. The fluorescence intensity at O reflects the minimal fluorescence yield when all molecules of the primary quinone acceptor (Q_A) are in the oxidized state. The fluorescence intensity at P corresponds to the state in which all molecules of Q_A are in the reduced state (Q_A^-). Steps J and I occur at about 2ms and 30ms, respectively, between these two extremes (Lu & Zhang, 1999). The fluorescence rise from O to J results from the reduction of Q_A to Q_A^- and is representative of the primary photochemical reactions of PSII. The intermediate step I reflects the existence of fast and slow reducing plastoquinone (PQ) centres as well as the different redox states of PSII reaction centres (Strasser *et al.*, 1995). Thus, the polyphasic chlorophyll fluorescence transient is rich in information about overall PSII photochemistry. A number of studies have employed the OJIP fluorescence transient to investigate the effects of drought stress on PSII function (e.g. Lu & Zhang (1999). Recently, Lu *et al.* (2003) investigated the effect of drought stress on PSII function in the CAM-succulent *Kalanchoë daigremontiana*. They did not observe significant changes in the kinetics of OJIP fluorescence transients when the plants were exposed to simultaneous drought stress and high light intensity. This finding implicates that drought stress had no negative effect on the reduction of Q_A to Q_A^- or the redox states of PSII reaction centers.

1.2.3 Ultrastructural changes in response to drought stress

In a study performed on lavender (*Lavendula stoechas* L.), Pastor *et al.* (1999) indicated that mesophyll cells of well-watered plants contained chloroplasts with well-developed grana and stroma thylakoids. After drought stress was imposed for four days at a Ψ_L of -2 MPa, ultrastructural alterations were observed. These included: plasmolysis and the formation of various cytoplasmic vesicles with electron dense

inclusions. Chloroplasts were irregular in shape and re-orientation of the thylakoids was observed. Dilatation of bent thylakoids was also observed. Under conditions of severe drought stress (Ψ_L = -3.2 MPa), the chloroplast envelope membranes were ruptured, with swelling of grana. As a consequence, the intra-thylakoid space increased. Cells were irregular in shape and the cell walls appeared undulated (Pastor *et al.*, 1999).

Munné-Bosch *et al.* (2001) observed clear symptoms of senescence, such as membrane whorls and condensation of chromatin in the nuclear matrix and nucleolus after severe drought stress in field-grown sage (*Salvia officinalis*). Swelling of chloroplasts, accumulation of plastoglobuli in the stroma and changes in the membrane system assemblage, such as loosening and distortion of thylakoids and much less granal stacking, were also noticed.

Sorghum bicolor plants grown under controlled conditions showed rearrangement of cell organelles within the mesophyll cells during drought stress. The tonoplast appeared to have fragmented but the only apparent chloroplast damage was the swelling of the outer membrane. A slight disarrangement of the stroma lamellae in some plastids occurred, but the general organization appeared to remain unchanged (Giles *et al.*, 1976). From the discussion it is clear that large variation in ultrastructural damage occurs in response to drought stress in different plant species.

1.3 Sulphur dioxide (SO_2) as an air pollutant in plants

In developing countries the emission of sulphur dioxide (SO_2), a phytotoxic by-product of fossil fuel burning, is rising progressively (Agrawal & Deepak, 2003). The industrial emission of SO_2 in developed countries, however, has been reduced dramatically during the last decade, mainly because of strict regulatory legislation and emission controls (Cape *et al.*, 2003).

Damage caused by SO_2 is not a new phenomenon - it has caused damage ever since the beginning of life on earth near volcanoes and ever since the beginning of the smelting of sulphur-containing ores over 4000 years ago (Larcher, 2003). Since the 1970's, there has been a dramatic decline in the health of forests (Guderian, 1985). Based on a series of facts and circumstantial evidence, the primary cause of the injury is due to ozone in connection, and in most locations in combination with SO_2.

Sulphur dioxide is a pollutant that can cause positive effects on physiological and growth characteristics of plants at very low concentrations, especially in plants growing in sulphur-deficient soils (Darrall, 1989). At the same time, increased uptake of SO_2 can cause toxicity and reduced growth and productivity of plants due to accumulation of sulfite and sulfate within the plant (Agrawal & Deepak, 2003). The main factors that determine the phytotoxicity of SO_2 are: environmental conditions, duration of exposure, atmospheric SO_2 concentration, sulphur status of the soil and the genetic constitution of the plant (Saxe, 1991).

Once SO_2 is emitted into the atmosphere it is converted into secondary pollution products such as sulphuric acid, which can easily dissolve in water, fog and clouds to form acid rain (Shvetsova *et al.*, 2002). Upon exposure to acid rain, plants respond on organ and whole-plant level. Acid rain adversely affects plant foliage (especially young leaves); leads to a loss of chlorophyll content; disrupts chloroplast ultrastructure and can lead to membrane lipid bi-layer reorganization (Chia *et al.*, 1984). Like other forms of oxidative stress, acid rain also causes an increase in the activity of anti-oxidant systems e.g. superoxide dismutase activity (Koricheva *et al.*, 1996).

1.3.1 Entry of SO_2 into the plant

Sulphur dioxide can enter the leaf of a plant as readily as CO_2 through the stomata. Even if the stomata are closed, SO_2 can easily enter the leaf by overcoming the cuticular resistance (Larcher, 2003). The diffusion pathway of SO_2 is similar to that of CO_2 and the concentration gradient between the atmosphere and the chloroplasts is just as steep. The solubility factor of SO_2 is almost 40 times higher than that CO_2 making it a stronger acid (Pfanz & Heber, 1986). This enables SO_2 to dissolve in the water occurring in the cell wall forming the byproducts, hydrogen sulfite (HSO_3^-) and sulfite (SO_3^{2-}) which are then distributed inside the cell between the chloroplasts, cytosol and vacuole in a 96:3:1% proportion (Larcher, 2003).

1.3.2 Visual symptoms of SO_2 pollution in plants

Atmospheric concentrations of SO_2 do not normally induce visual effects (such as chlorosis) in plants. The maximum SO_2 concentration (short term) recorded in central Europe is 150 ppb. At these concentrations, the effects of SO_2 are mostly limited to

enzymatic reactions and recovery of the reactions affected is also possible. Barley (*Hordeum vulgare* L.) exposed to a SO_2 concentration of 80 ppb showed no visible signs of injury (e.g. chlorosis) after 75 days of fumigation (Raneiri *et al.*, 1999). Only at higher concentrations can one observe visual, and mostly irreversible, symptoms (Beauregard, 1990). Rakwal *et al.* (2003) observed light brownish spots on the leaves of rice seedlings 60 h after exposure to SO_2. After 72 h, intensely reddish brown necrotic lesions and interveinal browning were apparent almost over the entire leaf surface. Maas *et al.* (1987) studied the response of *Spinacia oleracea* to H_2S and SO_2 fumigation. No effects on leaf morphology and appearance were observed when plants were fumigated with 0.10 or 0.25 ppm SO_2. An interesting observation is that plants, which take up SO_2 in the dark, sometimes experience greater leaf injury in the form of foliar necrosis relative to plants exposed to SO_2 under low to moderate light conditions (Nielsen, 1938; Davies, 1980; Jones & Mansfield, 1982). Olsyk & Tingey, (1984) also observed that SO_2 fumigations in the light were less toxic to plants than fumigation in the dark.

1.3.3 Physiological effects of SO_2 pollution

With continued uptake of SO_2 and increasing acidification, the cellular buffering capacity is exceeded, the sulfite level in the chloroplast rises, and SO_2 can even occupy the CO_2-binding sites of Rubisco (Larcher, 2003). This results in the inhibition of CO_2 assimilation and disruption of tertiary enzyme structure. Superoxide radicals, formed by photooxidation of sulfite to sulfate in the chloroplast, cause lipid peroxidation and destruction of chlorophyll. The scavenging mechanism by the enzyme superoxide dismutase (SOD) can be utilized to render these substances harmless (Larcher, 2003).

The C_4 syndrome promotes resistance to moderate SO_2 stress. The enzyme PEP-carboxylase is less sensitive to SO_2 than Rubisco (Larcher, 2003), and due to the CO_2 concentrating mechanism, there is less competitive inhibition of Rubisco. Thus, in general, C_4 plants are less sensitive to SO_2 than C_3 plants (Larcher, 2003). A study conducted by Olszyk & Bytnerowicz (1987) on CAM succulents revealed that these plants were not as sensitive to SO_2 under field conditions as other desert species. Their results suggest that the physiological mechanism of SO_2 toxicity may be different in CAM species compared to C_3 species. In agreement with the most common observations in other plant species, however, the toxicity in CAM-species,

such as *Opuntia basilaris*, is also enhanced during exposure to SO_2 in the light compared to the dark (Olszyk & Bytnerowicz, 1987). While their unique physiological adaptations to arid environments might render CAM plant species less sensitive to air pollutants than other plant species, these adaptations may maximize pollutant sensitivity during short transient periods of favourable environmental conditions when these plants are metabolically at their most active (Olszyk & Bytnerowicz, 1987).

Exposure of plants to SO_2 under certain conditions reversibly inhibits net photosynthesis. Both stomatal and mesophyll limitation of photosynthesis have been implicated in the inhibition. At the pH within the chloroplast, SO_2 is mainly converted to sulfite. Sulfite in isolated chloroplasts and thylakoids influences i) CO_2 assimilation, ii) the activity of the stromal bisphosphatases, iii) the activity of Rubisco, iv) photo-phosphorylation (Cerovic *et al.,* 1982) and v) the operation of the triose-phosphate translocator (Mourioux & Douce, 1979). Veljovic-Jovanovic *et al.* (1993) found an increased inhibition of FBPase activity and RuBP regeneration with increased SO_2 concentration. Nieboer *et al.* (1976) suggested that SO_2 and its oxidation products are capable of interfering with the electron flow through PSI and PSII. Thus, it is possible that SO_2 restricts the supply of reducing equivalents required for CO_2 assimilation.

It should be noted that different species, and even genotypes within a species, react differently to the same concentration of SO_2. Alcher *et al.* (1987) showed this clearly when they exposed two pea genotypes to the same concentration of SO_2. They showed different abilities in the genotypes to detoxify sulphite with higher levels accumulating in the sensitive genotype during exposure to SO_2.

The harmful effects of SO_2 pollution are generally more pronounced when the stomata are open, suggesting that the stomata are the main means of entry of SO_2 to the interior of the leaf. In an experiment where *Vicia faba* plants were fumigated with 0.025 and 1.0 ppm SO_2, the opening of stomata in the light was more rapid in treated than in control plants and stomatal conductance was also greater in treated plants (Majernik & Mansfield, 1970). The stomata in treated plants also took longer to close fully when transferred to darkness. Similar effects have been reported for barley and maize (Majernik & Mansfield, 1970). Injury occurred to the CAM-plant *Opuntia basilaris* only during SO_2 exposure in the light, even though that was the time of day that the stomata were closed (Olszyk & Bytnerowicz, 1987). There are a number of

unfavourable consequences of these effects on stomata. The access of SO_2 to the mesophyll cells is increased and thus greater damage can occur. In plants with a limited water supply (e.g. in desert environments), increased transpiration resulting from abnormally high stomatal conductance, might lead to damaging or even lethal drought stress (Majernik & Mansfield, 1970). Experiments have shown that those plant species with the highest stomatal conductances were likely to be less tolerant to SO_2. Similarly, plant species in which a stimulation of opening of stomata occurred in response to SO_2 were likely to be more sensitive (Darrall, 1989). A summary of typical symptoms, observed in a number of plant species after exposure to different SO_2 concentrations, are shown in Table 1.1. Similar information for succulent plant species could not be found in the scientific literature.

Table 1.1 Typical symptoms induced by SO_2 exposure in different plant species.

Species	SO_2 concentration	Symptoms
Triticum aestivum	0.06 ppm	• Minor inhibition of photosynthesis • Increased transpiration rate • Decrease in chlorophyll content (Agrawal & Deepak, 2003)
Glycine max	0.06 ppm	• Reduced plant growth, biomass and yield • Decline in foliar starch and protein content • Decrease in water use efficiency (Deepak & Agrawal, 2001)
	0.15 ppm	• Decline in photosynthetic pigments • Decrease in ascorbic acid • Decrease in biomass and productivity (Verma & Agrawal, 1996)
Oryza sativa	0.5 ppm	• Reddish brown necrotic spots • Induction of ascorbate peroxidases (Rakwal *et al.*, 2003)
Xanthoparmelia mexicana	0.5 ppm	• Decrease in chlorophyll and protein content (Kong *et al.*, 1999)

1.3.4 The effect of SO_2 on PSII function

The involvement of sulfite and sulfate anions in the inhibition of PSII function after excess SO_2 uptake has been demonstrated and several research groups have attempted to elucidate the mode of action of these anions on PSII.

The proteins of PSII are composed of an intrinsic (lipid-embedded) core complex, and the OEC, an extrinsic, lumen exposed ensemble of proteins. It is possible that the domains of PSII proteins exposed to the aqueous phase are the sites of action for sulfite and sulfate anions. Two areas are possible targets: 1) a portion of the core complex, exposed to the stromal aqueous phase, containing anion binding sites that determine the rate of charge transfer between the electron acceptors Q_A and Q_B; 2) the OEC, partially composed of polypeptides exposed to the lumen aqueous phase (Beauregard, 1990).

The effect of SO_2 on the photosynthetic apparatus is obviously dependent on the amount absorbed by the plant. Depending on the SO_2 treatment used, the effects range from "non visible" physiological disturbances (chloroplast swelling, inhibition of enzyme activity) to "visible" destruction of the photosynthetic pigments and proteins.

The *in vivo* mechanisms of SO_2 effects on photosynthetic electron transport were first provided by Shimazaki *et al.* (1984). They indicated that the oxidizing side of PSII was inhibited in leaves fumigated with SO_2 using an assay on isolated chloroplasts. This indicated inhibition of the OEC by SO_2. Decreases in PSII quantum yield have also been noticed (Schmidt *et al.* 1988). Although the OEC may also have played a role in the inhibition, slowed Q_A^- oxidation by SO_2 treatment was observed. This implies an impact on the bicarbonate site between Q_A and Q_B and thus a high sensitivity of the reducing side of PSII to SO_2 (Beauregard, 1990).

1.3.5 Ultrastructural changes as a result of SO_2 pollution

Several studies have reported a swelling of thylakoids as a consequence of altered osmotic conditions in the stroma and permeability changes in these membranes induced by SO_2 (Ranieri *et al.*, 1999). According to Stirban *et al.* (1979), SO_2 pollution induced similar changes in the leaves of various trees. They indicated disruption of the chloroplast envelope, a mixed cytoplasm containing plastidal ribosomes and vacuole content, and disintegration of the lipid membranes of the

granal and intergranal thylakoids. The stroma was also coagulated and the plastoglobuli lost its contents and appeared like vacuoles.

Exposure of *Vicia faba* leaves for 1 h to low SO_2 concentrations (0.25 ppm) resulted in slight swelling inside the stromal thylakoids. When the SO_2 concentration was increased to 1 ppm, or the exposure time prolonged to 2 h, the swelling increased and was also evident in granal thylakoids (Wellburn *et al.*, 1972). No alteration of the cytoplasm or the cell wall was observed.

Degradation of ribosomes and endoplasmic reticulum, agglutination of chromatin and plasmolysis have also been observed in beech and hornbeam leaves and buds (Stirban *et al.*, 1988). A loss of metabolic function for a large number of cells in the mesophyll tissue was observed as a result of disintegration. There were poorly developed chloroplasts, mitochondria and other organelles in a few mesophyll cells.

These ultrastructural effects observed in studies conducted in polluted environments can be related, to a certain extent, to the changes in the photosynthetic capacity of mesophyll cells. Such studies improve the information on the genetics of the population and the understanding of the resistance or sensitivity of species (Stirban *et al.*, 1988).

1.4 Research aims

The main aims of this study were to investigate the effects of water deprivation and SO_2 pollution, imposed separately or simultaneously, on the photosynthetic metabolism of *Augea capensis*, a representative succulent from the Namib Desert. The main driver for this investigation was the need to distinguish between the effects of these treatments on plants native to a desert environment, where water availability dominates, but where anthropogenic SO_2 pollution poses a new threat to the unique succulent vegetation.

To achieve these aims the following investigations were conducted:

a) Field experiments showing how rainfall (i.e. water availability) dictates the physiological status of the vegetation in the vicinity of Skorpion Zinc mine;

b) Comparative characterization (fingerprinting) of the effects of water deprivation and SO_2 pollution on photosynthesis and leaf ultrastructure under controlled laboratory conditions;

c) Laboratory experiments determining the effects of simultaneously imposed water deprivation and SO_2 pollution, a likely scenario in a desert environment, on photosynthesis.

1.5 Main research hypothesis

This investigation was premised on the idea that, due to the unique morphology and physiology of succulent plants, they will be affected differently by SO_2 pollution than mesophytic plants and that water deprivation during exposure to SO_2 will further modulate these effects.

CHAPTER 2

MATERIALS AND METHODS

2.1 Field measurements at Skorpion Zinc mine

Fifteen measuring sites were selected in the vicinity of Skorpion Zinc mine for the monitoring of the physiological status of two succulent species in the area of the mine (Fig. 2.1 and 2.2).

Figure 2.1 Skorpion Zinc Mine with the evaporation ponds in the foreground. The red arrow indicates the fume stack (point source) of the sulphuric acid plant.

Most of these sites coincided with the presence of SO_2 monitoring stations erected by the mine. The geographic coordinates of each measuring site were determined with a GPS and are listed in Table 2.1. The measuring sites were mostly located in a northwest-southeast transect (Fig. 2.3) with site No 1 (SZ04) situated the furthest northwest, and site No 15 (SZ11) the furthest southeast of the point source (the SO_2-emitting fume stack of the sulphuric acid plant at the zinc refinery). The prevailing wind direction in the vicinity of the mine is from the south-southeast (see wind roses – Fig. 2.3). Two succulent plant species, *Augea capensis* Thunb. (Fig. 2.4) and *Zygophyllum prismatocarpum* E. Meyer ex Sond. (Fig. 2.5) (both with C_3 photosynthetic pathways) were selected for the purpose of vegetation monitoring due to their wide-spread occurrence at the various measuring sites and physical features.

The measuring sites were visited during the period December 2002 – April 2004 on four occasions (at 3-4 month intervals) to assess the physiological status of the two species. Information regarding monthly rainfall and atmospheric SO_2 levels at each of the measuring sites was obtained from the mine. During the period of investigation, atmospheric SO_2 levels at these measuring sites were at or below baseline levels recorded at unpolluted areas in South Africa. However, emission of phytotoxic levels of SO_2 might occur in the event of a malfunction at the sulphuric acid plant, necessitating this investigation. During each visit, chlorophyll *a* fluorescence measurements (refer to section 2.8.2 for a full description of method) were conducted on leaves of both species. Due to the morphology of the plants, the relative long distances between some measuring sites, and difficult accessibility (some sites only accessible with four-wheel drive vehicles), small twigs containing sufficient leaf material were collected from the plants during the daytime (10h00 – 14h00) and placed in paper bags and immediately into a cooler box (kept at around 18°C). Measurements were done on the dark-adapted leaves within 2-4 h after sample collection in a darkened room at the mine. For each measuring site, chlorophyll fluorescence measurements were conducted on 30 leaves per species (collected from at least six individual plants). Above-mentioned procedure is acceptable and it was determined that time-related artefacts did not occur in the relatively short time between sample collection and measurement. This is especially true for leaves kept in the dark and at cool temperatures (Prof Reto Strasser, *personal communication*).

Figure 2.2 Typical vegetation in the vicinity of Skorpion Zinc Mine

Table 2.1 Site codes and coordinates for each of the 15 measuring sites in the vicinity of Skorpion Zinc mine. Sites are arranged in order of their location from northwest to southeast. Sites no. 1 – 7 are located northwest of the point source (fume stack of sulphuric acid plant), while sites no. 8 – 15 are located towards the east/southeast of the point source. Refer to Fig. 2.3 for a map showing the position of each measuring site in relation to the point source.

Site number	Site code	Site coordinates
1	SZ 04	S 27° 43' 11.8" / E 016° 31' 44.8"
2	SZ 19	S 27° 43' 27.8" / E 016° 32' 04.0"
3	SZ 18	S 27° 43' 34.1" / E 016° 30' 43.4"
4	SZ 17	S 27° 44' 58.4" / E 016° 31' 32.1"
5	SZ 20	S 27° 45' 14.7" / E 016° 33' 40.1
6	SZ 03	S 27° 46' 10.9" / E 016° 30' 39.5"
7	SZ 05	S 27° 46' 12.4" / E 016° 34' 32.7
8	SZ 07	S 27° 48' 27.2" / E 016° 37' 40.2
9	SZ 08	S 27° 49' 06.0" / E 016° 37' 22.8"
10	SZ 13	S 27° 49' 14.6" / E 016° 35' 45.9"
11	SZ 09	S 27° 49' 54.8" / E 016°37' 45.5"
12	SZ 02	S 27° 50' 16.8" / E 016° 36' 37.4"
13	SZ 22	S 27° 50' 38.9" / E 016° 37' 51.2"
14	SZ 21	S 27° 51' 37.2" / E 016° 38' 51.1"
15	SZ 11	S 27° 53' 02.1" / E 016° 39' 13.8"

2.2 Species selection for water deprivation and SO_2 fumigation experiments under controlled conditions

Based on the information obtained during the field visits to Skorpion Zinc mine, the evergreen succulent, *Augea capensis* (family Zygophyllaceae), was selected for detailed water deprivation and SO_2 fumigation experiments under controlled conditions (Fig. 2.4). This species was selected because of its abundance in the

vicinity of the mine and also because of low mortality rates following transplantation from the natural environment to plastic pots. Thirty potted plants were transported by road from Skorpion Zinc mine to a temperature-controlled glasshouse at North-West University (Potchefstroom) during April 2004. Additional plants were again obtained during September 2004 and April 2005.

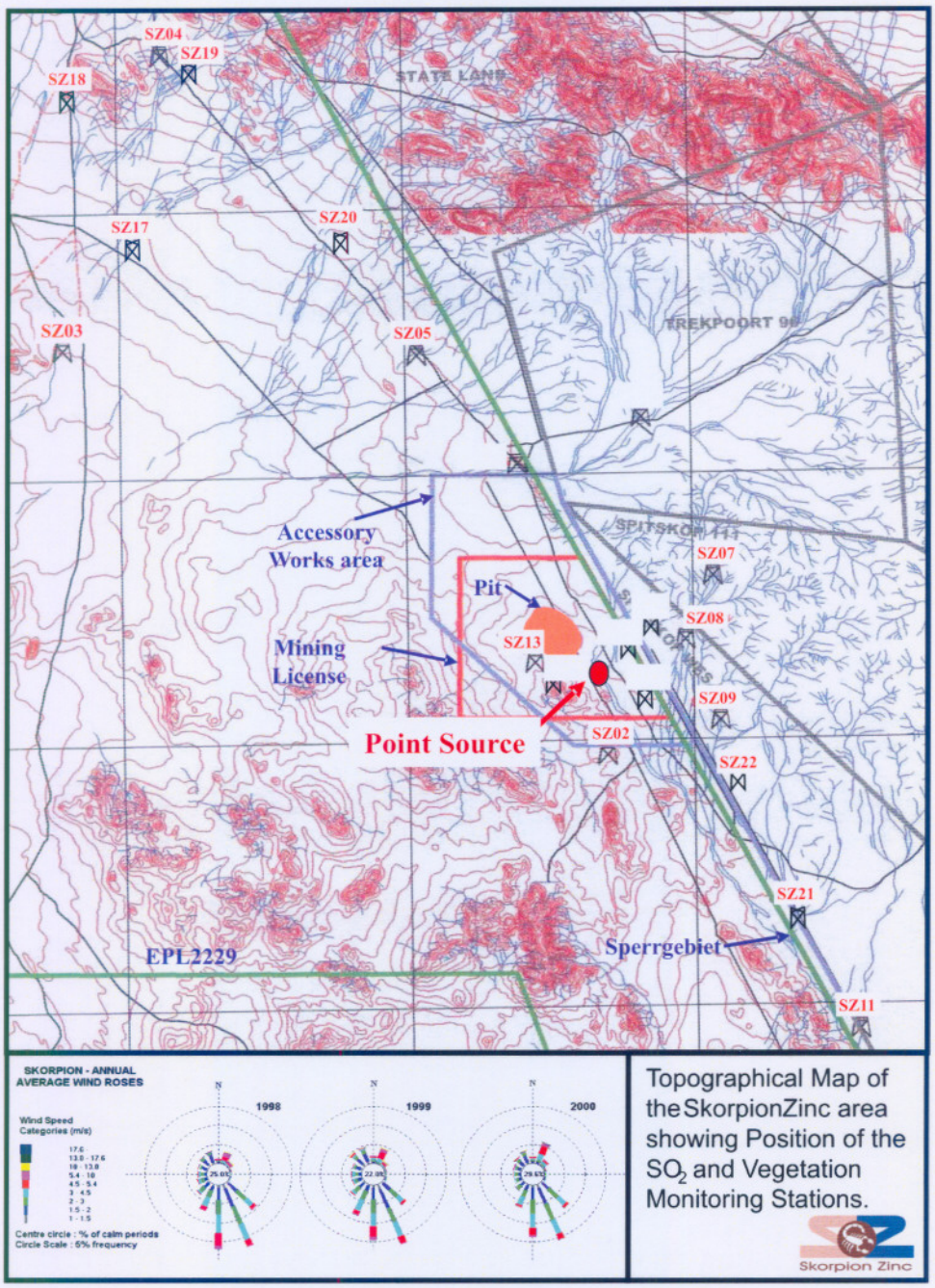


Figure 2.3 Topographical map of the Skorpion Zinc area showing the position of the SO_2 and vegetation monitoring sites. Also see table 2.1 for geographic coordinates of individual sites. The wind roses indicate the predominant wind directions during 1998 – 2000. Map kindly provided by Skorpion Zinc mine.

Figure 2.4 *Augea capensis*, a representative species of the succulent vegetation in the vicinity of Skorpion Zinc mine, was used for field monitoring purposes and in all laboratory experiments investigating the effects of water deprivation and SO_2 pollution under controlled growth conditions.

Figure 2.5 *Zygophyllum prismatocarpum*, a representative species of the succulent vegetation in the vicinity of Skorpion Zinc mine was used for field monitoring purposes.

2.3 Controlled growth conditions

After transfer from Skorpion Zinc mine, potted *A. capensis* plants were acclimated to the growth conditions in the glasshouse at North-West University for a period of two

months prior to the start of SO_2 fumigation and water deprivation experiments. Plants were watered twice weekly with distilled water and received granular fertilizer (50 g per pot once a month) containing 143.8 g kg^{-1} N, 17.9 g kg^{-1} P, 89.3 g kg^{-1} K and 18.8 % (w/w) lime (Wonder Rose Fertilizer, Agroserve, P.O. Box 912-787, Silverton, South Africa). Plants were routinely treated at prescribed dilutions with a systemic insecticide containing 400 g l^{-1} dimethoate (Aphicide, Agroserve, P.O. Box 912-787, Silverton, South Africa). The temperature inside the glasshouse was controlled between 18°C (night) and 28°C (day). Following the two-month acclimation period, plants selected for SO_2 fumigation or water deprivation experiments were transferred to a Conviron PGV 36 growth room under a 15h/9h and 26°C/20°C day/night cycle with an irradiance intensity of 600 photons µmol m^{-2} s^{-1}. Artificial illumination was provided by a combination of fluorescent (Sylvania Cool White VHO, 215W) and incandescent lamps (General Electric, Neodynium R80, 100W). Prior to the start of experiments these plants were acclimated to the conditions within the growth chamber for a period of one week. Chlorophyll *a* fluorescence and CO_2 assimilation measurements were conducted regularly on the plants during the acclimation period to determine their photosynthetic activity and stability under the controlled growth conditions.

2.4 Overview of stress treatments and experimental procedures

A number of water deprivation and SO_2 fumigation treatments were conducted on *A. capensis* plants under controlled growth conditions. In all cases non-destructive measurements (chlorophyll *a* fluorescence and CO_2 assimilation) were employed to quantify treatment effects on photosynthesis. In selected cases, destructive measurements (Rubisco activity and ultrastructure investigations) were also employed. A summary of the various stress treatments conducted, and experimental procedures employed in each case, are shown in Table 2.2. All experiments were repeated two - three times.

Table 2.2 Summary of water deprivation and SO_2 fumigation treatments conducted on *A. capensis*. In each case the experimental procedures employed are indicated with Y or N (Yes or No). Plants were exposed to two SO_2 concentrations (0.6 ppm and 1.2 ppm) either in the light or in the dark. In the case of simultaneous exposure to water deprivation and SO_2 fumigation (1.2 ppm in the light), plants were first exposed to moderate water deprivation for a period of 40 days,

where after SO_2 fumigation was introduced for a further 9 days together with water deprivation . The sections in the text referring to the stress treatments and experimental procedures are indicated in parenthesis.

Stress treatment	CO_2 assimilation (2.8.1)	Chlorophyll fluorescence (2.8.2)	Rubisco activity (2.9.1)	Ultrastructure (TEM) (2.9.2)
Water deprivation **(2.5)**	Y	Y	Y	Y
SO_2 fumigation in the dark (2.6)				
0.6 ppm	Y	Y	N	N
1.2 ppm	Y	Y	N	N
SO_2 fumigation in the light (2.6)				
0.6 ppm	Y	Y	N	N
1.2 ppm	Y	Y	N	Y
Simultaneous long-term water deprivation **and SO_2 fumigation (2.5 & 2.7)**	Y	Y	N	Y

2.5 Short-term and long-term water deprivation treatments

Six *A. capensis* plants were selected for each short-term water deprivation experiment. The three control plants were watered twice a week to field capacity, while water-deprived plants received no water for up to fifteen days. Because potted plants were used in all experiments, the loss of available soil water tends to be more rapid than under natural growth conditions. To minimize this effect, the exposed soil surface of all plants was covered with tin foil to slow water loss due to evaporation from the soil. Following the water deprivation period, the normal watering schedule was resumed in order to quantify the recovery potential of the plants. Long-term water deprivation experiments were also conducted to simulate the situation in the natural environment more closely. In these experiments plants were maintained at moderate water deprivation levels for up to 49 days. Plants were watered on

alternate days with small volumes of water (100 ml per pot) ensuring the presence of chronic mild water deprivation but preventing plant mortality. The presence of mild water deprivation response in the plants was routinely verified by measurement of the decrease in stomatal conductance relative to control plants.

2.6 Sulphur dioxide fumigation

For the purpose of SO_2 fumigation experiments, airtight glass chambers, capable of accommodating two – three potted plants, were manufactured (Fig. 2.6). These chambers were placed in the controlled growth room containing the *A. capensis* plants.

A concentrated gas mixture containing SO_2 (certified at 1388 ppm), N_2 and CO_2 was purchased (Afrox - Special Gasses Division, Germiston, South Africa). Plants were placed in each chamber and concentrated SO_2 gas injected through a small port containing an airtight silicon septum to give average chamber SO_2 concentrations of 0.6 ppm or 1.2 ppm (Fig. 2.7). A small electric fan was also positioned inside the chamber to ensure thorough distribution of the SO_2 gas.

Figure 2.6 Glass chambers used for the fumigation of *A. capensis* plants with SO_2.

Figure 2.7 Injection of concentrated SO_2 gas into the glass chambers containing *A. capensis* plants. Plants were fumigated at chamber concentrations of 0.6 ppm and 1.2 ppm applied either in the light or dark. The SO_2 concentrations in the chambers were constantly monitored with a SO_2 spectrometer and concentrated gas injected when required.

The exact volume of SO_2 gas required for injection into the chambers was determined with an ultraviolet fluorescence SO_2 spectrometer (MLR 9850B, Monitor Europe) kindly provided by SASOL. In Fig. 2.8 the oscillations in chamber SO_2 concentration, as measured with the SO_2 spectrometer, during a typical fumigation experiment is shown.

Following injection into the glass chamber, the SO_2 concentration rapidly declined because of SO_2 uptake by the plants. Repeated injections of SO_2 (Fig. 2.8, arrows) were required to maintain the average chamber concentration (see black regression line) at the required level. For both SO_2 concentrations, the fumigation was first done during the night period. These experiments were later repeated on new sets of plants, but with SO_2 fumigation occurring during the light period. This allowed direct comparison of the physiological effects induced by night and daytime fumigation.

Pots were sealed with aluminium foil and parafilm before the start of each experiment to minimize the uptake of SO_2 by the soil.

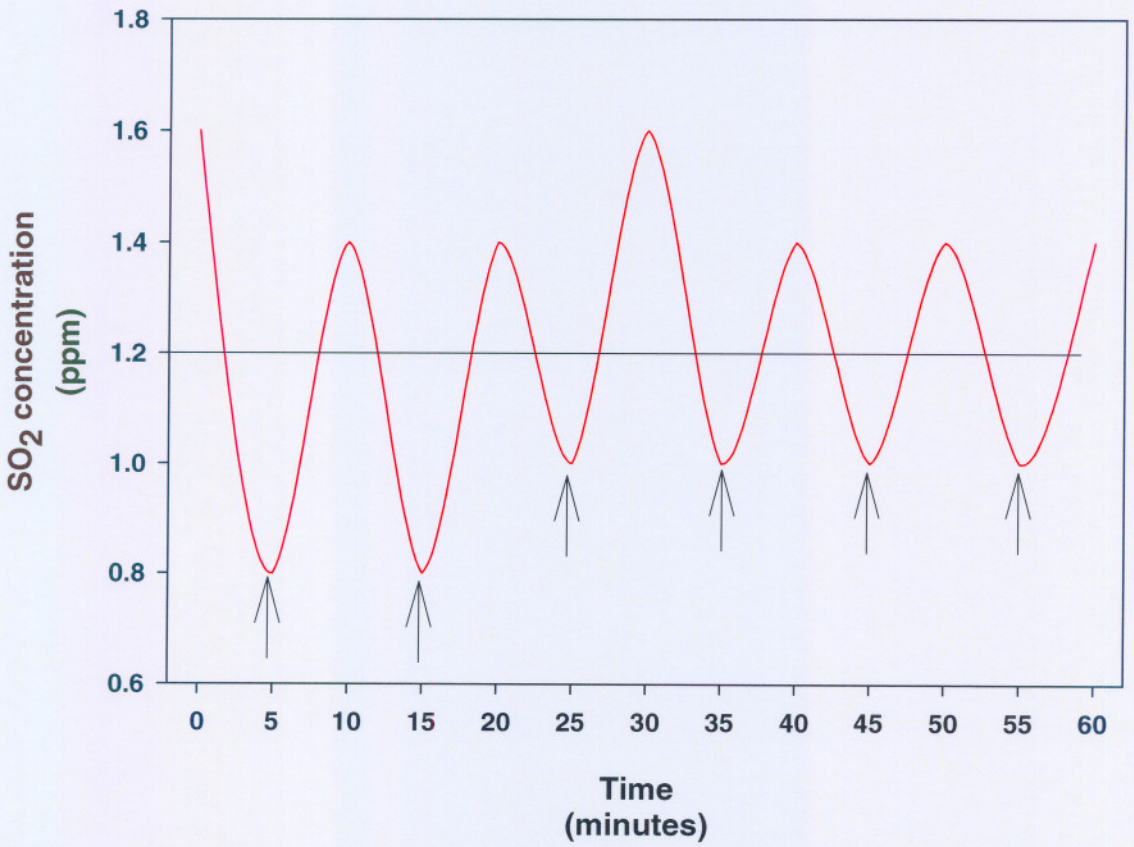


Figure 2.8 Typical oscillation in SO_2 concentration inside the glass chambers during a fumigation period of one hour. Arrows indicate the time points where concentrated SO_2 gas was injected into the chamber. The black regression line indicates the average SO_2 concentration (±1.2 ppm) maintained inside the chamber.

For the 0.6 ppm fumigation experiments, plants were exposed to SO_2 for up to three weeks for 3 h/day either during the dark or light period. After each 3-hour treatment, plants were removed from the glass chambers. Appropriate control plants were also incubated in a glass chamber but without exposure to SO_2, to ascertain any chamber effects on photosynthesis irrespective of the SO_2 treatment. For the fumigation experiments at 1.2 ppm SO_2, the same procedure was followed, except that the exposure time was reduced to one week for 3 h/day either during the dark or light period. In all experiments, the recovery capacity of the plants following the SO_2 fumigation period was also determined. At regular intervals during and following the

SO_2 fumigation treatments, the experimental procedures listed in table 2.2 were performed.

Because the sensitivity of *A. capensis* to SO_2 was completely unknown, and because of the reasonably basic (compared to open top chambers) SO_2 fumigation system used in these experiments, the effectiveness of the system was first verified using soybean as a model species. The effects of SO_2 fumigation on soybean are well known and detailed in the scientific literature (e.g. Verma & Agrawal, 1996; Deepak & Agrawal, 2001). The South African soybean genotype PAN 809 was used for this verification experiment. Soybean plants were cultivated in a growth chamber as described by Van Heerden *et al.* (2004). Following SO_2 fumigation, CO_2 assimilation and chlorophyll *a* fluorescence measurements were conducted on the soybean plants to assess the phytotoxic effects (see Chapter 4).

2.7 Simultaneous exposure to long-term water deprivation and SO_2

For the simultaneous exposure to water deprivation and SO_2, long-term mild water deprivation was first induced to simulate the situation in the natural environment more closely (see section 2.5 for method). After 40 days of water deprivation, three plants were selected and exposed to 1.2 ppm SO_2 in the light for 9 consecutive days as described in section 2.6. Another group of three plants were maintained in the water-deprived condition but without exposure to SO_2. The response to both these stress treatments was compared to that of well-watered plants (control treatment).

2.8 Non-destructive experimental procedures

2.8.1 Measurement of CO_2 assimilation

2.8.1.1 Overview of CO_2 assimilation kinetics

To study CO_2 assimilation in plants, a number of measurements, terms and units are employed (Von Caemmerer & Farquhar, 1981): The CO_2 assimilation rate (A) is expressed as the amount of CO_2 assimilated per unit leaf area and time (µmol CO_2 m^{-2} s^{-1}). The stomatal conductance (g_s) represents the flux of CO_2 through the stomata, with the same units as for A.

When a portable open-circuit photosynthesis system is used to measure CO_2 assimilation, air is pumped from the photosynthetic leaf chamber (PLC) enclosing a leaf into an infrared gas analyzer that continuously measures the CO_2 concentration in the air stream. The CO_2 concentration of the air stream will decrease if the leaf inside the PLC is assimilating CO_2. The CO_2 assimilation rate equals the change in the amount of CO_2 in the air stream per unit time. Changes in temperature and pressure are compensated for in the calculation of the CO_2 assimilation rate, but humidity has to be controlled, since a rise in transpiration will cause an increase in the amount of water vapour, resulting in dilution of CO_2 in the air stream (Long & Hällgren, 1993).

Carbon dioxide must first diffuse through the boundary layer on the leaf surface before it is able to enter the leaf. The boundary layer conductance will be at least an order of a magnitude greater than the highest possible g_s under field conditions (Long, 1985). In the Parkinson-type PLC (used in most portable photosynthesis systems) the boundary layer resistance is minimised and kept constant by the fan of the PLC, which keeps the turbulence high.

If only diffusion is taken into consideration, the intercellular CO_2 concentration (c_i) can be determined by:

$$c_i = c_a - A/g_1$$

where g_1 represents the total conductance, i.e. boundary layer as well as stomatal, and c_a is the atmospheric CO_2 concentration.

For a leaf enclosed in the PLC, CO_2 ($A:c_i$) response curves can be constructed by plotting A against c_i at a range of different c_a values (Fig. 2.9).

To determine the degree of stomatal limitation of photosynthesis, the following equation is employed:

$$l = (A_0 - A)/A_0$$

where A = the CO_2 assimilation rate at ambient CO_2 concentration (c_a = 350 µmol mol^{-1}) and A_0 = the CO_2 assimilation rate where no stomatal limitation is present ($c_i \geq$ 350 µmol mol^{-1}) (Farquhar & Sharkey, 1982). Stomatal limitation (l) represents the proportionate decrease in CO_2 assimilation rate that may be attributed to stomatal restrictions.

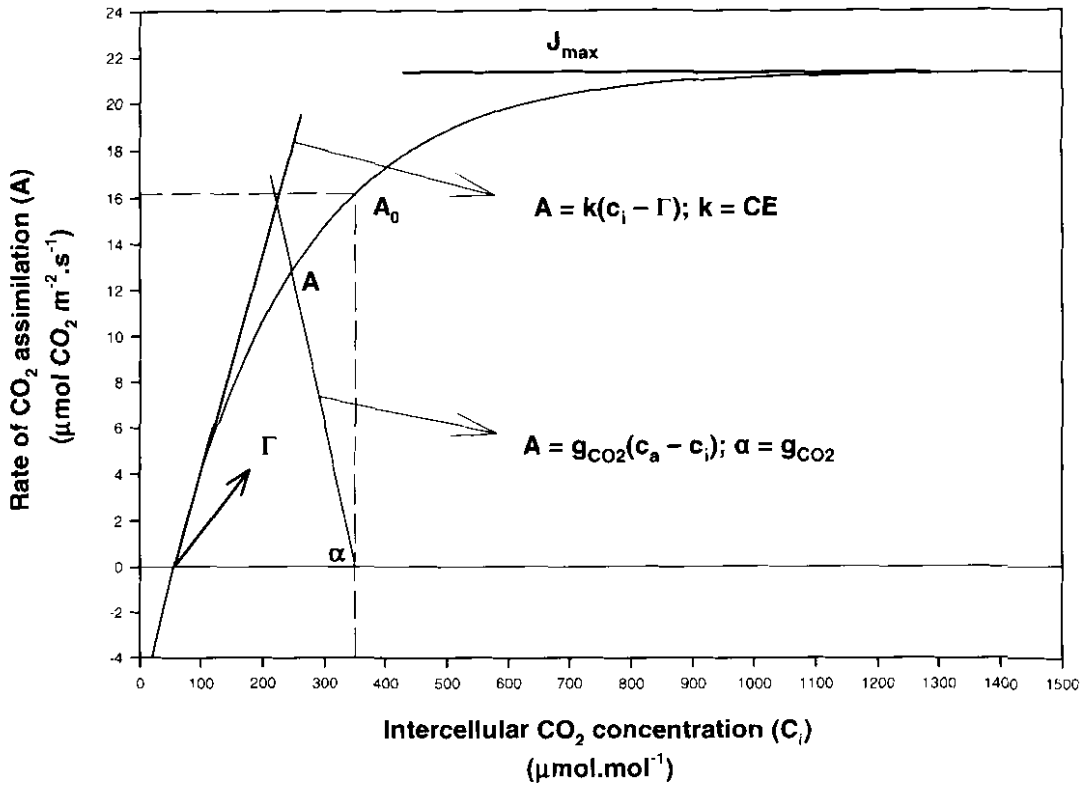


Figure 2.9 Response of CO_2 assimilation rate (A) against intercellular CO_2 concentration (c_i). A is the CO_2 assimilation rate under the given conditions, i.e. the point of simultaneous solution of the demand and supply functions. Carboxylation efficiency (CE) is represented by the initial slope of the demand function, the CO_2 compensation concentration (Γ) is the intercellular CO_2 level where the net usage of CO_2 equals zero, the maximum rate of assimilation (J_{max}) represents the rate of CO_2 assimilation at saturated levels of CO_2, and A_0 is the rate of assimilation that occurs when no stomatal limitations are present (interpolation of the value of A from the response curve at c_i = 350 µmol mol^{-1}).

The carboxylation efficiency of photosynthesis can be deduced from the initial linear response ($\delta A/\delta c_i$) of the A:c_i response curve which is an *in vivo* measure of Rubisco activity (Bolhàr-Nordenkampf & Öquist, 1993). The maximal CO_2 assimilation rate at saturating c_i (J_{max}) can be regarded as a reliable indicator of the RuBP regeneration capacity of the leaf.

2.8.1.2 CO_2 assimilation measurements on *A. capensis*

Carbon dioxide assimilation was measured at regular intervals on leaves of *A. capensis* plants to assess the effects of the various stress treatments (Table 2.2).

Measurements were conducted with an open-circuit portable photosynthesis system (CIRAS-I, PP-Systems, Hertz, UK). Due to the succulent morphology of the plants, measurements on individual leaves with a standard broad-leaf PLC were not possible. Instead, measurements were conducted on an attached twig (carrying several succulent leaves) of each plant. For this purpose a modified conifer-type PLC (Fig. 2.10), capable of accommodating small twigs, was custom manufactured (PP-systems, Herts, UK).

Figure 2.10 Custom-manufactured modified conifer-type photosynthetic leaf chamber and portable photosynthesis system used for measuring CO_2 assimilation in *A. capensis*

Measurements were conducted at a leaf temperature of 26°C. A twig of each plant was clamped in the PLC with built-in light and temperature control. A small thermistor was carefully attached (with a small piece of clear adhesive tape) to a leaf within the PLC for the purpose of accurate leaf temperature determinations. Humidity in the PLC was maintained close to ambient conditions. Irradiance during measurements was controlled at 1200 photons µmol m^{-2} s^{-1} to ensure full activation of Rubisco (the primary enzyme involved in CO_2 assimilation). Whilst in the PLC, each twig was exposed to a series of increasing c_a levels. At each c_a level the ratio of A to c_i was automatically recorded. By increasing c_a at 6-min intervals from 0 to 2000 µmol mol^{-1}, A:c_i response curves were generated for each twig. The initial slope of the demand function ($\delta A/\delta c_i$) was computed by linear regression analysis. All other calculations were done according to Farquhar & Sharkey (1982) and Chaves (1991) as explained

in section 2.8.1.1. The total surface area of the cylindrical leaves clamped in the PLC was estimated with the following formula and used in the calculation of the various CO_2 assimilation parameters:

$$\text{Surface area } (cm^2) = 2\Pi rl$$

where r = the average radius of each leaf (cm) and l = length of each leaf (cm)

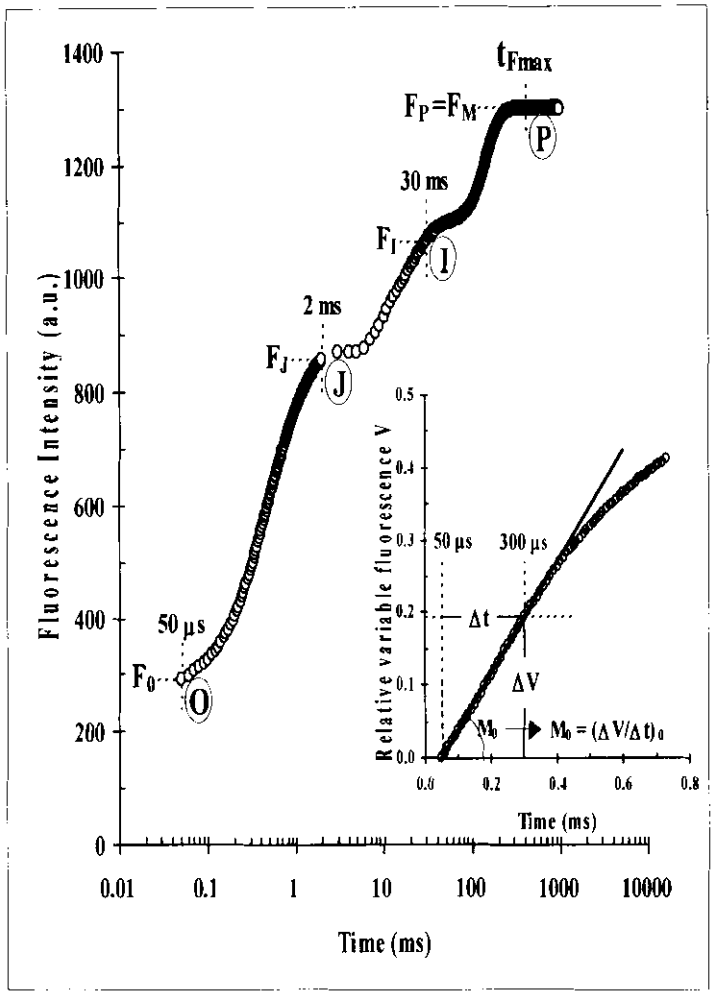
2.8.2 Measurement of chlorophyll *a* fluorescence

2.8.2.1 The polyphasic chlorophyll *a* fluorescence transient

When a dark-adapted leaf is illuminated with a saturated light pulse, characteristic changes in the intensity of chlorophyll *a* fluorescence, known as the Kautsky transient, are observed (Kautsky & Hirsch, 1931). The Kautsky transient shows a fast rise completed in less than one second, with a subsequent slower decline towards a steady state. It is postulated that the rising phase of the transient reflects the primary reactions of photosynthesis (Krause & Weis, 1991). With the development of fluorimeters with high time resolution, additional and more accurate information about the kinetics of these transients was obtained (Schreiber & Neubauer, 1987; Strasser & Govindjee, 1992; Strasser *et al.*, 1995). For example, it was demonstrated that the fluorescence rise kinetics of the Kautsky transient is polyphasic when plotted on a logarithmic time scale (Fig. 2.11), clearly exhibiting the steps J and I (Strasser & Govindjee, 1992) or I_1 and I_2 (Schreiber & Neubauer, 1987) between the initial O (F_0) and maximum P level ($F_P = F_M$).

Upon excitation with a saturated light pulse, there is a rapid initial rise in fluorescence intensity from O to the first intermediate step J within ca. 2 ms. This phase is followed by a further rise to the second intermediate step I within ca. 30 ms and to the final peak P in ca. 200 ms. The O-J-I-P fluorescence transient reflects the filling up of the electron acceptor side of PSII (Q_A, Q_B and PQ pool) with electrons from the donor side of PSII (Papageorgiou, 1975; Lavorel & Etienne, 1977; Strasser & Govindjee, 1992). The relationship of these events to the O-J-I-P fluorescence transient was suggested by Strasser *et al.* (1995) to be the following: O, minimal Chl *a* fluorescence yield (highest yield of photochemistry); O to J, reduction of Q_A to Q_A^- (photochemical phase, light intensity dependent); J to I to P, reduction of the PQ pool (non-photochemical phase). Since the O-J-I-P fluorescence transient reflects the kinetics

and heterogeneity involved in the filling up of the PQ pool with electrons, it can be used as a sensitive tool to investigate the photosynthetic apparatus *in vivo* (Strasser *et al.*, 1995). The shape of the O-J-I-P fluorescence transient has been found to be very sensitive to various types of stress (Krüger *et al.*, 1997; Lazár & Ilík, 1997; Tsimilli-Michael *et al.*, 1999; Strauss *et al.*, 2006).



Figue 2.11 An example of a typical polyphasic chlorophyll *a* fluorescence transient O-J-I-P emitted by higher plants. The transient is plotted on a logarithmic time scale from 50 µs to 1 s. The labels refer to the fluorescence data used by the JIP-test (see section 2.8.2.2) for the calculation of various parameters quantifying PSII structure and function. The labels are: the fluorescence intensity F_0 (at 50 µs); the fluorescence intensity F_J (at 2 ms); the fluorescence intensity F_I (at 30 ms) and the maximal fluorescence intensity $F_P = F_M$. The figure insert shows the transient expressed as the relative variable fluorescence, $V = (F - F_0)/(F_M - F_0)$, on a linear time-scale and demonstrates how the initial slope (M_0) is calculated: $M_0 = (D_V/D_t)_0 = (V_{300\mu s})/(0.25\ ms)$. (From Tsimilli-Michael *et al.*, 2000).

2.8.2.2 Analysis of the chlorophyll *a* fluorescence transient by the JIP-test

The O-J-I-P fluorescence transient is rich in information and can be used to derive a number of parameters (refer to Table 2.3) by the so-called JIP-test. The following data from the original measurements are used by the JIP-test: maximal fluorescence intensity (F_M); fluorescence intensity at 50 µs (considered as F_0); fluorescence intensity at 300 µs ($F_{300\mu s}$) required for calculation of the initial slope (M_0) of the relative variable fluorescence (V) kinetics; and the fluorescence intensity at 2 ms (the J step) denoted as F_J (refer to Fig. 2.11 for an explanation).

The JIP-test represents a translation of the original fluorescence data to biophysical parameters that quantify the stepwise flow of energy through PSII at the reaction center (RC) as well as excited cross-section (CS) level (Strasser & Strasser, 1995; Force *et al.*, 2003; Strasser *et al.*, 2004). The parameters which all refer to time zero (onset of fluorescence induction) are: (i) the specific energy fluxes (per reaction centre) for absorption (ABS/RC), trapping (TR_o/RC), dissipation at the level of the antenna chlorophylls (DI_o/RC) and electron transport (ET_o/RC); (ii) the flux ratios or yields, i.e. the maximum quantum yield of primary photochemistry ($\varphi_{Po} = TR_o/ABS = F_V/F_M$), the efficiency ($\psi_o = ET_o/TR_o$) with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-, the quantum yield of electron transport ($\varphi_{Eo} = ET_o/ABS = \varphi_{Po} \cdot \psi_o$); (iii) the phenomenological energy fluxes (per excited cross section, CS) for absorption (ABS/CS), trapping (TR_o/CS), dissipation (DI_o/CS) and electron transport (ET_o/CS). The fraction of active PSII reaction centres per excited cross section (RC/CS) is also calculated. The formulae in Table 2.3 illustrate how each of the above-mentioned biophysical parameters can be calculated from the original fluorescence measurements.

The initial stage of photosynthetic activity of a RC complex is regulated by three functional steps namely absorption of light energy (ABS), trapping of excitation energy (TR) and conversion of excitation energy to electron transport (ET). Strasser *et al.* (2000) introduced a multi-parametric expression of these three independent steps contributing to photosynthesis, the so-called performance index (PI_{ABS}):

$$PI_{ABS} = \frac{\gamma}{1-\gamma} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_o}{1-\psi_o}$$

where γ is the fraction of reaction centre chlorophyll (Chl_{RC}) per total chlorophyll ($Chl_{RC+Antenna}$). Therefore $\gamma/(1-\gamma) = Chl_{RC}/Chl_{Antenna}$ = RC/ABS. This expression can be de-convoluted into two JIP-test parameters and estimated from the original fluorescence measurements as $RC/ABS = RC/TR_o \bullet TR_o/ABS = [(F_{2ms} - F_{50\mu s})/4(F_{300\mu s} - F_{50\mu s})] \bullet F_V/F_M$. The factor 4 is used to express the initial fluorescence rise per 1 ms. The expression RC/ABS shows the contribution to the PI_{ABS} due to the RC density on a chlorophyll basis. The contribution of the light reactions for primary photochemistry are estimated according to the JIP-test as $[\varphi_{Po}/(1-\varphi_{Po})] = TR_o/DI_o = k_P/k_N = F_V/F_o$. The contribution of the dark reactions are derived as $[\psi_o/(1-\psi_o)] = ET_o/(TR_o - ET_o) = (F_M - F_{2ms})/(F_{2ms} - F_{50\mu s})$. The JIP-test reveals changes in the behaviour of PSII that cannot be detected by the commonly used $\varphi_{P0} = F_V/F_M$, which is the least sensitive of all parameters.

Table 2.3 Summary of the JIP-test formulae using data extracted from the chlorophyll *a* fluorescence transient O-J-I-P (From Strauss *et al.*, 2006) (also see Fig. 2.11).

Extracted and Technical Fluorescence Parameters		
F_0	=	**$F_{50\mu s}$, fluorescence intensity at 50µs**
$F_{100\mu s}$	=	**fluorescence intensity at 100µs**
$F_{300\mu s}$	=	**fluorescence intensity at 300µs**
F_J	=	**fluorescence intensity at the J-step (at 2ms)**
F_I	=	**fluorescence intensity at the I-step (at 30ms)**
F_M	=	**maximal fluorescence intensity**
t_{FM}	=	**time to reach F_M, in ms**
V_J	=	**relative variable fluorescence at the J-step = $(F_{2ms} - F_0) / (F_M - F_0)$**
$(dV / dt)_0 = M_0$	=	**fractional rate of PS II reaction centre closure = 4 . $(F_{300} - F_0)/(F_M - F_0)$**
Quantum Efficiencies or Flux Ratios or yields		
$\varphi_{Po} = TR_0 / ABS$	=	$[1 - (F_0 / F_M)] = F_V / F_M$
$\varphi_{Eo} = ET_0 / ABS$	=	$[1 - (F_0 / F_M)] . \Psi_0$
$\Psi_0 = ET_0 / TR_0$	=	$(1 - V_J)$

Specific Fluxes or Specific Activities		
ABS / RC	=	$M_0 . (1 / V_J) . (1 / \varphi_{Po})$
TR_0 / RC	=	$M_0 . (1 / V_J)$
ET_0 / RC	=	$M_0 . (1 / V_J) . \Psi_0$
DI_0 / RC	=	(ABS / RC) - (TR_0 / RC)
Phenomenological Fluxes or Phenomenological Activities		
ABS / CS	=	ABS / CS_{Chl} = Chl / CS or ABS / CS_0 = F_0 or ABS / CS_M = F_M
TR_0 / CS	=	$\varphi_{Po} . (ABS / CS)$
ET_0 / CS	=	$\varphi_{Po} . \Psi_0 . (ABS / CS)$
DI_0 / CS	=	(ABS / CS) - (TR_0 / CS)
Density of Reaction Centres		
RC / CS	=	$\varphi_{Po} . (V_J / M_0) . ABS / CS$
Performance Indexes		
PI_{ABS}	=	$(RC / ABS) . [\varphi_{Po} / (1 - \varphi_{Po})] . [\Psi_0 / (1 - \Psi_0)]$

ABS, absorption energy flux; CS, excited cross section of leaf sample; DI, dissipation energy flux at the level of the antenna chlorophylls; ET, flux of electrons from Q_A^- into the electron transport chain; φ_{Do}, quantum yield of dissipation; φ_{Eo}, probability that an absorbed photon will move an electron into electron transport further than Q_A^-; φ_{Po}, maximum quantum yield of primary photochemistry; PI_{ABS}, performance index; ψ_o, efficiency by which a trapped exciton, having triggered the reduction of Q_A to Q_A^-, can move an electron further than Q_A^- into the electron transport chain; RC, reaction centre of PSII; RC/CS, fraction of active reaction centres per excited cross section of leaf; TR, excitation energy flux trapped by a RC and utilized for the reduction of Q_A to Q_A^-.

2.8.2.3 Chlorophyll *a* fluorescence measurements on *A. capensis*

Chlorophyll *a* fluorescence was measured at regular intervals on young fully expanded leaves of *A. capensis* plants to assess the effects of the various stress treatments (Table 2.2). Polyphasic OJIP fluorescence transients were recorded during the dark period in the growth chamber at 20°C under dim green light with a Plant Efficiency Analyser (PEA, Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 30 4NE, UK). The transients were induced by a red light (peak at 650 nm) of 3200 µmol photons m^{-2} s^{-1} (sufficient excitation intensity to ensure closure of all PSII reaction centers to obtain a true fluorescence intensity of F_M) provided by the PEA through an array of six light-emitting diodes. All measurements were conducted on fully dark-adapted attached leaves. In the case of field measurements, the same measuring protocol was followed, but detached twigs containing sufficient leaves

were used (as explained in section 2.1). The recorded OJIP transients were subsequently analysed by the JIP test (explained fully in section 2.8.2.2).

2.9 Destructive experimental procedures

2.9.1 Measurement of Rubisco activity

Leaves were harvested at regular intervals to assess the effects of the various stress treatments on Rubisco activity (Table 2.2). Leaves were harvested under full illumination and immediately frozen in liquid nitrogen. Special care was taken not to shade the leaves during harvesting thereby preventing deactivation of Rubisco. Initial and total Rubisco activity was determined in harvested leaves according to the method of Keys & Parry (1990). Initial activity is the activity of the enzyme under the growth conditions at the time of sampling; total activity is obtained after prior activation of the extracted enzyme with bicarbonate.

Each frozen leaf sample was weighed and ground to a fine powder with liquid nitrogen in a pre-cooled mortar and rapidly extracted with 3ml ice-cold extraction buffer containing 100 mM Bicine-NaOH (pH 8.0), 20 mM $MgCl_2$, 50 mM β-mercaptoethanol, 5 mM phenylmethylsulfonyl fluoride (PMSF) and 30 mg insoluble polyvinylpolypyrrolidone (PVPP). An aliquot of the crude extract was transferred to a pre-cooled micro-centrifuge tube and centrifuged at 10 000 x g at 4 °C for 1 min.

The clarified supernatant was immediately used for the measurement of initial Rubisco activity. The time between extraction and the start of the measurement was not more than 2 min. Initial Rubisco activity was measured in a total volume of 0.5 ml containing 100 mM Bicine-NaOH (pH 8.2), 20 mM $MgCl_2$. 10 mM $NaH^{14}CO_3$ (0.5 μCi μmol^{-1}), 400 μM RuBP and 25 μl clarified supernatant. After 1 min the reaction was terminated by addition of 0.2 ml 10 M formic acid. Total Rubisco activity was measured after a 3 min incubation of 25 μl clarified supernatant in the above reaction mixture in the absence of RuBP to fully activate the enzyme. The reaction was then started by the addition of RuBP and terminated after 1 min with formic acid. Acidified samples were evaporated to dryness in an oven at 60 °C. After addition of 3.5 ml scintillation cocktail to each vial, the ^{14}C incorporated into 3-phosphoglycerate was determined using a liquid scintillation counter (Beckmann, Model LS6000TA). The

soluble protein content of the supernatant was determined according to the method of Bradford (1976).

2.9.2 Leaf ultrastructure

Leaves were collected and cut into small sections. For transmission electron microscopy, sections of young fully expanded leaves were fixated in glutaraldehyde (Karnovsky, 1965) in cacodilate buffer (pH 7.4) for 4 h followed by secondary fixation in 0.5% aqueous osmium tetroxide for 1 h. After pre-casting with 2% uranyl acetate for 30 min and dehydration in a series of acetone solutions, the material was infiltrated with resin (Spurr, 1969). Ultra thin sections was made with a Reichert Ultracut E microtome and contrasted with uranyl acetate and lead citrate (Reynolds, 1963). Sections were investigated with a Philips CM 10 transmission electron microscope.

2.10 Statistical analysis

Statistical analysis was conducted with the software package Statistica for Windows version 6 (StatSoft, Inc. 2300 East 14th Street, Tulsa OK 74104, USA). Normal distribution of data was determined using the Shapiro-Wilk W test (Shapiro *et al.*, 1968). In data sets with parametric distribution, significant differences between treatment means were determined using Student's t-test. In data sets with non-parametric distribution, significant differences between treatment means were determined with the Mann-Whitney U-test (Mann and Whitney, 1947).

CHAPTER 3

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES TO CHANGES IN WATER AVAILABILITY IN TWO NAMIB DESERT SUCCULENTS

3.1 Introduction

Concern was expressed about the possible impacts of mining activity (SO_2 pollution, excessive dust deposition etc.) on the vegetation in the vicinity of Skorpion Zinc mine. A long-term ecophysiological monitoring program was establised to investigate this possibility. The first step, however, was to characterize the effects of water availability on the vegetation. This information was required to enable distinction between effects caused by low rainfall on the one hand, and mining activity on the other hand. Fifteen measuring sites were selected in the vicinity of Skorpion Zinc mine. During each of four visits, chlorophyll *a* fluorescence measurements were conducted on *Augea capensis* and *Zygophyllum prismatocarpum* plants at each of these sites. For laboratory investigations, *A. capensis* plants were transplanted from the desert environment to pots and transferred to a growth chamber. Potted plants received no water for a period of up to fifteen days for assessment of short-term water deprivation effects. Besides chlorophyll *a* fluorescence, photosynthetic gas exchange parameters and Rubisco activity were also measured in these plants. After the water deprivation treatment, plants were re-watered and their recovery capacity assessed. Experiments where plants were exposed to long-term water deprivation were also conducted to simulate the situation in a desert environment more closely.

3.2 Results

3.2.1 Water availability under field conditions

3.2.1.1 Effects on PS II function

The changes that occurred in the PI_{ABS} in leaves of *A. capensis* and *Z. prismatocarpum* plants in the vicinity of Skorpion Zinc Mine during the period December 2002 – April 2004 are shown in Figure 3.1. The data shows that both species had low PI_{ABS} values during periods of low rainfall (e.g. December 2002, May 2003 & April 2004) and that values in *Z. prismatocarpum* were consistently lower than for *A. capensis*. One month following 31.6 mm of rainfall (September 2003), however, the PI_{ABS} values in *A. capensis* and *Z. prismatocarpum* were respectively 231% and 507% higher than those recorded during May 2003. Subsequently, in the absence of further significant rainfall (September 2003 – April 2004), the PI_{ABS} values in both species decreased again over time.

To determine the precise effects of rainfall on PS II function, data collected at time points representative of dry (Fig. 3.1, May 2003 - red arrows) and wet periods (Fig. 3.1, September 2003 - green arrows) were analyzed in more detail. In Figure 3.2 A & E original O-J-I-P fluorescence transients recorded in leaves of *A. capensis* and *Z. prismatocarpum* plants during May 2003 (red transients) and September 2003 (green transients) are shown. The transients recorded during May differed in two ways from those recorded during September. Firstly, the actual fluorescence intensities of the transients were different. For example, in both species the fluorescence intensities between 0.05 ms and 30 ms were considerably higher in the May transients. Beyond 30 ms this effect was maintained in *A. capensis*, whereas in *Z. prismatocarpum*, fluorescence intensities were lower than those of the September transients. Secondly, the fast fluorescence rise kinetics of the May and September transients are different.

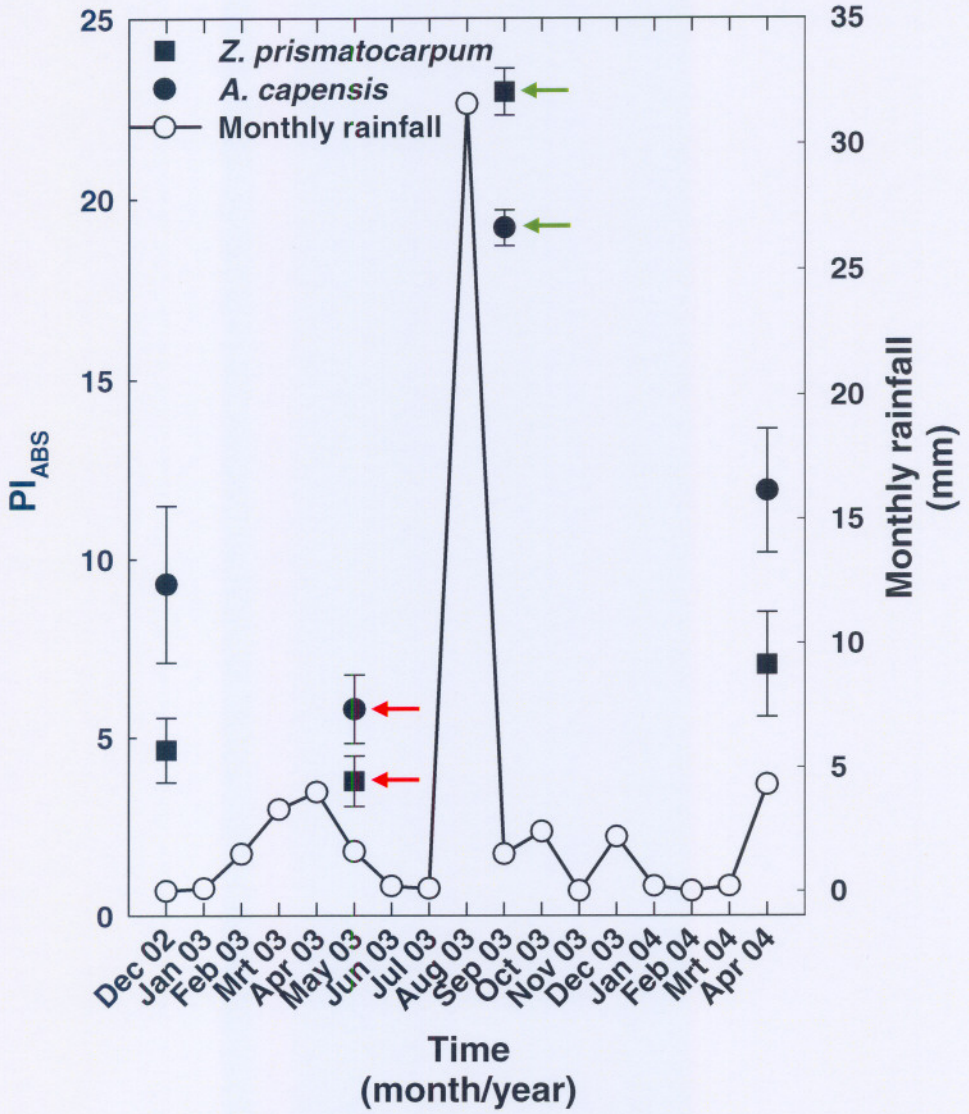


Figure 3.1 Performance index (PI_{ABS}) values recorded for *A. capensis* (closed circles) and *Z. prismatocarpum* (closed squares) in the vicinity of Skorpion Zinc mine during December 2002 – April 2004. Values shown represent the mean ± S.E. of 450 measurements conducted at the 15 measuring sites (30 measurements at each site). Monthly rainfall values during the period of investigation are also shown (open circles). Red (low rainfall period) and green (wet period) arrows indicate the measurements used for a detailed investigation about the effects of rainfall (31.6 mm during August 2003) on PS II function (Figures 3.2 and 3.3).

To reveal changes in the fluorescence rise kinetics more clearly, the original O-J-I-P transients were normalised between the two fluorescence extremes O (F_o) and P (F_M). In both species the normalised transients recorded during May were

characterized by higher fluorescence intensities over the entire time range between O and P (Fig. 3.2 B & F). Since the J-step (2 ms) represents the transition point between the light-dependent (O to J) and light-independent (J to I to P) reactions involved in PS II photochemistry (Strasser *et al.*, 1995; Lazár 2006), transients were also normalized between O and J (Fig. 3.2 C & G). Normalisation of transients in this fashion revealed that drought modified the shape of the transients not only between O and J but also between J and P, and that in both species, these modifications were remarkably similar.

Difference in variable fluorescence (ΔV) curves were constructed by subtraction of the normalised (O to P) fluorescence intensity values of the September transients from the May transients (Fig. 3.2 D & H). The green zero lines represent the values obtained at each time point after subtraction of the fluorescence intensities of September transients from them self. The very large positive deviations (increases in fluorescence intensity) in the May transients (red) of both species can clearly be seen. In particular the distinct increase at ca. 300 μs (ΔK peak, Strasser *et al.* 2000; Lazár 2006) must be noted. Although both species responded similarly, the ΔK peak was more pronounced in *Z. prismatocarpum*, and in addition, another peak (between 5 – 10 ms), of similar magnitude to the ΔK peak, could also be seen (Fig. 3.2 H).

The O-J-I-P fluorescence transients shown in Fig. 3.2 were analysed further according to the JIP-test protocol. The effects of water availability on the maximum quantum yield of primary photochemistry ($F_V/F_M = \varphi_{Po}$) and the specific (per RC) and phenomenological (per excited CS) energy fluxes for light absorption, excitation energy trapping and electron transport are presented in the form of radar plots (Fig. 3.3 A & B). In these plots all the parameter values calculated from the September 2003 measurements were normalised to the numeric value of one (green line) in order to show the fractional increase or decrease in the parameter values of the May 2003 measurements (red line). During periods of low rainfall PS II function was down-regulated in both species through deactivation of reaction centers (RC/CS) with associated decreases in excitation energy trapping (TR_o/CS) and electron transport (ET_o/CS) per excited cross section of leaf. The maximum quantum yield of primary photochemistry (F_V/F_M) decreased to a lesser extent. Acclimation responses to water availability were observed in the form of large increases in apparent antenna size (ABS/RC) and excitation energy trapping (TR_o/RC). These compensatory mechanisms resulted in maintenance of normal electron transport flux per remaining

functional (active) reaction center (ET_o/RC) in both species. The pattern and magnitude of low rainfall-induced changes in these parameters were very similar for both species.

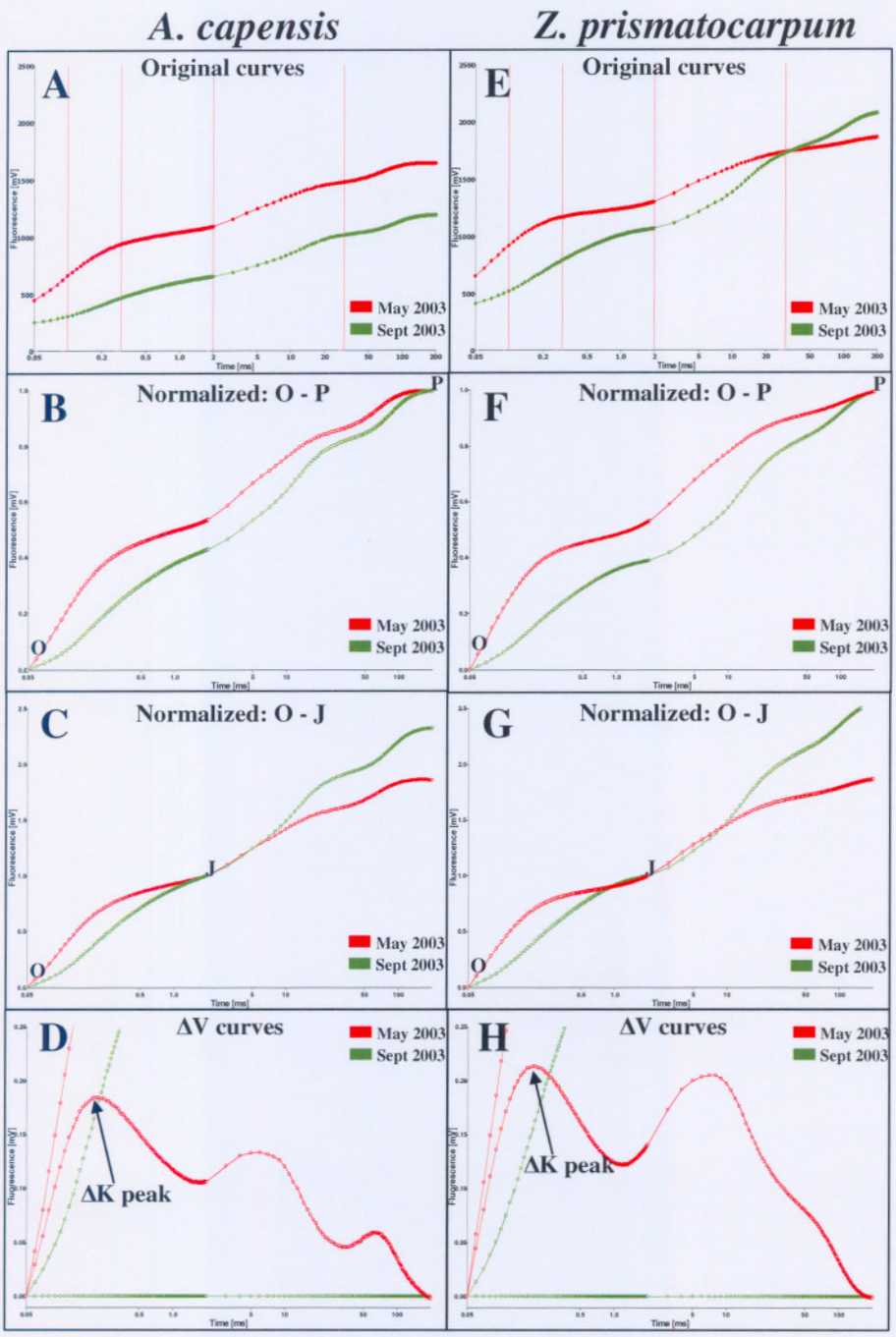


Figure 3.2 **A & E:** Polyphasic chlorophyll *a* fluorescence O-J-I-P transients recorded in the two species *A. capensis* (left hand) and *Z. prismatocarpum* (right hand) during May 2003 (red transients, low rainfall period) and September 2003 (green transients, wet period). Each transient represents the mean of 450 measurements conducted at the 15 measuring sites (30 measurements at each site). For the sake of clarity, S.E. bars are not shown but variation did not

exceed 20% of the mean values. **B & F:** Transients normalised between the fluorescence extremes O (F_o) and P (F_M). **C & G:** Transients normalised between O and J (2 ms). **D & H:** ΔV curves constructed by subtraction of the normalised (O to P) fluorescence values recorded during September 2003 from those recorded during May 2003. The green zero lines represent the values obtained at each time point after subtraction of the fluorescence intensities of September transients from them self. The position of the ΔK peak (at ca. 300 µs), which appeared in both species in response to low rainfall, is indicated by the arrows.

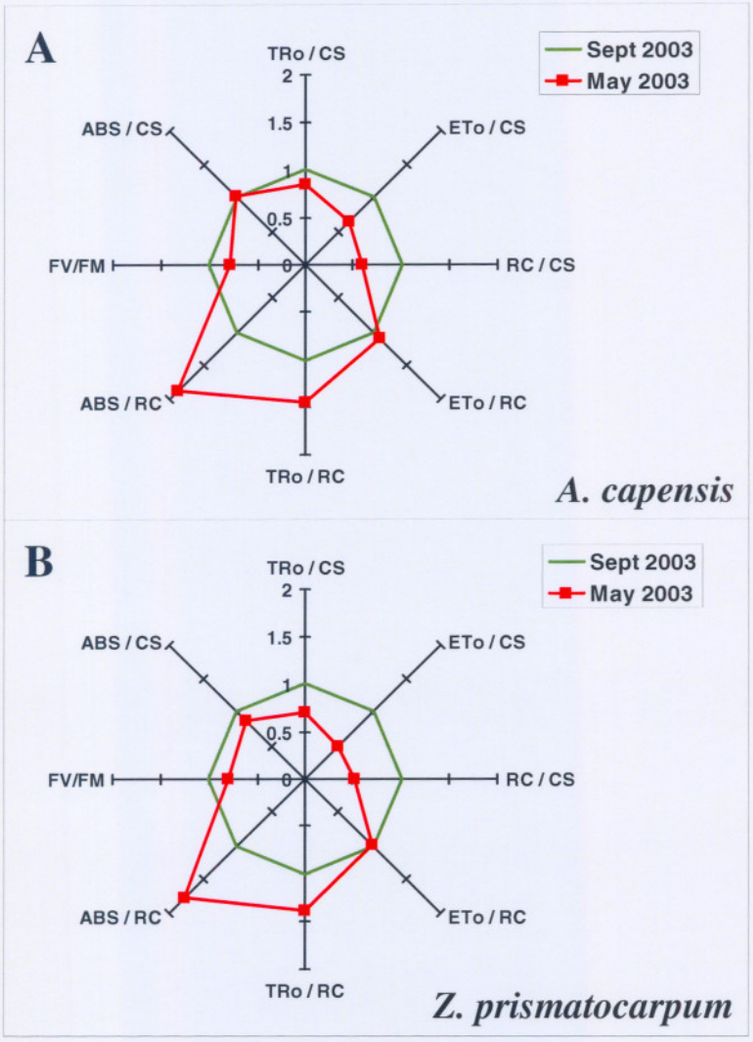


Figure 3.3 Radar plots visualising the fractional changes that occurred in the May 2003 values (red lines) of RC/CS, ET_o/CS, TR_o/CS, ABS/CS, F_V/F_M, ABS/RC, TR_o/RC and ET_o/RC relative to the September 2003 values (green lines). **(A)** *A. capensis*; **(B)** *Z. prismatocarpum*.

3.2.2 Short-term water deprivation under laboratory conditions

3.2.2.1 Effects on PS II function

Transplantation of *Z. prismatocarpum* from the desert environment to pots proved to be difficult and the survival rate of potted plants were also very low. On the other hand, *A. capensis* readily grew in pots. As a consequence all laboratory investigations were conducted on *A. capensis* only.

In Fig. 3.4 the PI_{ABS} values (red circles) measured in potted *A. capensis* plants after 0 (last day of watering), 5, 10 and 15 days of water deprivation, and again 7 days (day 22) after re-watering, are shown. The PI_{ABS} values (green circles) in plants that received water regularly (control) are also shown. The PI_{ABS} values measured in both sets of plants at the beginning of the experiment (day 0), and in control plants throughout the experiment, were of similar order of magnitude to values measured in *A. capensis* under field conditions during September 2003 (Fig. 3.1, wet period). Similarly, the PI_{ABS} values in potted plants after 15 days of water deprivation were similar to the May 2003 (Fig. 3.1, low rainfall period) values measured in the field. The data illustrates clearly how the induction of short-term water deprivation under laboratory conditions resulted in a progressive decline in PI_{ABS} values with increasing time duration, and how the values in re-watered plants increased again to levels comparable to those measured at the beginning of the experiment (day 0). A statistically significant reduction in the PI_{ABS} values of 26% and 63% was only observed after 10 and 15 days of water deprivation respectively.

For comparison of short-term water deprivation effects in the laboratory experiments with those under field conditions, O-J-I-P fluorescence transients recorded for control and water-deprived plants at the respective time points (days 0, 5, 10, 15 and 22) were analyzed in the same fashion as for the field experiments (see Fig. 3.2). In Figure 3.5 A the original O-J-I-P fluorescence transients recorded at each time point are shown. The increase in fluorescence intensities in plants after 10 (dark-brown transient) and 15 (red transient) days of water deprivation, and the large opposite response after re-watering (light-green transient), can clearly be seen. The original O-J-I-P transients were also normalised between the two fluorescence extremes O and P (Fig. 3.5 B). Similar to the field observations (Fig. 3.2 B), short-term water deprivation under laboratory conditions led to increases in fluorescence intensities over the entire time range between the two fluorescence extremes. A clear pattern

was visible with the smallest, intermediate and largest increases in fluorescence intensity obtained after 5 (light-brown transient), 10 (dark-brown transient) and 15 (red transient) days of water deprivation respectively. Normalisation of transients between O and J (Fig. 3.5 C) revealed that water deprivation only slightly modified the shape of transients before the J step. Similar to observations under field conditions (Fig. 3.2 C), the main effects of short-term water deprivation were also localized after the J step. In this respect, a clear time-dependent increase (day 5 < day 10 < day 15) in the deviation of maximal fluorescence intensity values from those of control plants (dark-green transient) was observed. The large degree of recovery in re-watered plants (light-green transient) can also be seen.

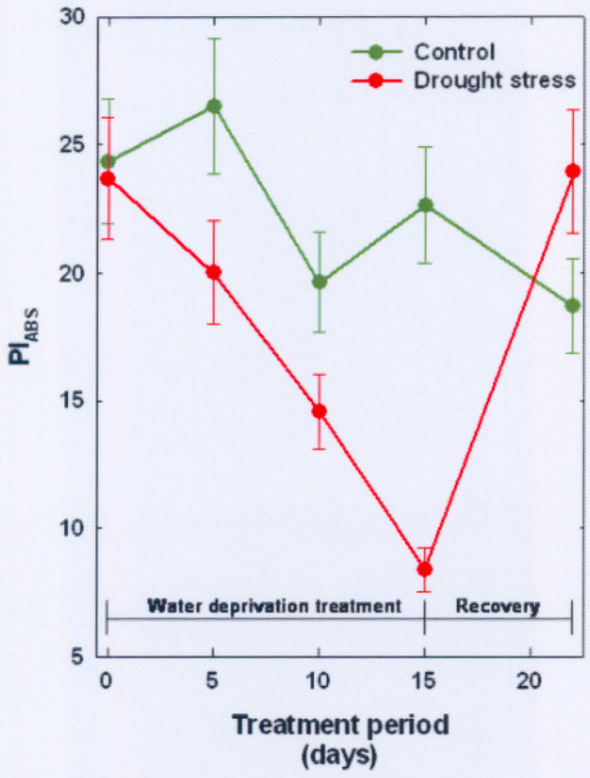


Figure 3.4 Performance index (PI_{ABS}) values recorded in control (green circles) and water-deprived (red circles) plants of *A. capensis*. Values after 0, 5, 10 and 15 days of water deprivation and 7 days following re-watering (day 22) are shown. Each value represents the mean ± S.E of measurements conducted in three individual plants. The water deprivation and recovery (following re-watering) periods are indicated by labels above the x-axis.

Difference in variable fluorescence (ΔV) curves were again constructed by subtraction of the normalised (O to P) fluorescence intensity values of the transients recorded on day 0 from those recorded after 5, 10 and 15 days of water deprivation (Fig. 3.5 D). The green zero line represents the values obtained at each time interval after subtraction of the fluorescence intensities at day 0 from them self. The positive deviations (increases in fluorescence intensity), which again occurred in a time-dependent fashion in response to water deprivation, can clearly be seen. Although there was a general (between 0.05 ms and 200 ms) increase in fluorescence intensity in water-deprived plants, the degree of deviation was smaller than under field conditions. In addition, no clearly defined peaks in fluorescence intensity (e.g. ΔK peak at ca. 300 μs), as observed under field conditions (Fig. 3.2 D), could be distinguished.

The effects of short-term water deprivation under laboratory conditions on the maximum quantum yield of primary photochemistry ($F_V/F_M = \varphi_{Po}$) and the specific and phenomenological energy fluxes for light absorption, excitation energy trapping and electron transport are also presented in the form of a radar plot (Fig. 3.6). In this plot all the parameters values calculated from the measurements conducted in control plants (15 days after initiation of experiment) were normalised to the numeric value of one (green line) in order to show the fractional increase or decrease in the parameter values of the measurements conducted in plants after 15 days of water deprivation (red line). Water deprivation resulted in the down-regulation of PS II function. This down-regulation is indicated by the large deactivation of reaction centers (RC/CS), decreased excitation energy trapping (TR_o/CS) and electron transport (ET_o/CS) with an accompanying small decrease in F_V/F_M. Increases in apparent antenna size (ABS/RC) and excitation energy trapping (TR_o/RC) enabled maintenance of normal electron transport flux per functional (active) reaction center (ET_o/RC). Of major significance is the observation that the effects of water deprivation under laboratory conditions on these JIP-test parameters were very similar to those under field conditions (Fig 3.3 A). Basically, the only difference was that the changes observed under field conditions were more pronounced than under laboratory conditions.

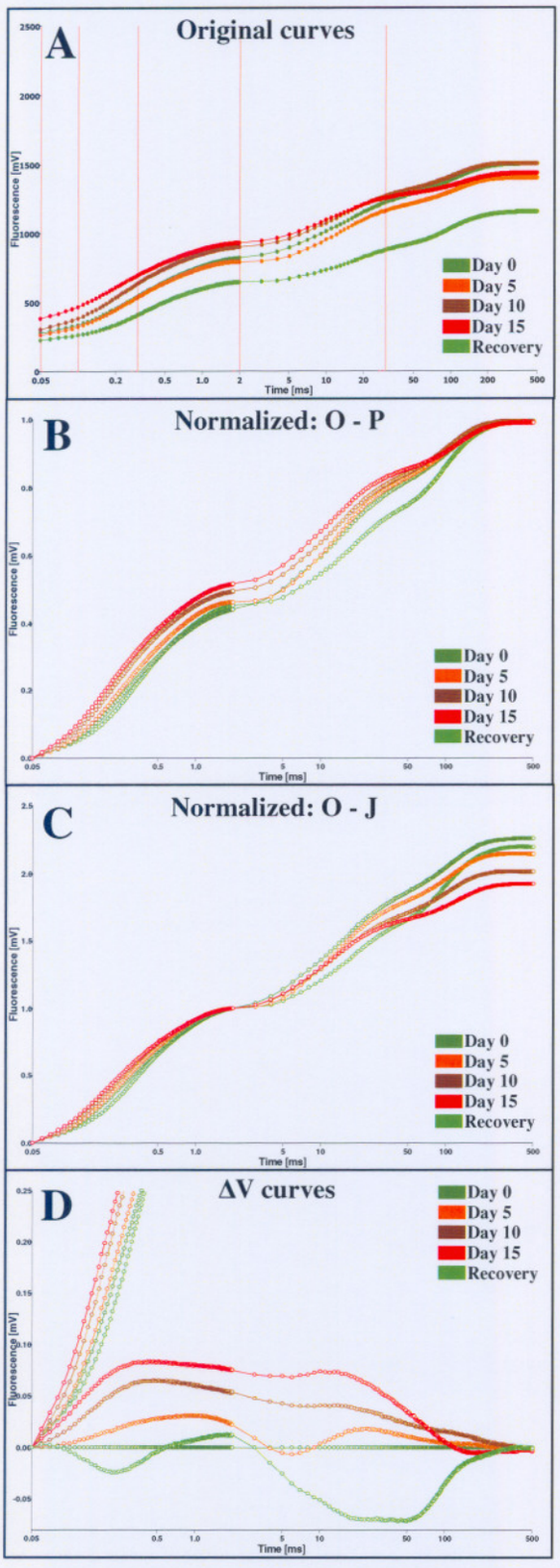


Figure 3.5 **A:** Polyphasic chlorophyll *a* fluorescence O-J-I-P transients recorded in water-deprived plants of *A. capensis*. Transients after 0 (dark-green), 5 (light-brown), 10 (dark-brown) and 15 (red) days of water deprivation and 7 days following re-watering (light-green) are shown. Each transient represents the mean of

measurements conducted in three plants (20 measurements per plant). **B:** Transients normalised between the fluorescence extremes O (F_o) and P (F_M). **C:** Transients normalised between O and J (2 ms). **D:** ΔV curves constructed by subtraction of the normalised (O to P) fluorescence values recorded on day 0 from those recorded after 5, 10, and 15 days of water deprivation, as well as after re-watering. The dark green zero lines represent the values obtained at each time point after subtraction of the fluorescence intensities of day 0 transients from them self.

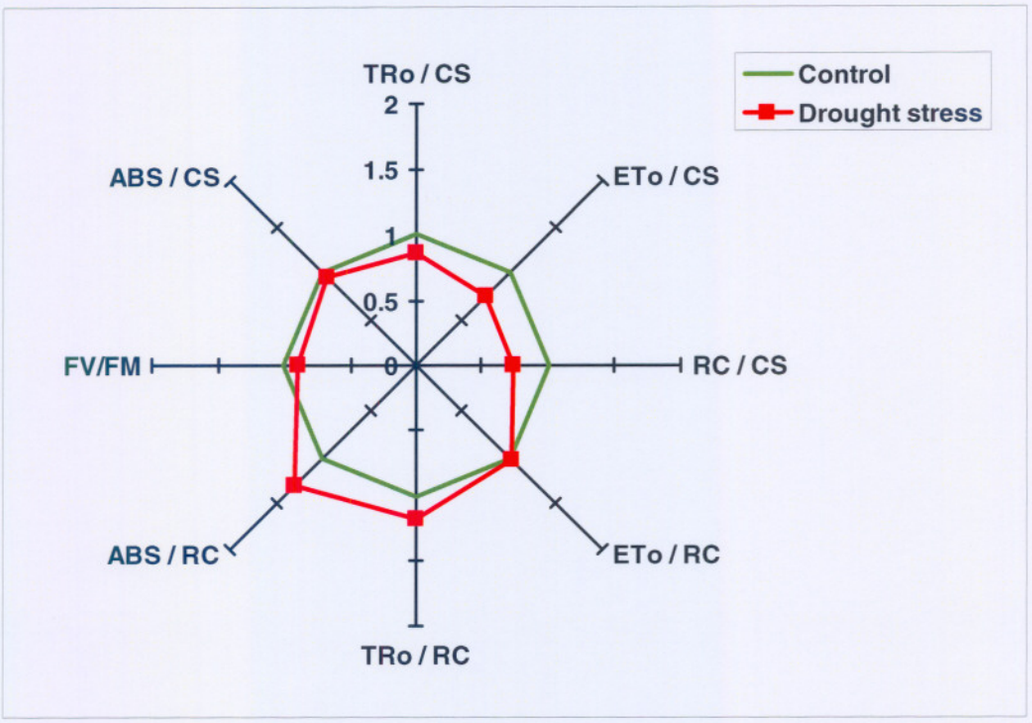


Figure 3.6 Radar plot visualising the fractional changes that occurred in the values of RC/CS, ET_o/CS, TR_o/CS, ABS/CS, F_V/F_M, ABS/RC, TR_o/RC and ET_o/RC after 15 days of water deprivation (red line) relative to the values of control plants (green line). Values represent the mean of measurements conducted in three plants (20 measurements per plant).

3.2.2.2 Effects on CO_2 assimilation

The effects of short-term water deprivation under laboratory conditions on CO_2 assimilation capacity in *A. capensis* were evaluated by construction of A:c_i response curves. During water deprivation a progressive decrease of CO_2 assimilation rates were observed (Fig. 3.7 A). After 5, 10 and 15 days of water deprivation, CO_2 saturated rates of photosynthesis (J_{max}) were inhibited by 5%, 67% and 91% respectively compared to values at the beginning of the experiment (day 0). At the

same time carboxylation efficiencies (CE) decreased by 15%, 35% and 69% respectively (Table 3.1). As expected, water deprivation led to large decreases in stomatal conductance (g_s). After 15 days of water deprivation g_s values were reduced by more than 90% (results not shown). Contrary to this, however, intercellular CO_2 concentrations (c_i) remained similar or increased slightly by up to 15% and relative stomatal limitation of photosynthesis (l) decreased by as much as 56% (Table 3.1). Water deprivation also resulted in increases in the CO_2 compensation concentration (Γ) by up to 113% (Table 3.1). Following re-watering all parameters, except J_{max}, recovered to levels similar to those measured in well-watered plants at the beginning of the experiment (day 0). The fact that J_{max} was still inhibited by 19% suggests that some inhibition of photosynthetic capacity remained when measured at saturated CO_2 concentrations. At ambient CO_2 concentrations (350 µmol mol^{-1}), however, where c_i is normally below 300 µmol mol^{-1}, plants showed complete recovery of CO_2 assimilation rates (Fig. 3.7 A; c_i < 300 µmol mol^{-1}).

Table 3.1 Changes in the carboxylation efficiency (CE, mol m^{-2} s^{-1}); CO_2 compensation concentration (Γ, µmol mol^{-1}); relative stomatal limitation of photosynthesis (l, %); CO_2 saturated rate of photosynthesis (J_{max}, µmol m^{-2} s^{-1}) and intercellular CO_2 concentration (c_i, µmol mol^{-1}) after 5, 10 and 15 days of water deprivation, and again 7 days after re-watering (recovery). Actual mean values ± S.E for each of these parameters, measured/calculated in well-watered plants at the beginning of the experiment (day 0), are shown in bold font. All other values are percentages that represent changes (positive or negative relative to day 0 values) that occurred in CE, Γ, l, J_{max} and c_i during the water deprivation and recovery period. Significant differences ($p < 0.05$ and $P < 0.01$) relative to control (day 0) values are indicated by * and ** respectively. Non-significant differences ($p>0.05$) are indicated by NS.

Day	CE	Γ	l	J_{max}	c_i
0	**0.05 ± 0.006**	**52 ± 1**	**36 ± 2**	**23 ± 2**	**214 ± 38**
5	-15^{NS}	$+28^{**}$	-17^{*}	-5^{NS}	$+15^{*}$
10	-35^{*}	$+20^{**}$	-56^{**}	-67^{**}	$+9^{**}$
15	-69^{**}	$+113^{**}$	-44^{**}	-91^{**}	$+3^{*}$
Recovery	$+7^{NS}$	$+4^{NS}$	-17^{NS}	-19^{*}	$+7^{NS}$

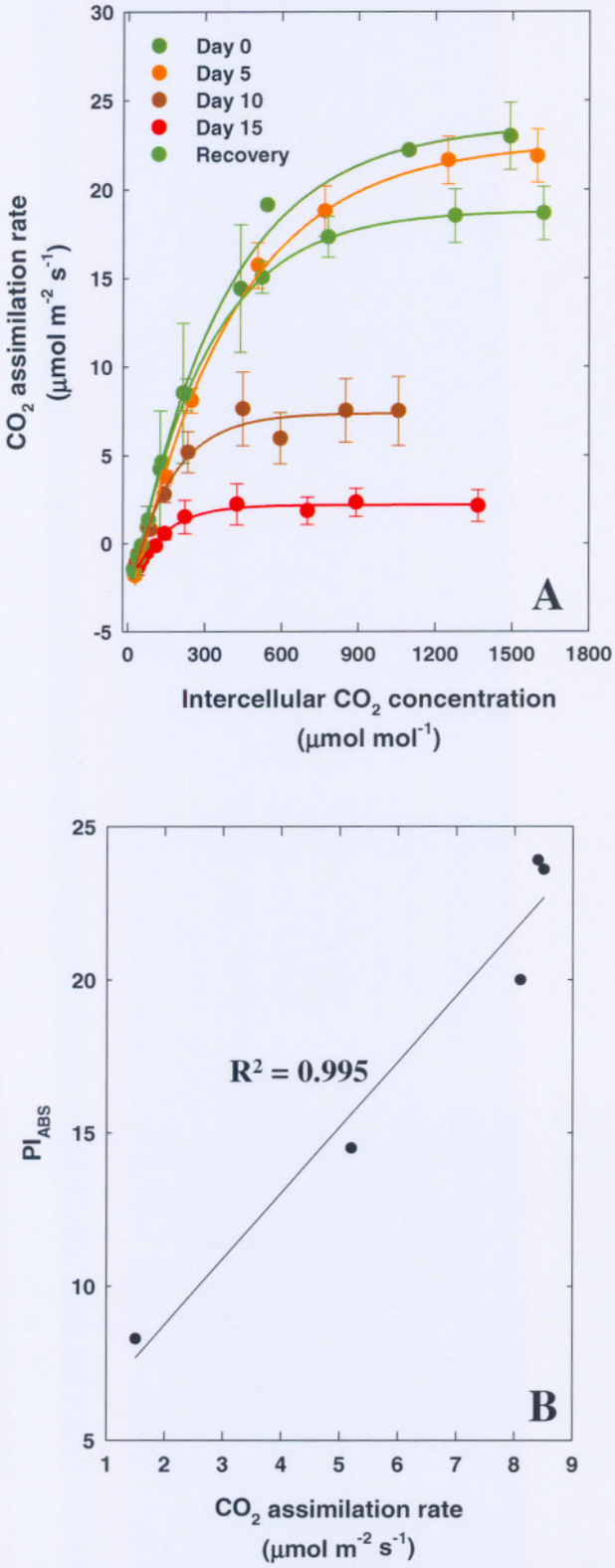


Figure 3.7 **A:** A:c_i response curves recorded in leaves of *A. capensis* plants after 0 (dark-green), 5 (light-brown), 10 (dark-brown) and 15 (red) days of water deprivation as well as 7 days after re-watering (recovery, light-green). Plotted values represent the mean ± S.E of measurements conducted in three plants. **B:** The

relationship between PI_{ABS} values measured on day 0, 5, 10, 15 and 22 in water-deprived plants (Fig. 3.4, red circles) and CO_2 assimilation rates (at ambient CO_2 concentration) in the same plants on each of these days (Fig. 3.7 A).

In Fig. 3.7 B the relationship between decreases in the PI_{ABS} values during short-term water deprivation (Fig. 3.4, red circles), and decreases in CO_2 assimilation rate at ambient CO_2 concentrations in the same plants (Fig. 3.7 A), are shown. A very good relationship (R^2 = 0.995) existed between these two parameters in the laboratory experiments, which suggest that the PI_{ABS} is potentially a valuable JIP-test parameter for rapid quantification of water deprivation effects on photosynthesis in *A. capensis*.

3.2.2.3 Effects on *in vitro* Rubisco activity

In Fig. 3.8 the effects of short-term water deprivation under laboratory conditions on initial and total extractable Rubisco activity in *A. capensis* are illustrated. After 15 days of water deprivation, initial and total Rubisco activity was respectively 44% and 59% lower than activities measured in well-watered plants at the beginning of the experiment (day 0). The decrease in *in vitro* Rubisco activity compared well with the decrease of CE (Table 3.1), which is regarded as a good indicator of *in vivo* Rubisco activity. The inhibition of Rubisco activity was however fully reversible since complete recovery of both initial and total Rubisco activity, as well as CE (Table 3.1), occurred 7 days after re-watering (day 22).

3.2.3 Long-term mild water deprivation under laboratory conditions

In experiments where potted plants are completely deprived of water, the loss of available soil water is often more rapid than under field conditions. This often leads to unrealistic laboratory artefacts making extrapolation to the situation under field conditions difficult. In an attempt to simulate the situation in the natural environment more closely, long-term water deprivation experiments, in addition to the short-term experiments detailed in section 3.2.2, were conducted. In these experiments, plants were maintained at mild water deprivation levels for up to 40 days by watering plants only occasionally with small volumes of water. When CO_2 assimilation rates were measured in these plants after 40 days of water deprivation (Fig. 3.9), inhibition of CE and J_{max} values very similar to those observed in plants under much more severe conditions of total water deprivation (Fig. 3.7 A, day 15) were found. Besides these

similarities, the recovery (following re-watering) of plants under short and long term water deprivation conditions were also very similar. This data suggests that, in the short-term experiments, the water deprivation effects on photosynthesis were not caused by unusually high desiccation rates.

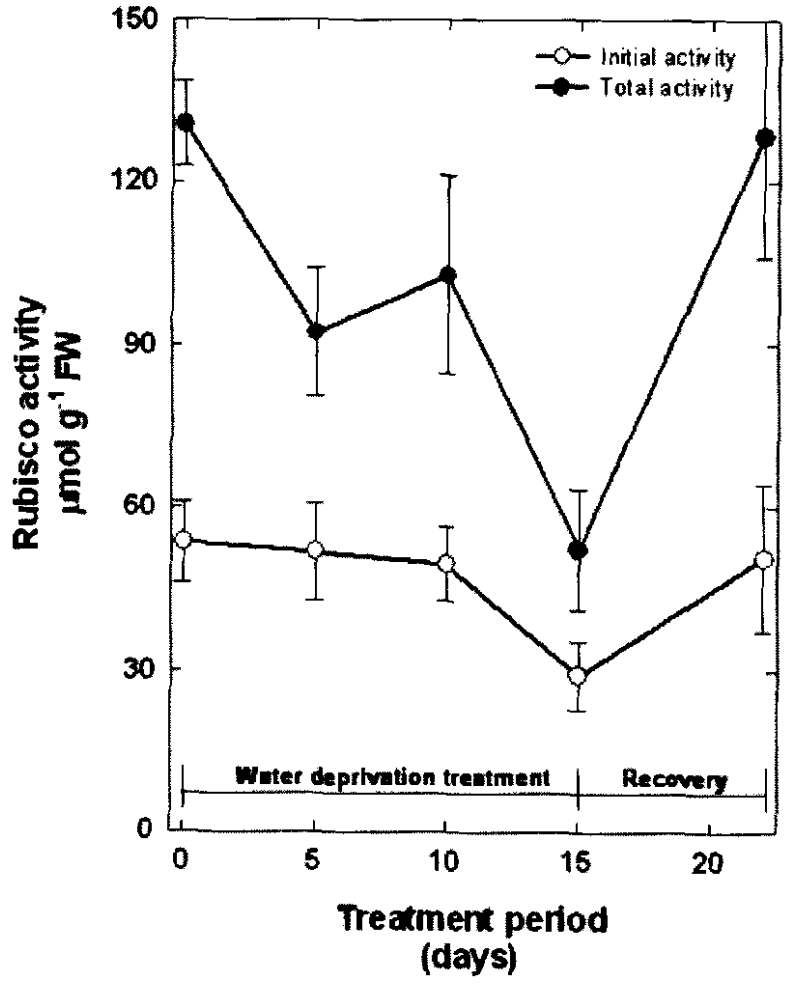


Figure 3.8 Initial (open circles) and total (closed circles) Rubisco activity after 0, 5, 10 and 15 days of water deprivation and 7 days following re-watering (day 22). Each value represents the mean activity ± S.E of six leaves. The water deprivation and recovery (following re-watering) periods are indicated by labels above the x-axis.

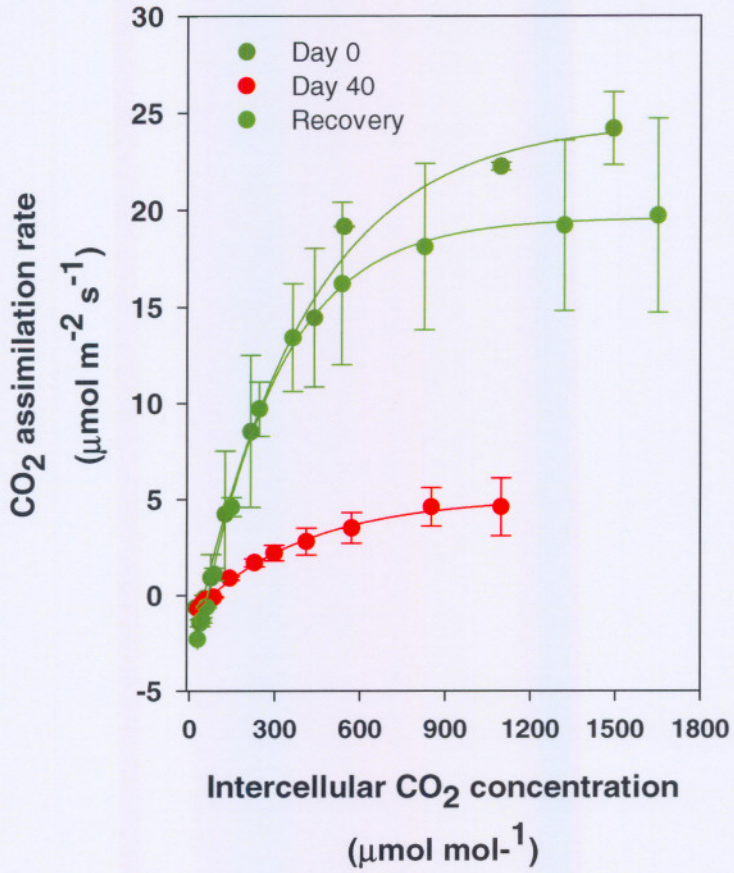


Figure 3.9 A:c_i response curves recorded in leaves of *A. capensis* plants after 0 (dark-green) and 40 (red) days of long-term water deprivation as well as after re-watering (recovery, light-green). Plotted values represent the mean ± S.E of measurements conducted in three plants.

3.3 Discussion

According to Von Willert *et al.* (1992) a succulent is a plant that possesses at least one succulent tissue. Succulent tissue guarantees a temporary storage of utilizable water when the roots are no longer able to obtain sufficient water. Keeping this definition in mind, it is evident, that succulents possess remarkable strategies, both morphological and physiological, that enable survival during periods of low water availability and extreme temperatures.

During the investigation of water availability effects under field conditions on *A. capensis* and *Z. prismatocarpum*, the PI_{ABS}, a JIP-test derived parameter, proved to be a very sensitive indicator of the physiological status of the plants. Its sensitivity is reflected by the fact that both species had low PI_{ABS} values during periods of low rainfall but very high values following relatively high rainfall. This in effect means that

PS II function was down regulated during periods of low rainfall in the field and that in both species, the occurrence of significant rainfall resulted in considerable stimulation of PSII function. The PI_{ABS} was much more sensitive for the detection of changes in PS II function than the commonly used F_V/F_M ratio. The main reason for this is the fact that the PI_{ABS} responds to changes in the fast fluorescence rise kinetics between the two fluorescence extremes (F_o and F_M), while the F_V/F_M ratio [$F_V/F_M = (F_M-F_o)/F_M$] only considers changes in the values of F_o and F_M. The F_V/F_M ratio is known to be insensitive to plant water deficit as observed in another succulent species *Kalanchoe daigremontiana* (Lu *et al.*, 2003). The effects of rainfall on the PI_{ABS} values were very similar in *A. capensis* and *Z. prismatocarpum* mainly because of very similar changes in the fast fluorescence rise kinetics in both species. Analysis of O-J-I-P fluorescence transients revealed a general increase in fluorescence intensities during low water availability in both species. Increases in fluorescence intensity at 2 ms (J-step) and beyond are normally believed to be the result of accumulation of the reduced Q_A pool, mainly because of a decreased capacity to reoxidate Q_A^- (Neubauer & Schreiber, 1987; Strasser *et al.*, 2000).

Another interesting similarity between the two species was a large increase in fluorescence intensity at around 300µs (K-peak). Therefore, although the leaf morphology of *Z. prismatocarpum* (dorsiventrally flattened sclerophyllous leaves) and *A. capensis* (fleshy cylindrical leaves) is very different, they responded remarkably similar, suggesting common components in the modulation of photosynthesis by water availability in these species. The appearance of a K peak in both species indicates alterations to the oxygen-evolving complex (OEC) (Guissé *et al.*, 1995). Interestingly, the appearance of a K-peak is typically a symptom observed in leaves exposed to heat stress (Guissé *et al.*, 1995; Srivastava *et al.*, 1997), suggesting that high leaf temperatures, in addition to decreased water availability, might have affected PSII function in both species. Transpiration can play an important role in the cooling of plant surfaces (Von Willert *et al.*, 1992; Hopkins, 1999). Because lowered water availability often leads to reduced stomatal conductance and transpiration rates, this effective cooling mechanism may not be at the disposal of desert succulents, leading to higher leaf temperatures.

The K-peak was more pronounced in *Z. prismatocarpum*. Possible morphological and physiological adaptations may have resulted in different heat responses in the two species. *Zygophyllum prismatocarpum*, a C_3 plant (Wand *et al.*, 2001), has small

oval-shaped leaves (giving it the common name "dollarbush") while *A. capensis*, also a C_3 plant (Von Willert *et al.,* 1992), has elongated cylindrical leaves. In contrast to *Z. prismatocarpum*, leaves of *A. capensis* contain large amounts of water and are characterized by the absence of clearly defined petioles. This feature enables desert plants to support heavy succulent leaves (Von Willert *et al.*, 1992). Anatomical investigations revealed large colorless central water storage cells (see Chapter 5). The high water content of leaves may contribute substantially to heat dissipation during periods of low water availability when reduced transpiration rates do not allow effective evaporative cooling of leaves (Von Willert *et al.*, 1992). The leaves of *Z. prismatocarpum* are not as succulent as the leaves of *A. capensis*, and may thus be less effective in terms of heat dissipation during periods of low water availability. In theory, smaller leaves have thinner boundary layers and show greater heat loss through convection (Smith & Nobel, 1977). *Zygophyllum prismatocarpum* has smaller leaves than *A. capensis*, but appears to have lower levels of heat tolerance. This implicates that the degree of leaf succulence, rather than leaf size, might have been a more important factor contributing to differences in heat tolerance between the two species. Several other mechanisms are also involved in conveying heat tolerance in desert succulents. Studies conducted on cacti and agaves by Nobel (1988) revealed that the fatty acid content of membranes has major implications for heat tolerance. More fatty acid saturation leads to less membrane fluidity and is advantageous for high temperature tolerance. Although this feature was not investigated in *A. capensis* and *Z. prismatocarpum*, it could have played a role in the way these species tolerated heat. Another physiological mechanism employed to resist heat stress is the formation of so-called heat-shock proteins. The *de novo* synthesis of these proteins under conditions of heat stress has been recorded in *Agave deserti*, *Carnegiea gigantean* and *Ferocactus acanthodes* from the Sonoran Desert in California (Nobel, 1988).

Taken together, the changes in fast fluorescence rise kinetics observed in both species under field conditions suggest considerable modulation of PS II function by water availability with concomitant involvement of heat stress as well. During dry periods the down regulation of PS II function was characterized by decreased excitation energy trapping (TR_0/CS) and electron transport (ET_0/CS) on the one hand, and large compensatory mechanisms (increased antenna size, ABS/RC), with associated maintenance of normal electron transport flux per remaining active

reaction centers (ET_0/RC), on the other hand. Both species responded remarkably similar in terms of changes in fluorescence rise kinetics and the down regulation of PS II electron transport although *A. capensis* appears to be more tolerant to low water availability -associated increases in leaf temperatures.

Besides the field experiments, the short-term water deprivation experiments under laboratory conditions also identified the PI_{ABS} as a very sensitive indicator of physiological status of the plants. The PI_{ABS} values in leaves of *A. capensis* decreased almost linearly from day 0 through to day 15 of water deprivation. Upon re-watering, there was a rapid increase in the PI_{ABS} values, indicating that the water deprivation effects on PS II function were fully reversible. The changes in PI_{ABS} values during water deprivation, and upon re-watering, correlated well with the observations under field conditions, where rainfall induced a large increase in the PI_{ABS} values. Moreover, under laboratory conditions, a good correlation (R^2 = 0.995) existed between the water deprivation-induced decline in photosynthetic rates and the decline in PI_{ABS} values. This suggests that the PI_{ABS} is not only a sensitive indicator of PS II function during water deprivation, but also of overall photosynthetic capacity. The JIP-test in general, and the PI_{ABS} in particular, therefore shows considerable potential for application in ecophysiological investigations on desert ecosystems. Out of a practical perspective this would be very desirable because chlorophyll fluorescence measurements with the PEA are quick and easy to perform in desert environments where more time-consuming measurements with less portable equipment are often impractical and difficult.

In general the fluorescence rise kinetics of the O-J-I-P transients recorded under laboratory conditions suggest that water deprivation had similar effects to those observed in the field. Interestingly, in the laboratory experiments no clearly defined K-peak was induced, indicating that heat stress did not play a significant role as additional stressor in the down regulation of PS II electron transport. This difference is maybe not surprising considering the moderate growth temperatures (26°C) in the growth chambers compared to scorching hot summer days in the Namib Desert. The slight $-\Delta K$ peak that appeared in leaves after re-watering (Fig 3.5D) suggests stimulation of electron donation from H_2O to PSII indicating complete recovery of OEC function (Lu & Zhang, 1999). Considering the absence of heat stress in the laboratory experiments, water deprivation induced remarkably similar, albeit smaller, effects on PS II function than those observed under field conditions. Similar to dry

periods in the field, water deprivation treatments in potted plants also led to decreased excitation energy trapping (TR_0/CS) and electron transport (ET_0/CS). Moreover, similar compensatory mechanisms (predominantly an increase in antenna size, ABS/RC) also allowed maintenance of normal electron transport flux per remaining active reaction centers (ET_0/RC). These results suggest similar responses to water deprivation under both sets of growth conditions (laboratory *versus* field) and intensification of these responses by heat stress.

It has long been established that g_s decreases during water deprivation, reducing water loss from the leaf (Slatyer, 1967). Such a decrease in conductance will produce stomatal limitation of photosynthesis, causing c_i to decrease (Farquhar & Sharkey, 1982). Traditionally, stomatal responses were regarded as the main limitation of photosynthesis during water deprivation (Sharkey, 1990; Chaves 1991), but knowledge has been gained during the last decade, which indicates contribution of other, non-stomatal (mesophyll), factors in the limitation of photosynthesis as well (Lawlor, 2002; Medrano *et al.*, 2002).

Mesophyll limitation of photosynthesis is indicated by an unchanged or elevated c_i, even in the presence of substantial stomatal closure (Brodribb, 1996). In the laboratory experiments with *A. capensis*, water deprivation caused stomatal closure but also a slight elevation in c_i indicating that mesophyll limitation was the dominant factor contributing to the decrease in CO_2 assimilation rates. There is evidence that the decrease in CO_2 assimilation rates in leaves of water-deprived plants cannot always be reversed by increasing the external CO_2 supply above the point where stomatal limitation of photosynthesis can be ignored. This acts as indirect evidence that water deprivation often also inhibit the mesophyll reactions of photosynthesis (Lawlor, 1995, 2002). The involvement of mesophyll limitation as the main limiting factor of photosynthesis in *A. capensis* during water deprivation is supported *in vivo* by the observed decline in J_{max} and CE, and *in vitro*, by the decline in extractable Rubisco activity. The reduction in CE and J_{max} can be seen as symptomatic of decreased activity or activation state of Rubisco and RuBP regenerating capacity respectively (Medrano *et al.*, 2002). Water deprivation may have lowered RuBP regeneration capacity through the inactivation of PCR cycle enzymes involved in the regeneration phase of the cycle or through limitations imposed on the supply of reducing equivalents (ATP and NADPH) via electron transport reactions. In this context the down regulation of PS II function (indicated by the decrease in PI_{ABS})

could be linked directly to reduced RuBP regeneration capacity (indicated by the decrease in J_{max}). Direct effects on the stromal bisphosphatases, such as fructose-1,6-bisphosphatase (FBPase), with decreasing relative water content have been indicated by others (Haupt-Herting & Fock, 2002). Inhibition of FBPase also has implications on starch synthesis, causing alterations in inorganic phosphate (P_i) fluxes across the chloroplast membrane, which may inhibit photo-phosphorylation reactions (Reddy *et al.,* 2004).

Irrespective of the mechanisms involved, *A. capensis* showed remarkable recovery capacity following re-watering, not only after severe water deprivation induced in the short-term experiments, but also after extended periods (up to 40 days) of mild water deprivation. Both *in vivo* CE and *in vitro* Rubisco activity of the plants demonstrated this well. During water deprivation *in vitro* Rubisco activity and CE decreased in a similar fashion, but both showed full recovery seven days after re-watering.

Importantly, the response of plants during long-term mild water deprivation was very similar to the response observed under severe water deprivation in short-term experiments where watering was terminated completely. This indicates that the response of potted *A. capensis* plants to water deprivation under laboratory conditions was most probably realistic, making extrapolation of these findings to those expected under field conditions, likely.

CHAPTER 4

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF *Augea capensis* TO SO_2 FUMIGATION UNDER LABORATORY CONDITIONS

4.1 Introduction

Concern was expressed about the possible negative effects of SO_2 pollution on the vegetation surrounding Skorpion Zinc mine. The effects of SO_2 pollution on photosynthesis were investigated under controlled laboratory conditions using *A. capensis*, a representative species of the succulent vegetation in the vicinity of the mine. Potted plants were fumigated for three hours per day, either during the light or dark period, at two SO_2 concentrations (0.6 ppm and 1.2 ppm) for a period of up to three weeks. In another experiment, plants were first exposed to long-term water deprivation for 40 days prior to simultaneous fumigation with 1.2 ppm SO_2 (three hours per day in the light) for the next 9 days. Following this combined treatment, plants were re-watered and their recovery capacity assessed.

4.2 Results

4.2.1 Verification of the effectiveness of SO_2 fumigation system

To determine the effectiveness (e.g. control of SO_2 concentrations, leaks etc.) of the SO_2 fumigation system used in the experiments, *Glycine max* (soybean), a species with well-characterized sensitivity to SO_2, was fumigated in the glass chambers. The occurrence of known symptoms of SO_2 toxicity in soybean, such as necrotic lesions and inhibition of photosynthesis, would confirm the effectiveness of the system. Plants were fumigated for two consecutive days with 1.2 ppm SO_2 for three hours during the light period.

Even after a single day of SO_2 fumigation, necrotic lesions, discolouring of leaves and curling of leaf edges developed in treated plants. These effects were irreversible and ultimately led to the death of the entire plant (results not shown). After two days

of fumigation, a drastic decrease in CO_2 assimilation capacity was observed (Fig 4.1 A). Both CE and J_{max} were inhibited by more than 80%, indicating severe mesophyll limitation of photosynthesis.

The effects of SO_2 fumigation on PSII function are shown in Fig. 4.1 B. In the radar plot all the parameter values calculated in control plants (day 0) were normalised to the numeric value of one (green line) in order to show the fractional increase or decrease in the parameter values of the plants after two days of SO_2 fumigation (red line). Fumigation resulted in a large deactivation of PS II reaction centers (RC/CS) and reduction in trapping (TR_o/CS) and electron transport flux (ET_o/CS and ET_o/RC), while the maximum quantum yield of primary photochemistry (F_V/F_M) decreased to a much lesser extent. Fumigation also led to a slight increase in apparent antenna size (ABS/RC), which prevented significant change in trapping flux per remaining active RC (TR_o/RC). From above findings it was concluded that injection of SO_2 at regular intervals into the glass chambers, to maintain concentrations at required levels, was an effective method for the investigation of SO_2 effects on plants.

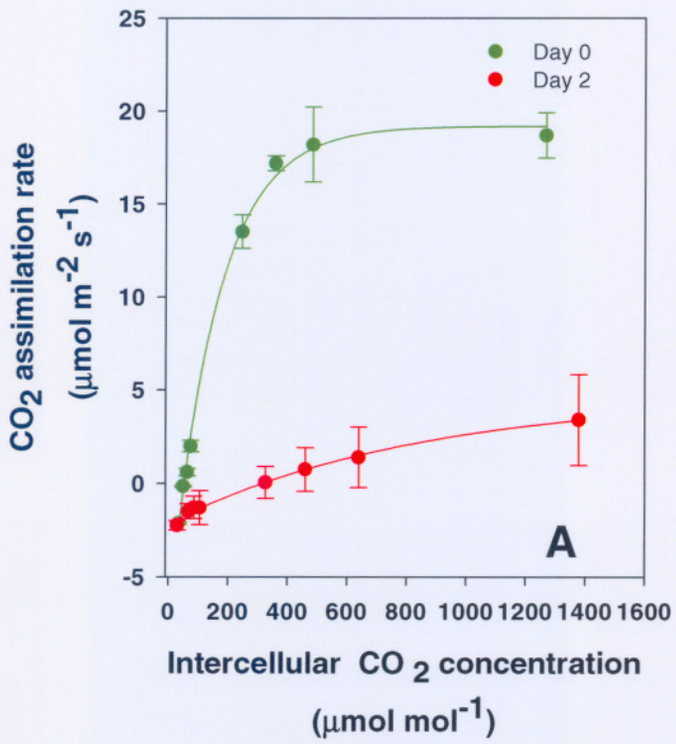


Figure 4.1 **A:** A:c_i response curves recorded in leaves of soybean plants before (day 0, green line) and after 2 days of fumigation with 1.2 ppm SO_2 in the light (day 2, red line). Plotted values represent the mean ± S.E. of measurements conducted in three plants.

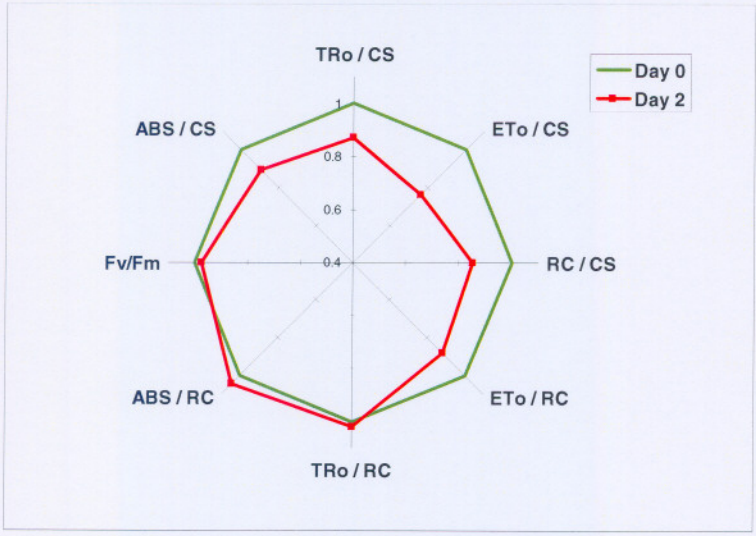


Figure 4.1 **B:** Radar plot visualising the fractional changes that occurred in the values of RC/CS, ET_o/CS, TR_o/CS, ABS/CS, F_V/F_M, ABS/RC, TR_o/RC and ET_o/RC in soybean plants after 2 days of fumigation with 1.2 ppm SO_2 in the light (red line) relative to the values recorded in plants before fumigation (day 0, green line)

4.2.2 The effects of SO_2 fumigation in the dark on *A. capensis*

The effects of SO_2 on *A. capensis* depended largely on the applied dosage during fumigation experiments in the dark. After three weeks of exposure to 0.6 ppm SO_2, there was no significant inhibition of photosynthesis (Fig 4.2 A). After one week of exposure to 1.2 ppm SO_2, however, an inhibition of 38% and 62% in CE and J_{max}, compared to values at the beginning of the experiment (day 0), was observed (Fig. 4.2 C and Table 4.1). At the same time, c_i remained similar or increased slightly (2%), while I decreased by as much as 26% (Table 4.1). There was also an increase in Γ by up to 13%. During these experiments, no water droplets formed on the leaf surfaces due to the higher humidity levels in the glass chambers at night. This suggests that the occurrence of acid rain (SO_2 gas dissolving in water) type of effects that could have resulted in additional damage to plants was unlikely. The absence of any visible damage, such as necrotic lesions or leaf discoloration, also supports this. The effects on photosynthesis were fully reversible, since all parameters recovered to levels similar or higher than those measured before SO_2 fumigation commenced (Fig. 4.2 C and Table 1).

Table 4.1 Changes in the carboxylation efficiency (CE, mol m^{-2} s^{-1}); CO_2 compensation concentration (Γ, µmol mol^{-1}); relative stomatal limitation of photosynthesis (l, %); CO_2 saturated rate of photosynthesis (J_{max}, µmol m^{-2} s^{-1}) and intercellular CO_2 concentration (c_i, µmol mol^{-1}) after one week of fumigation with 1.2 ppm SO_2 in the dark as well as during the recovery period after termination of fumigation. Actual mean values ± S.E for each of these parameters, measured/calculated in the same plants at the beginning of the experiment (day 0), are shown in bold font. All other values are percentages that represent SO_2-induced changes (positive or negative relative to day 0 values) that occurred in these parameters during the fumigation and recovery period.

Day	CE	Γ	l	J_{max}	c_i
0	**0.06 ± 0.01**	**77 ± 0.3**	**34 ± 3**	**38 ± 4**	**243 ± 9**
7 days of fumigation	-38	+13	-26	-62	+2
Recovery	+33	+6	-10	-1	-6

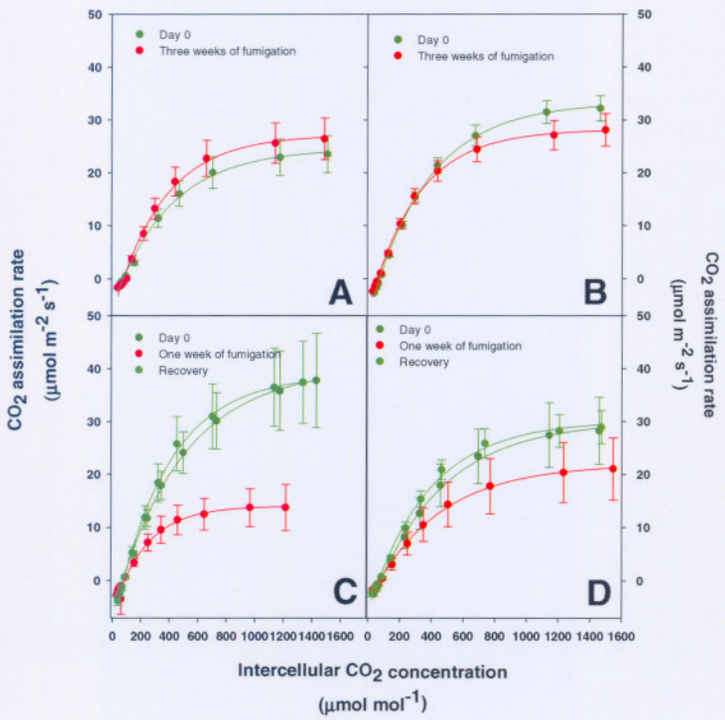


Figure 4.2 A:c_i response curves recorded in leaves of *A. capensis* plants before (dark green lines) and after fumigation with SO_2 (red lines). **A:** fumigation with 0.6 ppm SO_2 for 21 days in the dark; **B:** fumigation with 0.6 ppm SO_2 for 21 days in the light; **C:** fumigation with 1.2 ppm SO_2 for one week in the dark; **D:** fumigation with 1.2 ppm SO_2 for one week in the light. In the case of the 1.2 ppm treatments (C and D), the recovery of plants is also shown (light green

lines). Plotted values represent the mean ± S.E. of measurements conducted in three plants.

The effects of fumigation with 1.2 ppm SO_2 in the dark on PSII function are illustrated in Fig. 4.3. In the radar plot all the parameter values calculated in control plants (day 0) were normalised to the numeric value of one (green line) in order to show the fractional increase or decrease in the parameter values of the same plants after seven days of SO_2 fumigation (red line). Even though SO_2 fumigation under these conditions led to a large, albeit reversible, inhibition of photosynthetic rates (Fig. 4.2 C), it is clear that very little inhibition of PSII activity occurred in the same plants. There was only a very slight decrease in ET_o/CS and ET_o/RC without deactivation of RC/CS as compared to soybean, where deactivation of RC/CS occurred together with the large decrease in ET_o/CS (Fig. 4.1 B).

Figure 4.3 Radar plot visualising the fractional changes that occurred in the values of RC/CS, ET_o/CS, TR_o/CS, ABS/CS, F_V/F_M, ABS/RC, TR_o/RC and ET_o/RC in *A. capensis* plants after 7 days of fumigation with 1.2 ppm SO_2 in the dark (red line) relative to the values recorded before fumigation (day 0, green line)

4.2.3 The effects of SO_2 fumigation in the light on *A. capensis*

When plants were fumigated with 0.6 ppm SO_2 in the light (Fig 4.2 B), there was no significant inhibition of photosynthetic rates, not even after 21 days of fumigation. After one week of exposure to 1.2 ppm SO_2 in the light (Fig 4.2 D), there was only a

small decrease in J_{max}. When fumigation was terminated, plants rapidly recovered to levels similar to those measured before SO_2 fumigation commenced.

4.2.4 Effects of long-term mild water deprivation in combination with SO_2 fumigation (1.2 ppm in the light) on *A. capensis*

In this experiment long-term water deprivation of moderate intensity was induced in plants for 40 days prior to simultaneous fumigation with 1.2 ppm SO_2 (three hours per day in the light) for the next 9 days. Although the effect of SO_2 on *A. capensis* was shown to be more pronounced in the dark (Fig 4.2 C), this experiment was conducted in the light, since the effects on plant water status (often also in combination with heat stress) in desert environments are usually more severe during the day time. Results obtained from this experiment (Fig 4.4), indicate that SO_2 fumigation in combination with long-term water deprivation had no additional negative impacts on photosynthesis. In plants exposed only to water deprivation (red line with solid squares), the inhibition of J_{max} was very similar to that observed in plants exposed to water deprivation in combination with 1.2 ppm SO_2 (red line with solid triangles). Moreover, J_{max} values in both groups of plants recovered to levels similar to those measured in well-watered plants (dark green line with solid circles) following re-watering (light green lines with open squares and triangles).

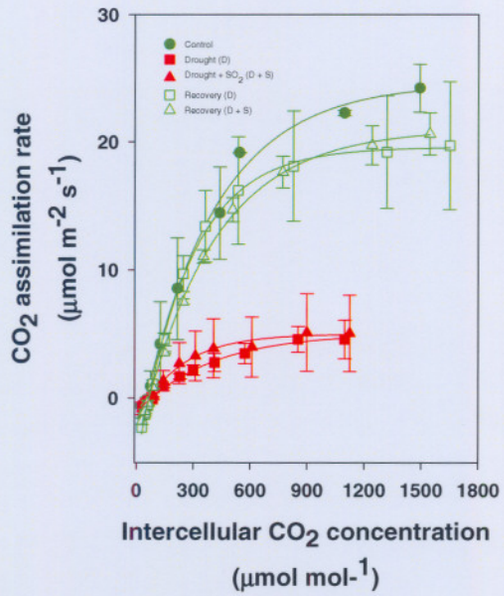


Figure 4.4 A:c_i response curves recorded in leaves of *A. capensis* plants during and after exposure to long-term mild water deprivation only or in combination with SO_2 fumigation. The dark green line with solid circles indicates well-watered control

plants. The red line with solid squares represents plants in which long-term mild water deprivation was maintained for a period of 49 days (D plants). The red line with solid triangles represents plants in which long-term mild water deprivation was induced for 40 days followed by fumigation with 1.2 ppm SO_2 in the light for 9 days in addition to ongoing water deprivation (D+S plants). The light-green lines with open squares and triangles represent the recovery response of D and D+S plants respectively, 7 days after re-watering and termination of SO_2 fumigation. Plotted values represent the mean ± S.E. of measurements conducted in three plants per treatment.

4.3 Discussion

The detrimental effects of SO_2 pollution on soybean are well described in the scientific literature (e.g. Verma & Agrawal, 1996; Deepak & Agrawal, 2001). At a very low concentration of 0.06 ppm, reduced growth, yield, foliar starch and protein content, and a decrease in water use efficiency were observed. In our experiments on soybean, the dosage was much higher (1.2 ppm). With this information in mind, severe inhibition of photosynthesis was expected under these experimental conditions. The objective to fumigate soybean with SO_2 was to verify the effectiveness of the fumigation system for subsequent use with *A. capensis*, a succulent species with unknown sensitivity towards SO_2 pollution. If soybean did not show severe symptoms of SO_2 fumigation, similar to those described in the literature, the effectiveness of the fumigation system could be questioned. Sulphur dioxide fumigation had a detrimental and irreversible effect on growth, CO_2 assimilation and PSII function in soybean, suggesting that the fumigation system could be employed successfully for further studies with *A. capensis*.

Fumigation of *A. capensis* with 1.2 ppm SO_2 in the dark or light revealed that the effects on CO_2 assimilation were much less pronounced than in the case of soybean. The inhibitory effects on photosynthesis were also fully reversible, indicating no permanent metabolic/structural damage. From these results it can be concluded that the succulent *A. capensis* is far less sensitive to SO_2 than soybean at the same SO_2 concentration. This is further supported by the fact that very little inhibition of photosynthesis occurred at SO_2 dosages below 1.2 ppm, even after prolonged periods of exposure for up to three weeks. For example, at a SO_2 concentration of 0.6 ppm, where other plants usually experience severe inhibition of photosynthesis

(see table 1.1, chapter 1), *A. capensis* appeared to be very resistant with little inhibition of photosynthesis.

When the SO_2 concentration was doubled to 1.2 ppm, inhibition of photosynthesis occurred both in the dark and light fumigation experiments. Interestingly, the effect on photosynthesis was much more pronounced when fumigation occurred in the dark. Stomatal conductance is normally seen as the primary factor controlling SO_2 absorption by leaves (Spedding, 1969; Majernik & Mansfield 1970). Based on this, it would be expected that SO_2 uptake and toxicity symptoms in non-CAM plants such as *A. capensis* would be greater during the day when stomata are open. The greater sensitivity of *A. capensis* towards SO_2 in the dark might be related to diurnal differences in cellular capacity for SO_2 detoxification. Figure 4.5 illustrates current understanding regarding metabolic pathways involved in SO_2 detoxification within plant cells. This scheme is based on concepts reported by Larcher (2003), but also includes more recent information obtained by Hänsch & Mendel (2005) and De Kok *et al.* (2005).

Other studies have also found that certain plants that take up SO_2 during the night experience greater leaf injury and reduced growth compared to plants that take up SO_2 during the day (Adams *et al.*, 1989). Usually the light-dependent, chloroplast-based sulfate assimilation pathway is favored above the peroxisomal sulfite detoxification pathway (DeCornis, 1968, Rennenberg, 1984). The greater inhibition of photosynthesis observed in response to dark fumigation in *A. capensis* could possible be because at night, the light-dependent detoxification pathway is switched off. In the dark, plants can only detoxify excess sulfur via the oxidation pathway with accumulation of SO_4^{2-} in the vacuoles, while during the day both pathways are in operation. It has been shown previously that the degree of resistance against SO_2 toxicity in certain plants is related to emission rates of H_2S (Adams *et al.*, 1989), suggesting that the light-dependent route (the source of H_2S) is the most dominant, and probably also the most effective pathway employed by plants for the purpose of SO_2 detoxification.

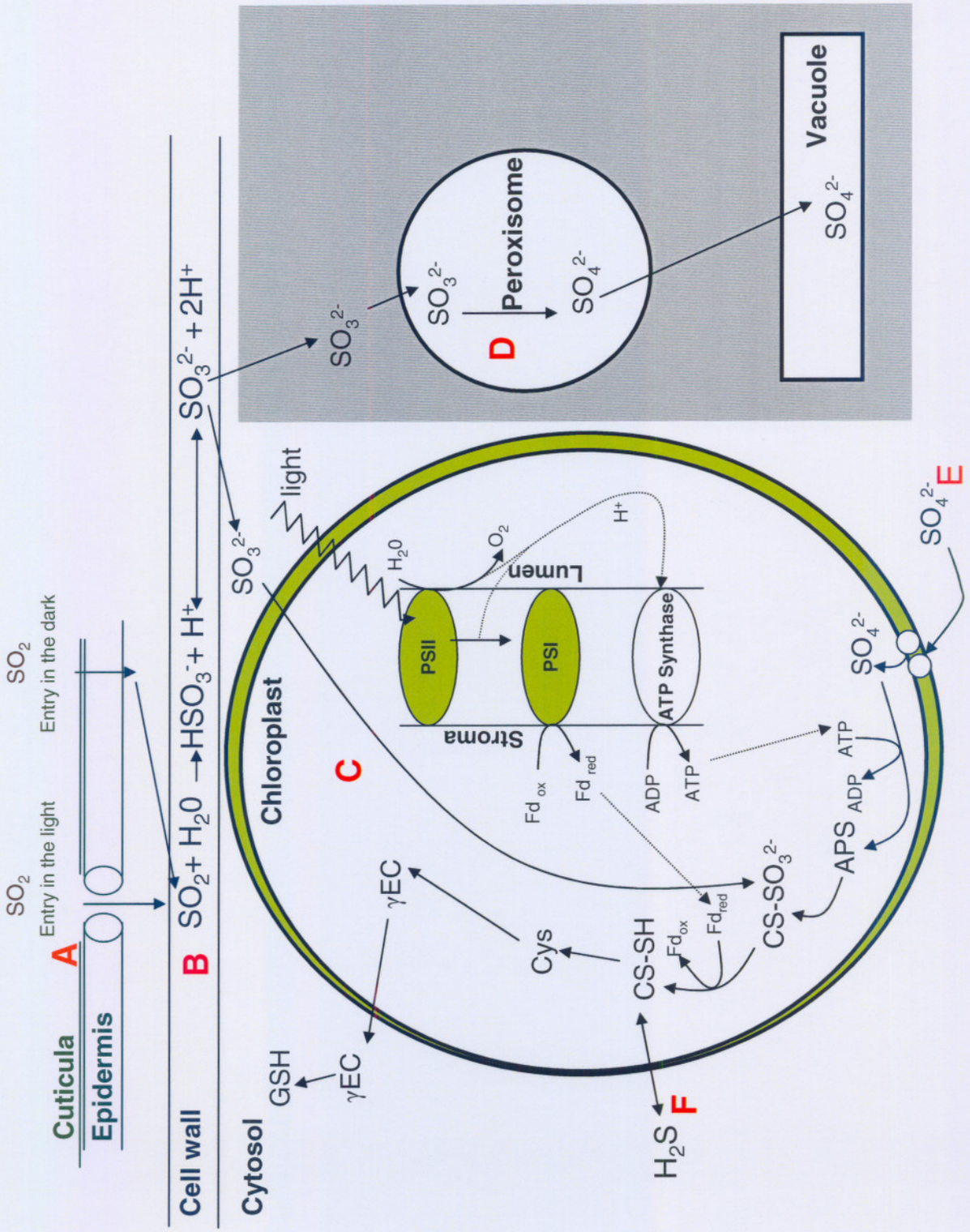


Figure 4.5 Scheme based on recent information obtained from the scientific literature explaining possible SO_2 detoxification pathways occurring during the day and night within mesophyll cells. Bold letters in the figure indicates: **A,** During the day, SO_2 diffuses through the stomata into the leaf (Sekiya *et al.*, 1982; Pfanz *et al.*, 1987). At night, when stomata are normally closed, SO_2 can easily overcome the cuticular resistance and still enter the leaf (Larcher, 2003); **B,**

After entering the leaf, SO_2 dissolves in water situated within cell walls forming hydrogen-sulfite (HSO_3^-) and sulfite (SO_3^{2-}) (Sekiya *et al.*, 1982; Laisk *et al.*, 1988; Larcher, 2003, Hänsch & Mendel, 2005). Sulfite then enters the cytosol where it may be detoxified by a light-dependent or light-independent route; **C,** In the light-dependent route, SO_3^{2-} enters the chloroplasts where it is metabolised by a reduction pathway involving the formation of a protein bound sulfite-complex ($CS\text{-}SO_3^{2-}$) and a protein-sulfide complex (CS-SH). Reduced ferredoxin (Fd_{red}) and ATP, obtained through light-dependent PSII/PSI electron transport, is required during this reduction process. One of the products of this reduction pathway is γ-glutamylcysteine (γEC), a precursor required for the synthesis of glutathione (GSH) in the cytosol of mesophyll cells (Larcher, 2003; Hänsch & Mendel, 2005); **D,** In the light-independent route (shaded area in figure), SO_3^{2-} enters the peroxisomes (Hänsch & Mendel, 2005), where it is oxidized to SO_4^{2-}, the main form in which sulfur is assimilated inside the leaf (Adams *et al.,* 1989). The SO_4^{2-} formed in the peroxisomes is then transported to the vacuole; **E,** Following the oxidation of SO_3^{2-}, SO_4^{2-} is metabolised via the light-dependent reduction pathway within the chloroplast (De Kok *et al.*, 2005, Hansch & Mendel, 2005); **F,** Excess H_2S formed during the light-dependent route is emitted to the atmosphere (Adams *et al.,* 1989).

Observations that SO_2 frequently inhibits electron transport through PSII (Chung, 1982; Schmidt *et al.*, 1988) were not seen in *A. capensis*. Characteristic decreases in PSII quantum yield (F_V/F_M) after SO_2 uptake (Schmidt *et al.*, 1988) were also not observed. In fact, there was no change whatsoever in the F_v/F_m ratio, even after one week of exposure to 1.2 ppm SO_2 in the dark.

When long-term moderate water deprivation was combined with simultaneous fumigation with 1.2 ppm SO_2 in the light, there was no additional inhibitory effect on photosynthesis. These findings suggest that water deprivation do not increase sensitivity towards SO_2 pollution in desert succulents such as *A. capensis*. In fact, the presence of water deprivation under field conditions might actually decrease SO_2 uptake by plants because of stomatal closure in response to low plant water status.

Winner & Mooney (1980) predicted that crops plants, in contrast to native species, might be less useful in predicting SO_2 resistance, as they present a narrower range of stomatal conductance values as a result of continual (optimal) water supply. Using native species (such as *A. capensis*), that represents the interaction of ecological, morphological and physiological characteristics more clearly, might be more useful in

predicting SO_2 resistance in plants. From the experiments reported in this study it is evident that *A. capensis* is remarkably resistant to SO_2 pollution even in the presence of water deprivation, which is a common phenomenon in desert ecosystems. However, it would seem that SO_2 exposure during the night, might pose a greater threat due to less effective and limited detoxification capacity. This might be especially true in the case of CAM-plants, which open their stomata at night allowing higher uptake rates of SO_2.

CHAPTER 5

ULTRASTRUCTURAL CHANGES IN RESPONSE TO WATER DEPRIVATION AND SO_2 POLLUTION IN *Augea capensis*

5.1 Introduction

To determine if any ultrastructural changes occurred in chloroplasts during exposure to water deprivation, fumigation with SO_2 and simultaneous exposure to water deprivation and SO_2, leaf samples of *A. capensis* were collected at various time-points during exposure to these treatments. Leaf samples were fixated accordingly and used for transmission electron microscopy.

5.2 Results

5.2.1 Leaf anatomy of untreated plants

Sections of adaxial leaf tissue were studied by light microscopy. Mesophyll cells have large water-filled vacuoles, thus the cells appear empty of cytoplasm, except for the thin layer of cytoplasm, where the nuclei, chloroplasts and other organelles are found. The mesophyll is comprised of three to four layers of palisade chlorenchyma and large colorless central water storage cells (results not shown).

5.2.2 Ultrastructural changes in response to water deprivation

Control material collected for electron microscopy was collected at about midday for preparation. Chloroplasts of untreated plants exhibited no granal stacks. The internal membranes of all the plastids were irregular and smaller or larger electron-lucent areas were observed (Fig. 5.1 & 5.2, arrows A). Electron dense lipidic globules were present in the chloroplasts (Fig. 5.1 & 5.2, arrows B). The presence of starch in the chloroplasts was conspicuous (Fig. 5.2, arrow C). The vacuoles of untreated plants often contained some fibrillose material (Fig. 5.2, arrow D).

Many of the chloroplasts, however, retained the shape of those of control material (Fig. 5.3). No obvious re-arrangement of internal membranes or changes in the size and number of starch granules were observed (Fig. 5.3). Cells of plants subjected to short-term water deprivation for 15 days, exhibited loss of turgidity (Fig. 5.4, arrow E) and a change in chloroplast shape from oval to more disc-shaped (Fig. 5.4). There was no noticeable increase in lipidic globules within the chloroplasts.

After subjection to long-term water deprivation of moderate intensity for up to 49 days, a loss of starch granules and an increase in the size and number of the electron-lucent areas within the chloroplasts were observed (Fig. 5.5 & 5.6, arrows A). Lipidic globules increased in number and size within the chloroplasts (Fig. 5.5 & 5.6, arrows B). An increase in fibrillose material as well as structures of membranous origin was observed in the vacuoles (Fig. 5.6, arrow D).

5.2.3 Ultrastructural changes in response to SO_2 fumigation

After fumigation with 1.2 ppm SO_2 (in the light), the chloroplast membranes showed re-arrangement with accompanying enlargement of electron-lucent areas (Fig. 5.7 & 5.8, arrows A). The chloroplasts were devoid of starch (Fig. 5.7). Compared to the control material, there was no noticeable increase in the size or number of the lipidic globules. The cells were still largely turgid when compared with those of water-deprived plants. The contents of the vacuoles also appeared similar.

5.2.4 Ultrastructural changes in response to simultaneous exposure to water deprivation and SO_2 fumigation

Simultaneous exposure to water deprivation of moderate intensity and SO_2 fumigation caused loss of turgidity and changes in chloroplast shape similar to those observed in plants during exposure to long-term water deprivation alone (section 5.2.2). An increase in lipidic globules within the plastids also occurred (Fig. 5.9 & 5.10, arrows B).

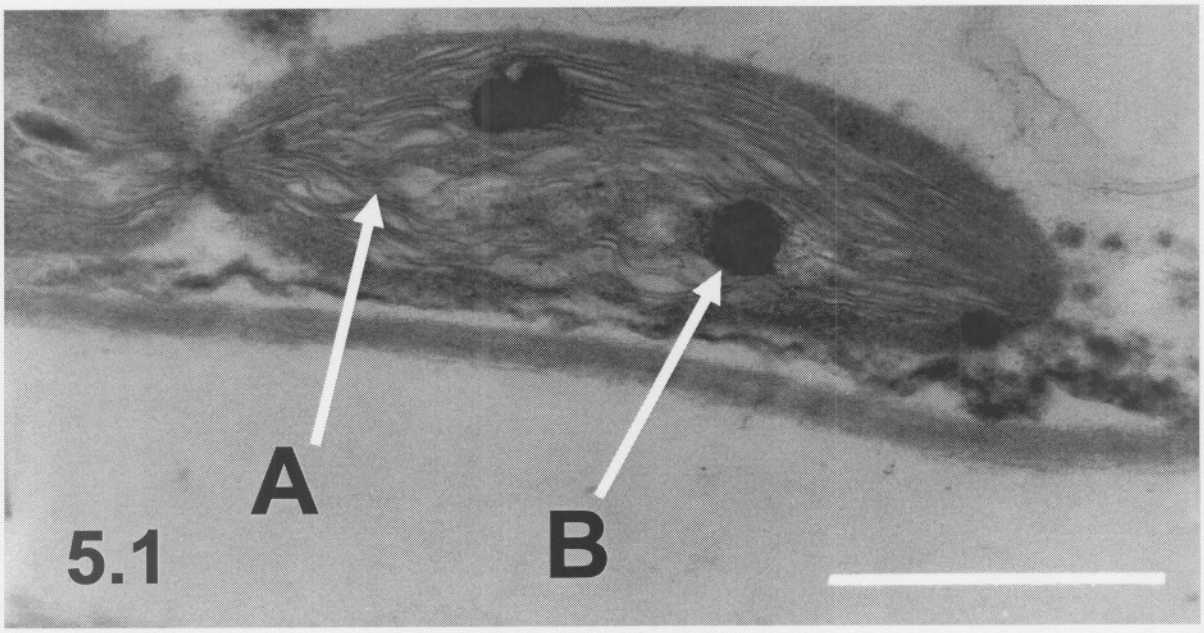
5.3 Discussion

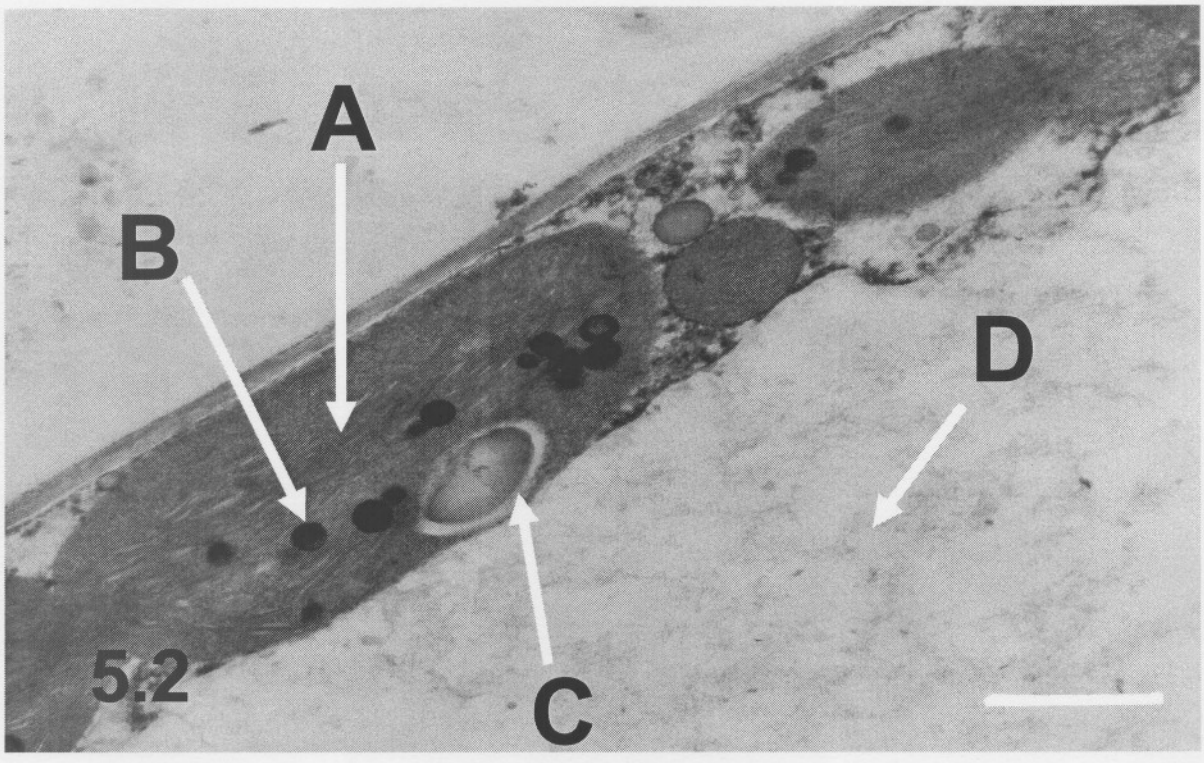
Ultrastructural investigations indicated that chloroplasts remained intact after subjection to water deprivation, fumigation with SO_2 and simultaneous exposure to water deprivation and SO_2. The most notable changes were with regard to

chloroplast shape, arrangement of inner chloroplast membranes and the size of starch granules and lipidic globules.

Plant species such as *A. capensis,* which grow naturally in arid environments, develop strategies that allow the plant to maintain photosynthesis under conditions of low water availability. One of these adaptations is leaf succulence, which allows maintenance of high water content. The water filled mesophyll (central water storage cells) of *A. capensis* might have imparted a greater SO_2 sink capacity, which would have resulted in less severe damage compared to a typical crop species such as soybean. These adaptive physiological changes in succulents may minimize the plant's sensitivity to pollutants (Taylor and Tingey, 1983). The data reported in chapter 3 and 4 showed that although photosynthetic rates were reduced by water deprivation and SO_2 fumigation, complete recovery was possible after re-watering or termination of SO_2 fumigation. This is in accordance with the results of the ultrastructural investigation reported in this chapter. The inhibition of photosynthesis by water deprivation or SO_2 fumigation could therefore be primarily ascribed to altered metabolic processes within the cell rather than changes in chloroplast ultrastructure.

None of the severe ultrastructural alterations normally observed in crop species (disruption of the chloroplast envelope; a mixed cytoplasm containing plastidal ribosomes and vacuole content; (Stirban *et al.*, 1979) and swelling and disintegration of thylakoid membranes (Wellburn *et al.*, 1972) occurred in *Augea capensis.*





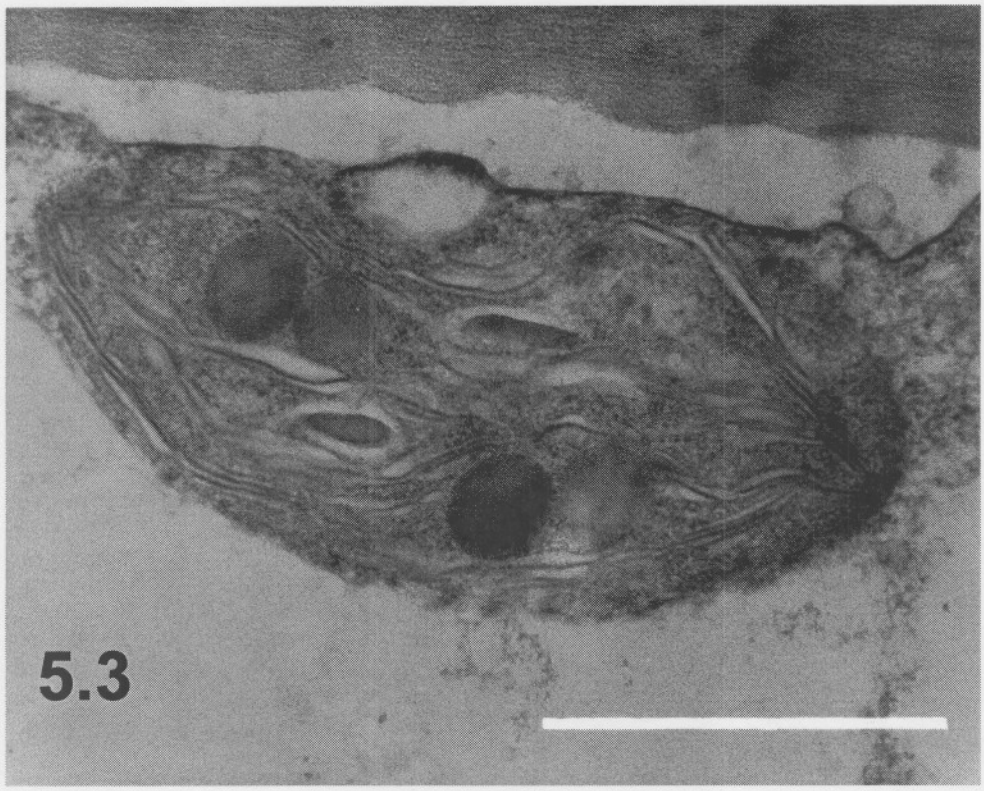
Figures 5.1 and 5.2 Transmission electron micrographs of adaxial mesophyll cells of leaves of untreated plants

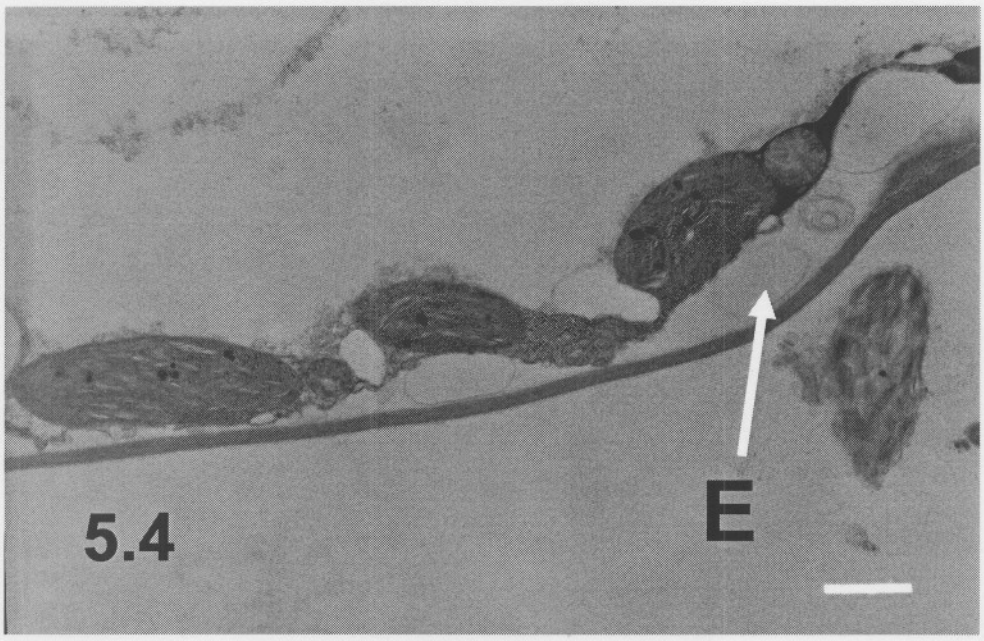
Figure 5.1 Chloroplasts with electron dense lipidic globules and electron-lucent areas

Scale bar = 1µm

Figure 5.2 Chloroplasts with starch granules and lipidic globules

Scale bar = 1µm





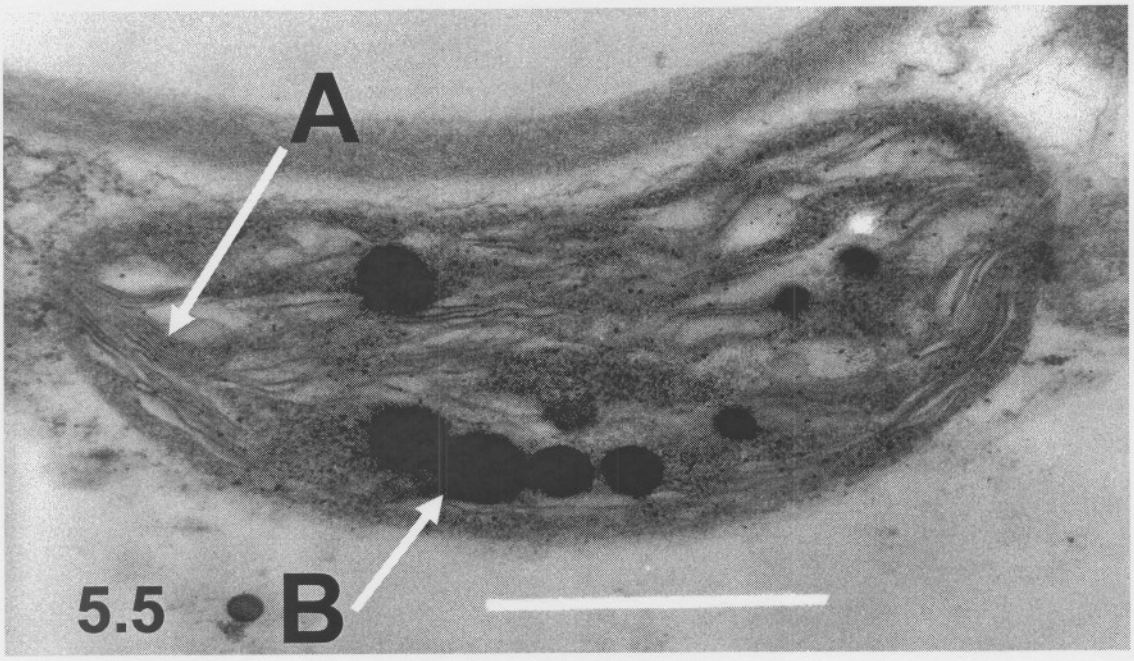
Figures 5.3 and 5.4 Transmission electron micrographs of adaxial mesophyll cells of leaves of plants subjected to short-term water deprivation

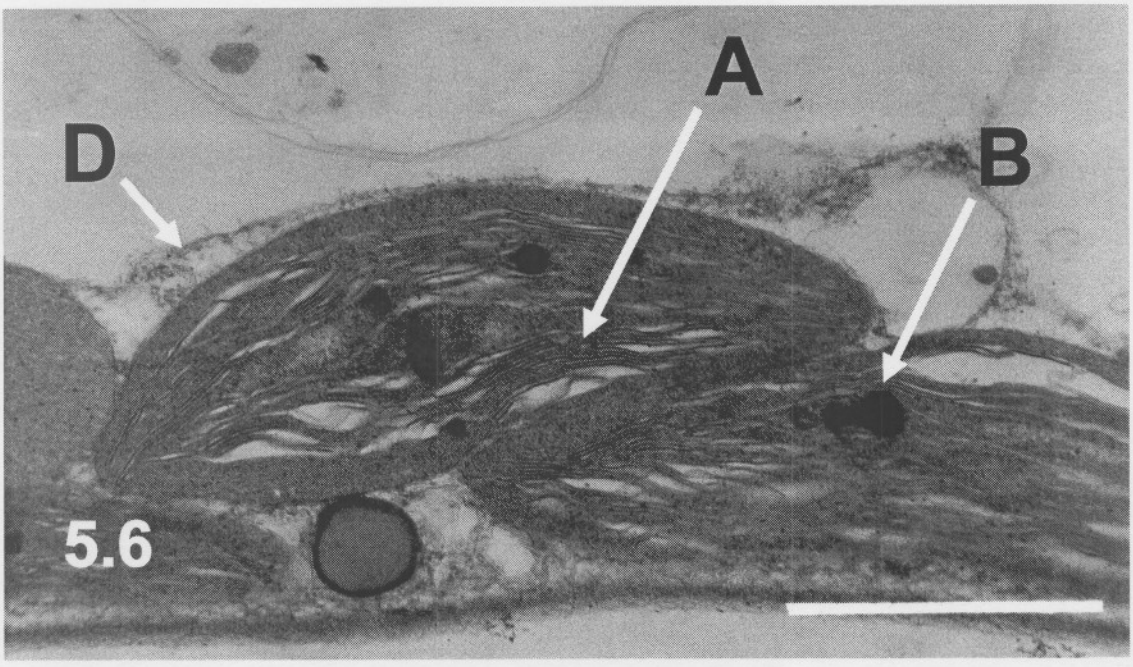
Figure 5.3 Chloroplast with lipidic globules and starch granules

Scale bar = 1µm

Figure 5.4 Cells have lost their turgidity and the chloroplasts have become disc-shaped

Scale bar = 1µm





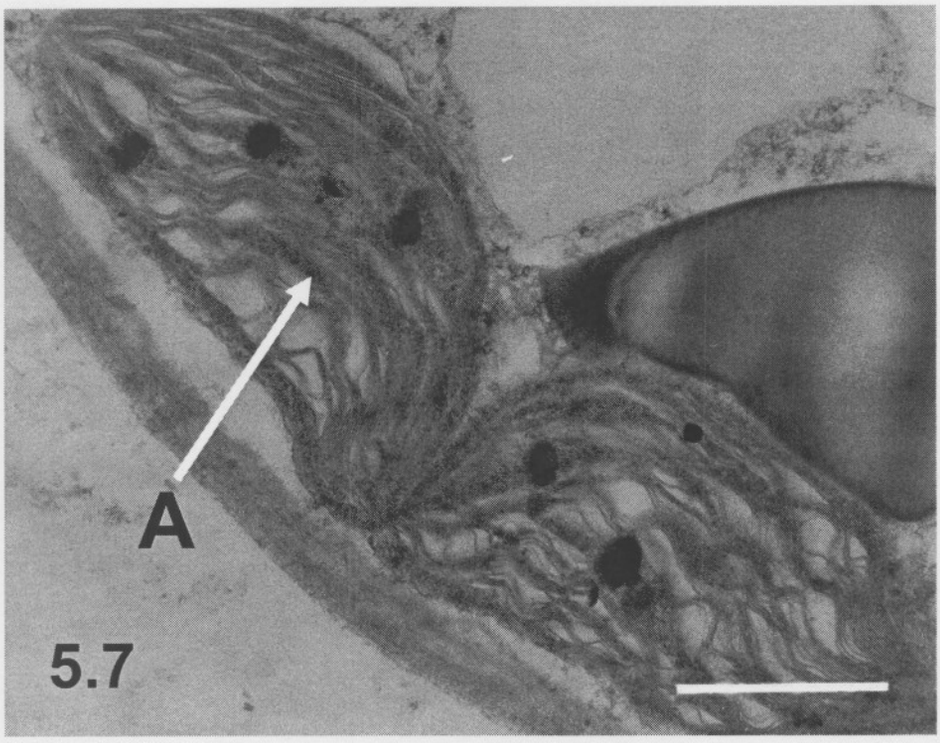
Figures 5.5 and 5.6 Transmission electron micrographs of adaxial mesophyll cells of leaves of plants subjected to long-term water deprivation

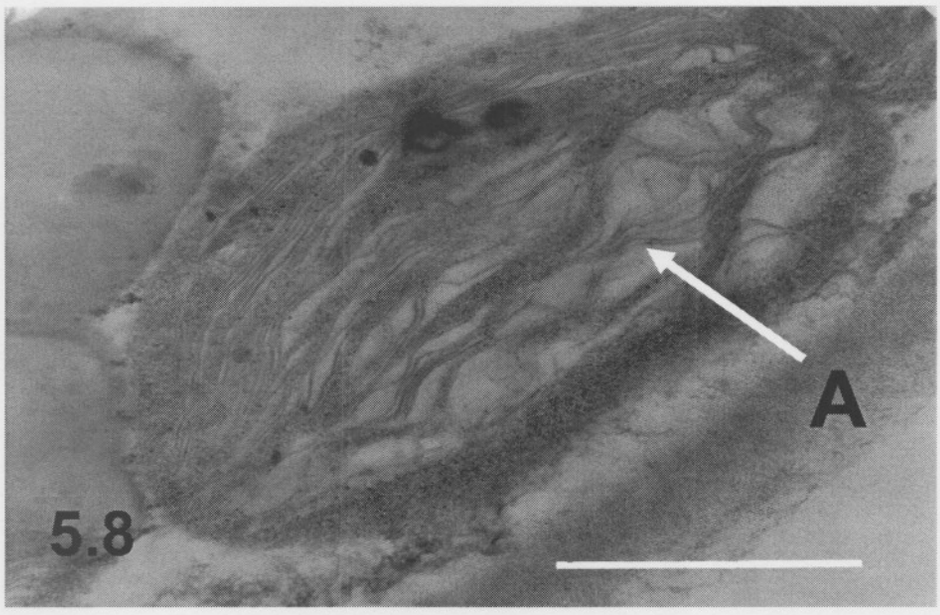
Figure 5.5 Chloroplast devoid of starch, but containing large lipidic globules

Scale bar = 1µm

Figure 5.6 Chloroplast with large electron-lucent areas

Scale bar = 1µm





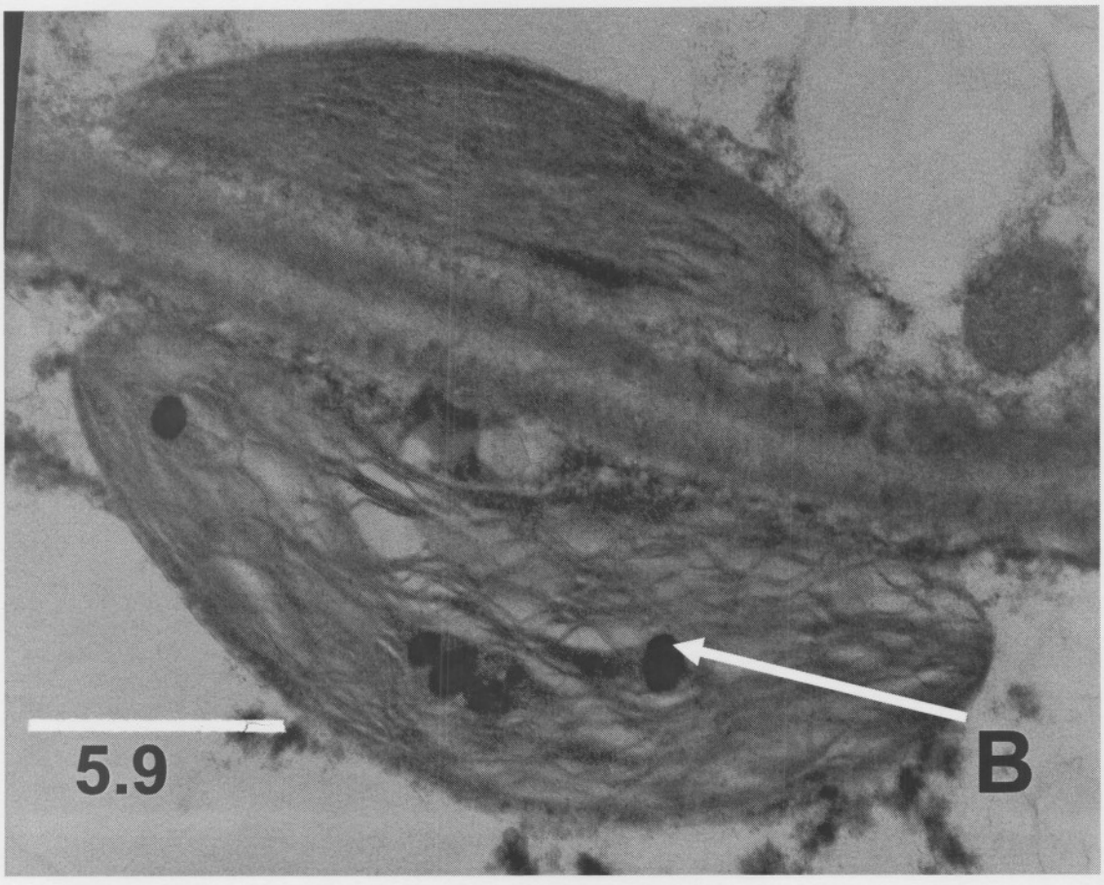
Figures 5.7 and 5.8 Transmission electron micrographs of adaxial mesophyll cells of leaves of plants subjected to 1.2 ppm SO_2 fumigation

Figure 5.7 Chloroplasts with rearranged internal membranes and large electron-lucent areas

Scale bar = 1µm

Figure 5.8 Chloroplast devoid of starch and with no noticeable size/number increase of lipidic globules

Scale bar = 1µm



Figures 5.9 Transmission electron micrographs of adaxial mesophyll cells of leaves of plants subjected to 1.2 ppm SO_2 together with long-term water deprivation

Figure 5.9 Chloroplasts showing increases in lipidic globules

Scale bar = 1μm

CHAPTER 6

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

We investigated the effects of water deprivation and SO_2 pollution, imposed separately or simultaneously, on a succulent species occurring in the vicinity of Skorpion Zinc Mine, Namibia. The main aim of this investigation was the need to distinguish between the effects of these stressors on plants native to a desert environment, where low water availability dominates plant response, but where possible anthropogenic SO_2 pollution poses a new threat to the unique succulent vegetation.

Field measurements in the vicinity of the mine provided valuable information about the effects of water availability on the photosynthetic capacity of two succulent species, *A. capensis* and *Z. prismatocarpum*. These measurements indicated remarkably similar responses in both species, although *Z. prismatocarpum* appeared to be slightly more sensitive. Additional heat stress effects, because of less effective leaf cooling, could have caused this greater sensitivity. In the field, chlorophyll *a* fluorescence measurements, followed by analysis with the JIP-test, was highly effective in demonstrating the modulation of photosyntehsis by water availability. One of the JIP-test parameters, the PI_{ABS}, proved to be particularly useful as a sensitive indicator of overall plant vitality.

For the purpose of laboratory experiments *A. capensis* was selected as test species, mainly because of its abundance in the vicinity of the mine, similar physiological responses to water availability under field conditions than another well-represented species (*Z. prismatocarpum*), and high survival rates following transplantation to pots (in contrast to *Z. prismatocarpum*). The response to water availability under both laboratory and field conditions appeared to be very similar in *A. capensis*. Both the short and long-term experiments revealed that photosynthesis was inhibited as a result of both stomatal and mesophyll restrictions. However, in all instances plants demonstrated a remarkable recovery capacity after re-watering suggesting that the effects of water deprivation were essentially fully reversible. Ultrastructural

investigations supported this since water deprivation resulted in very little impairment of chloroplast structure.

When *A. capensis* was fumigated with SO_2 at moderate concentrations (0.6 ppm), there was no inhibition of photosynthesis whatsoever. Only at a very high concentration of 1.2 ppm did plants show signs of photosynthetic inhibition. The fact that the inhibition of photosynthesis was greater when SO_2 fumigation occurred in the dark is noteworthy and is possibly due to lower sulfite detoxification capacity in the dark. Despite the fact that photosynthesis was inhibited under these conditions, recovery was rapid and complete. Fumigation with SO_2 also did not alter chloroplast ultrastructure. Taken together, all these results suggest that *A. capensis* is highly tolerant to SO_2 pollution, even at a concentration of 1.2 ppm, which proved to be lethal for soybean. In general, succulents such as *A. capensis* might be far less sensitive to SO_2 pollution than typical mesophytic species under the same experimental conditions.

In an attempt to simulate the situation in the desert environment more closely, *A. capensis* was also exposed simultaneously to water deprivation and SO_2 fumigation. These experiments revealed the typical response to water deprivation with apparently no additional SO_2-induced inhibitory effects. Ultrastructural investigations also indicated no additional perturbations besides those induced by water deprivation. It would appear as if water deprivation was by far the most important factor in the inhibition of photosynthesis in these experiments.

Because the findings reported in this study suggests that *A. capensis* is highly tolerant to SO_2, its suitability as an indicator species for the detection of SO_2 pollution effects at Skorpion Zinc mine might be questionable. There might be other species (e.g. CAM plants that open their stomata at night) in the vicinity of the mine with far less tolerance to SO_2. Since water availability dominates the physiological/biochemical response in this species, subtle SO_2 pollution effects might be difficult to detect against this dominant background. The high water content of *A. capensis*, might act as a substantial sink for SO_2 and could convey considerable tolerance against this form of air pollution. Because of the large differences in leaf morphology, *Z. prismatocarpum* might be a more suitable indicator species of SO_2 pollution than *A. capensis*. Chlorophyll fluorescence measurements showed that both species elicit remarkably similar "signatures" in response to changes in water

availability under field conditions. If *Z. prismatocarpum* is indeed more sensitive to SO_2 pollution, changes in this "signature", relative to that of *A. capensis*, are likely, making detection of SO_2 pollution effects possible.

As a next step the sensitivity of *Z. prismatocarpum* to SO_2 pollution should be assessed. We have recently discovered that it is possible to transplant young *Z. prismatocarpum* seedlings to pots. Mortality rates due to transplantation are much less than in larger individuals and the growth rates of the young seedlings under glasshouse conditions in Potchefstroom appear to be high. This holds promise for future laboratory investigations about SO_2 pollution effects in this species.

Recommendations to mine management:

a) The nursery at Skorpion Zinc mine is a very valuable and unique asset and must be maintained and even expanded further. A great variety of species (e.g. *Z. prismatocarpum* and CAM-plant species) could be transplanted to pots. It seems to be very important to transplant only young individuals because the chances of survival are much higher than for larger individuals. Special care must be taken to excavate the plants in such a way that the root systems are not unnecessary damaged. Those species that show high survival and growth rates in pots could then be used for further experimental work involving SO_2 pollution (see below);

b) The School of Environmental Sciences and Development, North-West University, Potchefstroom Campus will soon (October 2006) be in possession of an open top chamber (OTC) facility for the purpose of conducting research on the impacts of air pollution on plants. The construction costs of this facility (consisting of twelve OTC's) are estimated at R1 400 000 and will be the first of its kind in South Africa. Internationally, the OTC method is accepted and widely utilised, as OTC's allow plants to experience natural levels of light and temperature and at the same time realistic levels (in the parts per billion range) of SO_2 and other air pollutants can be introduced into the filtered air stream. This facility will enable us to investigate the effects of chronic long-term exposure to low concentrations of SO_2 on several species maintained at Skorpion Zinc nursery. The envisaged outcome would be the identification of one or more indicator species for use in long-term vegetation monitoring at the mine.

BIBLIOGRAPHY

ADAMS III, W.W, WINTER, K. & LANZL, A. 1989. Light and the maintenance of photosynthetic competence in leaves of *Populus balsamifera* L. during short-term exposures to high concentration of sulfur dioxide. Planta, 177:91-97.

AGRAWAL, M. & DEEPAK, S. 2003. Physiological and biochemical responses of two cultivars of wheat to elevated CO_2 and SO_2, singly and in combination. Environmental Pollution, 121:189-197.

ALSCHER, R.G., BOWER, J.L. & ZIPFEL, W. 1987. The basis for different sensitivities of photosynthesis to SO_2 in two cultivars of pea. Journal of Experimental Botany, 38:99-108.

ALSCHER, RG, CUMMINGS, JR. 1990. Stress Responses in Plants: Adaptations and Acclimation Mechanisms. Wiley-Liss, New York. pp. 57-86.

ANDERSSON, B. & STYRING, S. 1991. Photosystem II. Molecular organization, function and acclimation. Current Topics in Bioenergetics, 16:2-81.

BARTHOLOMEW, D.M., BARTLEY, G.E. & SCOLNIK, P.A. 1991. Absisic acid control of *rbc*S and *cab* transcription in tomato leaves. Plant Physiology, 996:291-296.

BEAUREGARD, M. 1990. Involvement of sulfite and sulfate anions in the SO_2-induced inhibition of the oxygen evolving enzyme photosystem II in chloroplasts: a review. Environmental and Experimental Botany, 31:11-21.

BOLHÁR-NORDENKAMPF, H.R. & ÖQUIST, G. 1993. Chlorophyll fluorescence as a tool in photosynthesis research. (*In*: Hall, D.O., Scurlock, J.M.O., Bolhár-Nordenkampf, H.R., Leegood, R.C. & Long, S.P., *eds*. Photosynthesis and Production in a changing Environment: a field and laboratory manual. Chapman & Hall: London. pp 193-206.)

BOYER, J.S. 1982. Plant productivity and Environment. Science, 218:443-448.

BOYER. J.S. & POTTER, J.R. 1973. Chloroplast response to low leaf water potential I. Role of turgor. Plant Physiology, 51:989-992.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72:248-254.

BRODRIBB, T. 1996. Dynamics of changing intercellular CO_2 concentration (c_i) during drought and determination of minimum functional c_i. Plant Physiology, 111:179-185.

CANAANI, C., HAVAUX, M. & MALKIN, S. 1986. Hydroxylamine, hydrazine and methylamine donate electrons to the photooxidising side of PSII in leaves inhibited in oxygen evolution due to water stress. Biochimica et Biophysica Acta, 851:151-155.

CAPE, J.N., FOWLER, D. & DAVISON, A. 2003. Ecological effects of sulfur dioxide, fluorides, and minor air pollutants: recent trends and research needs. Environmental International, 29:201-211.

CEROVIC, Z.G., KALEZIC, R. & PLESNICAR, M. 1982. The role of photophosphorolision in SO_2 and SO_2^{3-} inhibition of photosynthesis in isolated chloroplasts. Planta, 156:249-254.

CHIA, L.S., MAYFIELD, C.I. & THOMPSON, J.E. 1984. Simulated acid rain induces lipid peroxidation and membrane damage in foliage. Plant Cell and Environment, 7:333-338.

CHAITANYA, K.V., SUNDAR, D., JUTUR, P.P., RAMACHANDRA REDDY, A. 2003. Water stress effects on photosynthesis in different mulberry cultivars. Plant Growth Regulation, 40:75-80.

CHAVES, M.M. 1991. Effects of water deficits on carbon assimilation. Journal of Experimental Botany, 42:1-16.

CHAVES, M.M., PEREIRA, J.S., MAROCO, J., RODRIQUES, M.L., RICARDO, P.P, OSÓRIO, M.L., CARVALHO, I., FARIA, T. & PINHEIRO, C. 2002. How plants cope with water stress in the field- photosynthesis and growth. Annals of Botany, 89:907-916.

CHUNG, H.S. 1982. Effects of sulfur dioxide on pigments, protein content and photosystem II activity of barley and corn leaves. Korean Journal of Botany, 25:135-151.

CORNIC, G. 1994. Drought stress and high light effects on leaf photosynthesis. (*In*: Baker, N.R. & Boyer, J.R., *eds.* Photoinhibition of Photosynthesis. Bios Scientific Publishers Ltd: Oxford. pp 297-313.)

CORNIC, G. 2000. Drought stress inhibits photosynthesis by decreasing stomatal aperture not by affecting ATP synthesis. Trends in Plant Science, 5:187-188.

CORNIC, G. & BRIANTAIS, J.M. 1991. Portioning of photosynthetic electron flow between CO_2 and O_2 reduction in a C_3 leaf (*Phaseolus vulgaris* L.) at different CO_2 concentrations and during drought stress. Planta, 183:178-184.

COWLING, R.R., ESLER, K.J. & RUNDEL, P.W. 1999. Namaqualand, South-Africa-an overview of a unique winter-rainfall desert ecosysyem. Plant Ecology, 142:3-21.

DARRALL, N.M. 1989. The effect of air pollutants on physiological processes in plants. Plant, Cell and Environment, 12:1-30.

DAVIES, T. 1980. Grasses are more sensitive to SO_2 pollution in conditions of low irradiance and short days. Nature, 284:483-485.

DAVIES, W.J. & ZHANG, J. 1991. Root signals and the regulation of growth and development of plants in drying soil. Annual Review of Plant Physiology and Plant Molecular Biology, 42:55-76.

DE CORNIS, L. 1968. Dégagement d' hydrogene sulfuré par des plantes soumises á une atmosphere contenant de l'anhydride sulfureaux. Les Comptes rendus de l'Académie des sciences , 226:683-685.

DEEPAK, S. & AGRAWAL, M. 2001. Influence of elevated CO_2 on the sensitivity of two soybean cultivars to sulphur dioxide. Environmental and Experimental Botany, 46:81-91.

DE KOK, L.J., CASTRO, A., DURENKAMP, M., KORALEWSKA, A., POSTHUMUS, F.S., STUIVER, C.E.E., YANG, L & STULEN, I. 2005. Pathway of plant sulfur uptake and metabolism. Landbouforschung Völkenrode:Special Issue, 283:176-185.

DOWNTOWN, W.J.S., LOVEYS, B.R. & GRANT, W.J.R. 1988. Non-uniform stomatal closure induced by water stress causes putative non-stomatal inhibition of photosynthesis. New Phytologist, 110:503-509.

EPRON, D., DREYER, E. & AUSSENA, G. 1993. A comparison of photosynthetic responses to water stress in seedlings from 3 oak species: *Quercus patraea* (Matt) Liebl., *Q. rubra* L. and *Q. cerris* L. Annales des Sciences Forestieres, 50:48-60.

FARQUHAR, G.D., VON CAEMERER, S. & BERRY, J.A. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta, 149:78-90.

FARQUHAR, G.D. & SHARKEY, T.D. 1982. Stomatal conductance & photosynthesis. Annual Review of Plant Physiology, 33:317-345.

FORCE, L., CRITCHLEY, C. & VAN RENSEN, J.J.S. 2003. New fluorescence parameters for monitoring photosynthesis in plants. Photosynthesis Research. 78: 17-33.

FREDERIK, J.R., ALM, D.M., HESKETH, J.D. & BELOW, E.E. 1990. Overcoming drought-induced decrease in soybean leaf photosynthesis by measuring with CO_2-enriched air. Photosynthesis Research, 25:49-57.

GIARDI, M.T., CONA, A., GEIKEN, B., KUCERA, T., MASOJIDEK, J. & MATTOO, A.K. 1996. Long-term drought stress induces structural and functional reorganization of photosystem II. Planta, 199:118-125.

GILES, K.L., COHEN, D. & BEARDSELL, M.F. 1976. Effects of water stress on the ultrastructure of leaf cells of *Sorghum bicolor*. Plant Physiology, 57:11-14.

GIMENÉZ, C., MITCHELL, V.J. & LAWLOR, D.W. 1992. Regulation of photosynthesis rate of two sunflower hybrids under water stress. Plant Physiology, 98:516-524.

GOLDING, A.J. & JOHNSON, G.N. 2003. Down-regulation of linear and activation of cyclic electron transport during drought. Planta, 218:107-114.

GREEN, B.R. & DURNFORD, D.G. 1996. The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annual Review of Plant Physiology Plant Molecular Biology, 47:685-714.

GUDERIAN, R. 1985. Air Pollution by photochemical oxidants:formation, transport, control and effects on plants. Springer-Verlag: Berlin. 346p.

GUISSÉ, B, SRIVASTAVA, A. & R.J. STRASSER. 1995. The polyphasic rise of the chlorophyll a fluorescence (OKJIP) in heat-stressed leaves. Archives of Science, 48:147-160.

GUNASEKERA, D. & BERKOWITZ, G.A. 1992. Heterogeneous stomatal closure in response to leaf water deficits is not a universal phenomenon. Plant Physiology, 98:660-665.

GUNASEKERA, D. & BERKOWITZ, G.A. 1993. Use of transgenic plants with ribulose-1,5-biphospahte carboxylase oxygenase antisense DNA to evaluate the rate limitation of photosynthesis under water stress. Plant Physiology, 103:629-635.

GURALNICK, L.J. & TING, I.P. 1987. Physiological changes in *Portulacaria afra* (L.) Jacq. during a summer drought and rewatering. Plant Physiology,85: 481–486.

HÄNSCH, R. & MENDEL, R.R. 2005. Sulfite oxidation in plant peroxisomes. Photosynthesis Research, 86:337-343.

HANSCOM, Z. & I, TING. 1978. Responses of succulents to plant water stress. Plant Physiology, 61:327-330.

HAUPT-HERTING, S. & FOCK, H.P. 2002. Oxygen exchange in relation to carbon assimilation in water-stressed leaves during photosynthesis. Annals of Botany, 89:851-859.

HAVAUX, M., CANAANI, O. & MALKIN, S. 1986. Photosynthetic responses of leaves to water stress, expressed by photo-acoustic and related methods. Plant Physiology, 82:827-833.

HAVAUX, M., CANAANI, O. & MALKIN, S. 1987. Inhibition of photosynthetic activities under slow water stress measured in vivo by the photoacoustic method. Physiologia Plantarum, 70:503-510.

HE, J.X., WANG, J. & LIANG, H.G. 1995. Effect of water stress on photochemical function and protein metabolism of photosystem II in wheat leaves. Physiologia Plantarum, 93:771-777.

HOPKINS, W.G. 1999. Introduction to plant physiology. 2nd ed. New York:Wiley. 512p.

ISHIBASHI, M., USUDA, H. & TERASHIMA, I. 1996. The loss of ribulose-1,5-bisphosphate-carboxylase/oxygenase caused by 24-hour rain treatment fully explain the decrease in the photosynthetic rate in bean leaves. Plant Physiology, 111:635-640.

KARNOVSKY, M.J. 1965. a Formaldehyde-glutaraldehyde fixative of high osmality for use in electron microscopy. Journal of Cell Biology, 27:137-138.

KAUTSKY, H. & HIRSCH, A. 1931. Neue Versuche zur Kohlensäureassimilation. Naturwissenschaften, 19:96.

KEYS, A.J. & PARRY, M.A.J. 1990. Ribulose biphosphate carboxylase/oxygenase and carbonic anhydrase. Methods in Plant Biochemistry, 3:1-15.

KICHEVA, M.I., TSONEV, T.D. & POPOVA, L.P. 1994. Stomatal and non-stomatal limitations to photosynthesis in two wheat cultivars subjected to water stress. Photosynthetica, 30:107-116.

KOCHEVA, K., LAMBREV, P., GEORGIEV, G., GOLTSEV, V, & KARABALIEV, M. 2004. Evaluation of chlorophyll fluorescence and membrane injury in the leaves of barley cultivars under osmotic stress. Bioelectrochemistry, 63:121-124.

KONG, F.X., HU, W., CHAO, S.Y., SANG, W.L. & WANG, L.S. 1999. Physiological responses of lichen *Xanthoparmelia mexicana* to oxidative stress of SO_2. Environmental and Experimental Botany, 42:210-209.

KORICHEVA, J., ROY, S., VRANJIC, J.A., HAUKIOJA, E., HUGHES, P.R. & HÄNNINEN, O. 1996. Antioxidant responses to simulated acid rain and heavy metal deposition in birch seedlings. Environmental Pollution, 2:249-258.

KRAUSE, G.H. & WEIS, E., 1991. Chlorophyll fluorescence and photosynthesis: the basics. Annual Review of Plant Physiology, 42:313-349.

KRÜGER, G.H.J. & TSIMILLI-MICHAEL, M. & STRASSER, R.J. 1997. Light stress provokes plastic and elastic modifications in structure and function of photosystem II in camellia leaves. Physiologia Plantarum, 101:265-277.

LAISK, A., PFANZ, H., SCHRAMM, M.J. & HEBER, U. 1988. Sulfur-dioxide fluxes into different cellular compartments of leaves photosynthesizing in a polluted atmosphere. Planta, 173:230-240.

LARCHER, W. 2003. Physiological Plant Ecology: Ecophysiology and Stress Physiology of Functional Groups. 4Th Ed. Springer-Verlag , Heidelberg , New York. 513pp.

LAVOREL, J. & ETIENNE, A.L. 1977. In vivo chlorophyll fluorescence. Topics in Photosynthesis, 2:203-268.

LAW.LOR, D.W. 1995. The effects of water deficit on photosynthesis. (*In*: Smirnoff, N., *ed.* Environment and plant metabolism- flexibility and acclimation. BIOS Scientific Publishers: Oxford. pp 129-156.)

LAWLOR, D. 2002. Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. Annals of Botany, 871-885.

LAZÁR, D. 2006. The polyphasic chlorophyll a fluorescence rise measured under high intensity of exciting light. Functional Plant Biology, 33:9-30.

LAZÁR, D. & ILÍK, P. 1997. High-temperature induced chlorophyll fluorescence changes in barley leaves: Comparison of the critical temperatures determined from fluorescence induction and from fluorescence temperature curve. Plant Science, 124: 159-164.

LIANG, J., ZHANG, J. & WONG, M. 1997. Can stomatal closure caused by xylem ABA explain the inhibition of leaf photosynthesis under soil drying? Plant Physiology, 98:1010-1016.

LIMA, A.L.S, DaMATTA, F.M., PINHEIRO, H.A., TOTOLA, M.R. & LOUREIRO, M.E. 2002. Photochemical responses and oxidative stress in two clones of *Coffea canephora* under water deficit conditions. Environmental and Experimental Botany, 47:239-247.

LONG, S.P. 1985. Leaf gas exchange. (*In*: Barber, J. & Baker, N.R., *eds.* Photosynthetic Mechanisms and the Environment. Elsevier: Amsterdam. pp 453-500.)

LONG, S.P. & HÄLLGREN, J.E. 1993. Measurement of CO_2 assimilation by plants in the field and the laboratory. (*In*: Hall et al., *eds.* Photosynthesis and Production in a changing Environment: a field and laboratory manual. Chapman & Hall: London. pp 129-167.)

LU, C. & ZHANG, J. 1999. Effects of water stress on photosystem II photochemistry and its thermostability in wheat plants. Journal of Experimental Botany, 50:1199-1206.

LU, C., QIU, N., LU, Q., WANG, B. & KUANG, T. 2003. PSII photochemistry, thermal energy dissipation, and the xanthophylls cycle in *Kalanchoe daigremontiana* exposed to a combination of water stress and high light. Physiologia Plantarum, 118:173-182.

MAAS F.M., DE KOK , L.J., HOFFMANN, I. & KUIPER, P.J.C. 1987. Plant responses to H_2S and SO_2 fumigation. I. Effects on growth, transpiration and sulfur content of spinach. Physiologia Plantarum, 70:713-721.

MAJERNIK, O. & MANSFIELD, T.A. 1970. Direct effect of SO_2 pollution on the degree of opening of stomata. Nature, 227:377-378.

MAJUMBAR, S., GHOSH, S., GLICK, B.R. & DUMBROFF, E.B. 1991. Activities of chlorophyllase, phosphoenolpyruvate carboxylase and ribulose-1,5-biphosphate carboxylase in the primary leaves of soybean during senescence and drought. Physiologia Plantarum, 81:473-480.

MANN, H.B. & WHITNEY, D.R. 1947. On a test of whether one of two random variables is stochastically larger than the other. The Annals of Mathematical Statistics, 18:50-60.

MEDRANO, H., ESCALONA, J.M., BOTA, J., GULIAS, J. & FLEXAS, J. 2002. Regulation of photosynthesis of C_3 plants in response to progressive drought: stomatal conductances as a reference parameter. Annals of Botany, 89:895-905.

MEHTA, R.A., FAWCELL, T.W., PORATH, D. & MATTOO, A.K. 1992. Oxidative stress causes rapid membrane translocation and in vivo degradation of ribulose-1,5-

biphosphate carboxylase/oxygenase. Journal of Biological Chemistry, 267:2810-2816.

MOURIOUX, G. & DOUCE, R. 1979. Sulfate transport across the limiting double membrane or envelope of spinach chloroplasts. Biochimie, 61:1283-1292.

MUNNÉ-BOSCH, S., JUBANY-MARI, T. & ALEGRE, L. 2001. Drought-induced senescence is characterized by a loss of antioxidants defences in chloroplasts. Plant, Cell and Environment, 24:1319-1329.

NEUBAUER, C. & SCHREIBER, U. 1987. The polyphasic rise of chlorophyll fluorescence upon onset of strong continuous illumination: saturation characteristics and partial control by Photosystem II acceptor side. Zeitschrift Naturforschung, 42:1246-1254.

NIEBOER, E., RICHARDSON, D.H.S., PUCKETT, K.J. & TOMASSINI, F.D. 1976. Effect of air pollutants on plants. ED.T.A. Mansfield. Cambridge University Press. p. 61-85.

NOBEL. P.S. 1988. Environmental biology of Agaves and Cacti. Cambridge University Press:Cambridge. 270pp.

OLSZYK, D.M. & TINGEY, D.T. 1984. Phytotoxicity of air pollutants. Evidence for the photodetoxification of SO_2 but not O_3. Plant Physiology, 74:999-1005.

OLSZYK, D.M. & BYTNEROWICZ, A. 1987. Sulfur dioxide effects on plants exhibiting Crassulacean Acid Metabolism. Environmental Pollution, 43:47-62.

ORT, D.R., OXBOROUGH K. & WISE, R.R. 1994. Depressions of photosynthesis in crops with water deficits. (*In*: Baker, N.R. & Bowyer J.R., *eds*. Photoinhibition of photosynthesis: from molecular mechanisms to the field. BIOS Scientific Publishers: Oxford. pp 315-330)

OSMOND, C. B., AUSTIN, M. P., BERRY, J. A., BILLINGS, W. D., BOYER, J. S., J. DACEY, W. H., NOBEL, P. S., SMITH, S. D., WINNER, W.E. 1987. How Plants Cope: Plant Physiological Ecology. BioScience, 37: 38-48.

PAPAGEORGIOU, J. 1975. Chlorophyll fluorescence: an intrinsic probe of photosynthesis. (*In*: Govindjee., *ed.* Bioenergetics of Photosynthesis, Academic Press: New York. pp 319-371.)

PARRY, M.A.J., DELGADO, E., VADELL, E., KEYS, A.J. LAWLOR, D.W. & MEDRANO, H. 1993. Water stress and the diurnal activity of ribulose-1,5-biphosphate carboxylase in field grown *Nicotiana tabacum* genotypes selected for survival at low CO_2 concentrations. Plant Physiology and Biochemistry, 31:113-120.

PARRY, M.A.J., ANDRALOJC, P.J., KHAN, S., LEA, P.J. & KEYS, A.J. 2002. Rubisco activity: effects of drought stress. Annals of Botany, 89:833-839.

PASTOR, A., LÓPEZ-CARBONELL, M. & ALEGRE L. 1999. Abscicic acid immunolocalization and ultrastructural changes in water-stressed lavender (*Lavendula stoechas* L.) plants. Physiologia Plantarum, 105:272-279.

PFANZ, H. & HEBER, U. 1986. Buffer capacities of leaves, leaf cells and leaf organelles in relation to fluxes of potential acidic gases. Plant Physiology, 81:597-602.

PFANZ, H., MARTINOIA. E., LANGE, O.L. & HEBER, U. 1987. Flux of SO_2 into leaf cells and cellular acidification by SO_2. Plant Physiology, 85:928-933.

PIERCE, M. & RASCHKE, K. 1980. Correlation between loss of turgor and accumulation of absicic acid in detached leaves. Planta, 148:174-82.

RAKWAL, R., AGRAWAL, G.K., KUBO, A., YONEKURA, M., TAMOGAMI, S., SAJI, H. & IWAHASHI, H. 2003. Defense/stress responses elicited in rice seedlings exposed to the gaseous air pollutant sulfur dioxide. Environmental and Experimental Botany, 40:223-235.

RANEIRI, A., PIERUCETTI, F., PANIUCUCCU, A., CASTAGNA, A., LORENZINI, G. & SOLDATINI, G.F. 1999. SO_2-induced decrease in photosynthetic activity in two barley cultivars. Evidence against specific damage at the protein-pigment complex level. Plant Physiology and Biochemistry, 37:919-929.

REDDY, A.R., CHAITANYA, K.V. & VIVEKANANDAN, M. 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Journal of Plant Physiology, 161:1189-1202.

RENNENBERG, H. 1984. The fate of excess sulfur in higher plants. Annual Review of Plant Physiology, 35:121-153.

REYNOLDS, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology, 17:208-212.

SALISBURY, F.B. & ROSS, C.W. 1992. Plant Physiology. 4th Ed:Wadsworth, California. 682pp.

SAXE, H. 1983. Long-term effects of low levels of SO_2 on bean plants (*Phaseolus vulgaris*). II. Immission-response effects on biomass production: quantity and quality. Physiologia Plantarum, 57:108-113.

Saxe, H. 1991. Photosynthesis and stomatal response to pooluted air and the use of physiological and biochemical responses for easy detection and diagnostic tools. Advances in Botanical Research,18:1-128.

SCHMIDT, W., SCHREIBER, U. & URBACH, W. 1988. SO_2 injury in intact leaves as detected by chlorophyll fluorescence. Zeitschrift Naturforschung, 43c:269-274.

SCHREIBER, U. & NEUBAUER, C. 1987. The polyphasic rise of chlorophyll fluorescence upon onset of strong continuous illumination. II. Partial control by photosystem II donor side and possible ways of interpretation. Zeitschrift Naturforschung., 42c:1255-1264.

SEKIYA, J., WILSON, L.G. & FILNER, P. 1982. Resistance to injury by sulfur dioxide. Plant Physiology, 70:437-441.

SHAPIRO, S.S., WILK, M. & CHEN, H. 1968. A comparative study for various test of normality. Journal of the American Statistical Association, 63:1343-1372.

SHARKEY, T.D. 1990. Water stress effects on photosynthesis. Photosynthetica, 24:651.

SHVETSOVA, T., MWESIGWA, J., LABADY, K., KELLY, S., THOMAS, P., LEWIS, K. & VOLKOR, A.G. 2002. Soybean electrophysiology: effects of acid rain. Plant Science, 162: 723-731.

SHIMAZAKI, K., ITO, K., KONDO, N. & SUGAHARA, K. 1984. Reversible inhibition of the photosynthetic water-splitting enzyme system by SO_2 fumigation assayed by chlorophyll fluorescence and EPR signal *in vivo*. Plant and Cell Physiology, 25:337-341.

SMITH, W.K. & NOBEL. P.S. 1977. Influences of seasonal changes in leaf morphology on water-use efficiency for three desert broadleaf shrubs. Ecology, 58:1033-1043.

SMITH, T.L. & EICKEMEIER, W.G. 1983. Limited photosynthetic plasticity in *Sedum pulchellum* M. Oecologia, 65:374-380.

SLATYER, R.0. 1967. Plant-water relationships. Academic Press, New York. 336pp.

SPEDDING, D.J. 1969. Uptake of sulphur dioxide by barley leaves at low sulphur dioxide concentrations. Nature, 224:1229-1231.

SPURR, A.R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research, 26:31-43.

SRIVASTAVA, A., GUISSé, B., GREPPIN, H. & STRASSER, R.J. 1997. Regulation of antennae structure and electron transport in photosystem II of *Pisum sativum* under elevated temperature probed by the fast polyphasic chlorophyll *a* fluorescence transient OKJIP. Biochemica Biophysica Acta, 1320:95-106.

STIRBAN, M., SORAN, V., SPARCHEZ, C. & CRÄCIUN, C. 1979. The effect of atmospheric pollution on chloroplast ultrastructure under natural conditions. Ecotoxicology and Environmental Safety, 3:369-373.

STIRBAN, M., CRÄCIUN, C. & BATHORY, D. 1988. Ultrastructure in leaves of *Fagus silvatica* and *Carpinus betulus* individuals tolerant and susceptible to SO_2 and heavy metal pollution. Ecotoxicology and Environmental Safety, 16:45-56.

STRASSER, R.J. 7 GOVINDJEE, A. 1992. On the OJIP fluorescence transient in leaves and DI mutants of *Chlamydomonas reinhardtii.* (*In:* Murata, N., *ed.* Research in Photosynthesis. Kluwer Academic Publishers: Dordrecht. pp 29-32).

STRASSER B.J. & STRASSER, R.J. 1995. Measuring fast fluorescence transients to address environmental questions: The JIP-test. (*In*: Mathis, P., *ed.* Photosynthesis: From light to biosphere. Kluwer Academic Publishers: The Netherlands. pp 977-980.)

STRASSER, R.J., SRIVASTAVA, A. & GOVINDJEE, A. 1995. Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochemistry and Photobiology, 61:32-42.

STRASSER, R.J., SRIVASTAVA, A. & TSIMILLI-MICHAEL, M. 2000. The fluorescence transient as a tool to charactierise and screen photosynthetic samples. (*In*: Yunus, M., Pathre, U. & Mohanty, P., *eds.* Probing Photosynthesis: Mechanism, Regulation and Adaptation. Taylor &Francis: London. pp 443-488.)

STRASSER, R.J. TSIMILLI-MICHAEL, M. & SRIVASTAVA, A. 2004. Analysis of the chlorophyll a fluorescence transient. (*In*: Papageorgiou, G.C. & Govindjee, *eds.* Chlorophyll a fluorescence: a signature of photosynthesis. Springer: The Netherlands. pp 322-362.)

STRAUSS, A.J., KRÜGER, G.H.J., STRASSER, R.J. & VAN HEERDEN. P.D.R. 2006. Ranking of dark chilling tolerance in soybean genotypes probed by the chlorophyll a fluorescence transient O-J-I-P. Environmental and Experimental Botany, 56: 147 - 157

TAYLOR, G.E. & TINGEY, D.T. 1983. Sulfur dioxide flux into leaves of *Geranium carolinianum* L. Plant Physiology, 72:237-244.

TERASHIMA, I., WONG, S.C., OSMOND, C.B. & SHARKEY, G.D. 1988. Characterization of non-uniform photosynthesis induced by abscicic acid in leaves having different mesophyll anatomies. Plant and Cell Physiology, 29:385-394.

TEZARA, W. & LAWLOR, D.W. 1995. Effect of water stress on the biochemistry and physiology of photosynthesis in sunflower. (*In*: Mathis, P., *ed.* Photosynthesis: from light to bioshere IV. Kluwer Academic Publishers: Dordrecht. pp 625-628.)

TEZARA, W., MITCHELL, V.J., DRISCOLL, S.D. & LAWLOR, D.W. 1999. Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature, 1401:914-917.

TOIVONEN, P. & VIDAVER, W. 1988. Variable chlorophyll *a* fluorescence and CO_2 uptake in water stressed white spruce seedlings. Plant Physiology, 86:744-748.

TSIMILI-MICHAEL, M., PÊCHEUX, M. & STRASSER, R.J. 1999. Light and heat stress adaptation of the symbionts of coral reef and temperate foraminifers probed in hospite by the chlorophyll a fluorescence kinetics O-J-I-P. Zeitschrift Naturforsch. 54C: 671-680.

TSIMILI-MICHAEL, M., EGGENBERG, P., BIRO, B., KÖVES-PECKY, K., VÖRÖS, I. & STRASSER. R.J. 2000. Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and polyphasic fluorescence transient O-J-I-P. Applied Soil Ecology, 15:169-182.

VAN HEERDEN, P.D.R., STRASSER, R.J., KRÜGER, G.H.J. 2004. Reduction of dark chilling stress in N_2-fixing soybean by nitrate as indicated by chlorophyll a fluorescence kinetics. Physiologia Plantarum, 121, 239-249.

VELJOVIC-JOVANOVIC, S., BILGER, W. & HEBER, U. 1993. Inhibition of photosynthesis, acidification and stimulation of zeaxanthin formation in leaves by sulfur dioxide and reversal of these effects. Planta, 191:365-376.

VERMA, M & AGRAWAL, M. 1996. Alleviation of injurious effects of sulphur dioxide on soybean by modifying NPK nutrients. Agriculture Ecosystems & Environment, 57:49-55.

VON CAEMMERER, S. & FARQUHAR, G.D. 1981. Some ralatiobships between the biochemistry of photosynthesis and gas exchange of leaves. Planta, 153:376-387.

VON WILLERT, D.J., ELLER, B.M., WERGER, M.J.A., BRINCKMANN, E. & IHLENFELDT, H.D. 1992. Life strategies of succulents in deserts. Cambridge University Press:Cambridge. 340pp.

VU, JCV., GESCH, R.W., ALLEN, L.H., BOOTE, K.J. & BOWES, G. 1999. CO_2 enrichment delays a rapid, drought-induced decrease in Rubisco small subunit transcripts abundance. Journal of Plant Physiology, 155:139-142.

WAND, S.J.E., ESLER. K.J. & BOWIE, M.R. 2001. Seasonal photosynthetic temperature responses and changes in $\delta^{13}C$ under varying temperature regimes in leaf-succulent and drought deciduous shrubs from the Succulent Karoo. South African Journal of Botany, 67:253-243.

WEBBER, A.N., NIE, G.Y. & LONG, S.P. 1994. Acclimation of photosynthetic proteins to rising atmospheric CO_2. Photosynthesis Research, 39:413-425.

WELLBURN, A.R., MAJERNIK, O. & WELLBURN, F.A.M. 1972. Effects of SO_2 and NO_2 polluted air upon the ultrastructure of chloroplasts. Environmental Pollution, 3:37-49.

WILLIAMS, J., BULMAN, M.P., & NEILL, S.J. 1994. Wilt-induced ABA biosynthesis, gene-expression and down-regulation of *rbc*S messenger-RNA levels in *Arabidopsis thaliana*. Physiologia Plantarum, 91:177-182.

WINNER, W.E. & MOONEY, H.A. 1980. Ecology of SO_2 resistance: II. Photosynthetic changes of shrubs in relation to SO_2 absorption and stomatal behavior. Oecologia, 44:296-302