The Utility of *Brachylaena discolor* as a Bioindicator of Air Pollution within Selected Industrial Areas in KwaZulu-Natal

by

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A research report submitted in fulfilment of the academic requirements for the degree of Master of Science in Biological Sciences (Plant Ecophysiology).

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ABSTRACT

The negative impacts of air pollution have made monitoring of air quality increasingly important. This is primarily true for industrial areas such as the South Durban Basin (SDB) within the eThekwini Municipal Area (EMA), South Africa. Bioindicators can complement the process of monitoring air quality. For the establishment of *Brachylaena discolor* DC. tree leaves as a bioindicator of air pollution, this study investigated the effects of sulphur dioxide (SO_2) pollution on various biochemical (intracellular superoxide [$\cdot O_2^-$], hydrogen peroxide [H_2O_2] production, total aqueous [TAA] and enzymic antioxidants [superoxide dismutase and catalase], lipid peroxidation [LPO] and electrolyte leakage), physiological (leaf chlorophyll fluorescence and chlorophyll content) and morphological (leaf area [LA]) biomarkers of stress. Leaves were sampled from (four) trees growing at three industrial sites (Prospecton, Ganges and Southern Works) within the SDB and from greenhouse-grown trees that served as an *ex situ* control. The sampling (n=24, per parameter) accommodated directional and seasonal effects. Annual [SO_2] measured at all three treatment sites (Prospecton [4.39±3.92 ppb], Ganges [5.10±4.73 ppb] and Southern Works [6.71±5.47 ppb]) during the study were high compared to global standards. Values for all biomarkers did not differ significantly for leaves from different cardinal points within sites but seasonal differences were evident in some cases; $\cdot O_2^-$, LPO, electrolyte leakage, leaf chlorophyll fluorescence, and LA were significantly ($p<0.05$) correlated with seasonal [SO_2]. Except for $\cdot O_2^-$, superoxide dismutase and catalase, all other biomarkers investigated could differentiate between SO_2 exposed and unexposed leaves. However, only electrolyte leakage was sensitive enough to reflect differences in [SO_2] across the treatment sites. Qualitative data on land-use practices at each site suggests that the pollution sources/pollutants differed across the SDB and that the use of SO_2 as the sole proxy of air pollution may not be ideal. Actually, $\cdot O_2^-$, H_2O_2, lipid peroxidation and LA data suggested that trees at Ganges were exposed to the highest levels of stress, even though annual average [SO_2] was highest at Southern Works. Nevertheless, the investigated biomarkers provide motivation for the establishment of *B. discolor* a bioindicator of air pollution within the SDB. If the appropriate biomarkers are measured (e.g. LPO, electrolyte leakage, leaf chlorophyll fluorescence and LA in this study), *B. discolor* leaves can serve as reliable bioindicators complementing current air monitoring techniques within the EMA.

PREFACE

The experimental work described in this dissertation was carried out in the Plant Ecophysiology Laboratory, School of Life Sciences, University of KwaZulu-Natal, Westville campus, Durban, South Africa from February 2014 to February 2016, under the supervision of Drs Sershen Naidoo and Boby Varghese.

This study represents original work by the author and has not otherwise been submitted in any form of any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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DECLARATION 1-PLAGIARISM

I, **Candyce Ann Areington**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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DEDICATION

In loving memory of:

Alice May Areington (Nanny)

and

Petrus Cornelis Pelser (Uncle Spook)

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My parents, Arthur and Nora Areington, who acting selfless gave up so much for me to get this education, I love you both you.

And lastly my Heavenly Father, my Saviour Jesus Christ and the Holy Spirit my guide; You said "Go into all the world and preach the good news to all creation" Mark 16:15b. I am not a good speaker but I know my job is to protect and sustain the world long enough for the preachers and speakers to get your truth out. **Heavenly Father this is for your glory and yours alone.**

LIST OF ABBREVIATIONS

ABTS	2, 2'-azino-bis-3-ethylbbenzthiazoline-6-sulphonic acid
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
Car	Carotenoids
CAT	Catalase
CH_4	Methane
CO	Carbon monoxide
E	East
EDTA	Ethylenediaminetetraacetic acid
EMA	eThekwini Municipal Area
ETC	Electron transport chain
Fd_{red}	Ferredoxin
Fe_2^+	Iron II cation
F_m	Maximum value of chlorophyll fluorescence
F_o	Minimum value for chlorophyll fluorescence
F_v	Difference between F_o and F_m
FW	Fresh Weight
GDP	Gross domestic product
GPS	Global Positioning System
GPX	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione S-transferase

H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HSO_3^-	Bisulfite
LA	Leaf Area
LPO	Lipid peroxidation
LN	Liquid Nitrogen
M	Molar
mM	Millimolar
MDA	Malondialdehyde
mol g^{-1} FW	Mols per gram fresh weight
$NaNO_2$	Sodium nitrite
NBT	Nitroblue Tetrazolium
NO_2	Nitrogen dioxide
NO_x	Nitrogen oxides
NPQ	Non-photochemical quenching
$\cdot O_2^-$	Superoxide radical
$^1O_2$	Singlet oxygen
O_2	Oxygen
·OH	Hydroxyl radical
O_3	Ozone
·OH	Hydroxyl radical
PBS	Phosphate Buffer Saline
PII	Photosystem II

ppb	Parts per billion
PM	Particulate matter
PUFA	Polyunsaturated fatty acid
PVP	Polyvinylpyrrolidone
R·	Lipid alkye radical
ROO·	Lipid peroxy radical
ROS	Reactive oxygen species
S	South
SD	Standard Deviation
SDB	South Durban Basin
SO_2	Sulphur dioxide
$[SO_2]$	Sulphur dioxide concentration
SO_3^{2-}	Sulphite
SO_4^{2-}	Sulphates
SOD	Superoxide Dismutase
SO_x	Sulphur oxides
TAA	Total Aqueous Antioxidants
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
Trolox™	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UV	Ultraviolet

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CHAPTER 1 INTRODUCTION

1.1. Preamble

The unprecedented rate of climate change has led to uncertainty regarding the responses of ecosystems and the appropriate regulations that need to be established for mitigation of its consequences (Ramanathan and Feng, 2009). Air pollution and climate change have been acknowledged as related entities, yet, they are still often viewed as separate factors in terms of their effects (Swart *et al.*, 2004; Bytherowicz *et al.*, 2007; Paoletti *et al.*, 2007). Further separation between air pollution and climate change can be seen in the approach taken by policy-makers: policies for air pollution are usually short-term, whilst those for climate change are long-term (Swart *et al.*, 2004).

Air pollution and climate change share common drivers such as anthropogenic activities (mainly the burning of fossil fuels) that result in the emission of harmful gases, which react with other atmospheric constituents to produce air pollutants and greenhouse gases (Mickley *et al.*, 2004; Swart *et al.*, 2004; Paoletti *et al.*, 2007; Ramanathan and Feng, 2009). Air pollutants of concern include: carbon monoxide (CO), nitrogen oxides (NO_x), sulphur dioxide (SO_2), ozone (O_3), methane (CH_4) and particulate matter (Taylor *et al.*, 1994; Emberson *et al.*, 2001). By appreciating the responses of ecosystems to both air pollution and climate change the implementation of more appropriate and effective mitigation strategies may be possible (Swart *et al.*, 2004).

The current and future effects of air pollution, in particular, on different vegetation types remains uncertain but it is clear that biochemical, physiological and morphological responses of certain species to pollution are measurable (Conti and Cecchetti, 2001; Tiwari *et al.*, 2006; Tripathi and Gautam, 2007). This has created opportunities for the use of plants/trees as bioindicators of air pollution (Mičieta and Murín, 1998; Conti and Cecchetti, 2001; Novak *et al.*, 2003; Bermudez and Pignata, 2011). However, the establishment of pollutant-specific plant bioindicators has been challenging since plant responses to air pollution are dependent on many factors: rate/duration of exposure, concentration of the pollutant and the combination of pollutants acting together, amongst others (Winner, 1994; Novak *et al.*, 2003; Rai *et al.*, 2011). Another factor to consider is that the responses of plants to air pollution vary

across species and on temporal and spatial scales (Novak *et al.*, 2003; Rai *et al.*, 2011). Nevertheless, understanding the biochemical, physiological and morphological responses of specific species to air pollution, can lead to the establishment of reliable bioindicators (Taylor *et al.*, 1994; Rai *et al.*, 2011). Bioindicators, i.e. representative organisms that are used to evaluate human-induced stresses within the specific ecosystems (Conti and Cecchetti, 2001), can be useful for monitoring air quality, particularly in rapidly developing countries like South Africa (Emberson *et al.*, 2001). The establishment of bioindicators of air pollution is also essential for the development of suitable conservation and climate change mitigation strategies (Taylor *et al.*, 1994; Rai *et al.*, 2011). The present study focuses on the utility of *Brachylaena discolor* DC. as a bioindicator of air pollution within selected industrial areas in KwaZulu-Natal, South Africa.

1.2. Problem identification

Having established the link between air pollution and climate change above, it is evident that these factors impact negatively on ecosystems (Taylor *et al.*, 1994; Paoletti *et al.*, 2007) and human health (Matooane and Diab, 2001; 2003). The increasing need to monitor air pollution and understand its impacts on ecosystems has necessitated the use of active or passive air samplers (Taylor *et al.*, 1994; Paoletti *et al.*, 2007) in various parts of the world (also see Conti and Cecchetti, [2001] and Snyder *et al.*, [2013] for details). However, these techniques are often extremely costly to install/maintain, especially in developing countries where the cost is unjustifiable when compared to social and economic challenges (Tyson *et al.*, 1988; Conti and Cecchetti 2001; Emberson *et al.*, 2001; 2003; Moodley *et al.*, 2011; Rai *et al.*, 2011; Naiker *et al.*, 2012); one such developing country is South Africa (Naiker *et al.*, 2012; Venter *et al.*, 2012). Within South Africa, local municipalities have been tasked with monitoring air pollution, implementing and enforcing mitigation policies (Naiker *et al.*, 2012). However, eThekwini Municipality (Durban, South Africa) is one in which the collection of air pollution data, maintenance of monitoring stations and reporting are severely compromised due to the lack of financial resources and expertise (Diab *et al.*, 2002; Moodley *et al.*, 2011; Naiker *et al.*, 2012). This has resulted in poor enforcement of air pollution emission policies and as a result allowed for air pollution emissions to exceed global limits as well as those set out in the Air Quality Act of South Africa (Josipovic *et*

al., 2010; Naiker *et al.*, 2012). There is, therefore, a pressing need for the use of inexpensive and effective air pollution monitoring strategies such as bioindicators (Moodley *et al.*, 2011).

1.3. Motivation of the study

Still viewed as a third world country, South Africa's GDP is based largely on the industrial and mining sector (Tyson *et al.*, 1988; Naiker *et al.*, 2012; Venter *et al.*, 2012). For this reason, managing developmental needs and conserving the environment represents a great challenge for the country. Huge investment into the industrial processing of raw materials and increased urbanization has led to increased air pollution levels across the country (Tyson *et al.*, 1988; Diab *et al.*, 2002; Venter *et al.*, 2012). This threatens the country's rich biodiversity and emphasises the need for air quality monitoring and control (Winner, 1994). Reports of vegetation damage that are generally associated with air pollution in commercial forest species in South Africa has raised alarm over the potentially detrimental effects of air pollution on wild vegetation (Emberson *et al.*, 2001; 2003).

Increased atmospheric concentrations of SO_2, the major by-product of industrial hubs, are of immense concern given its negative impacts on human and environment health (Taylor *et al.*, 1994; Emberson *et al.*, 2001; Diab *et al.*, 2002; Naiker *et al.*, 2012). Industries within the eThekwini Municipal Area (EMA) have been reported to produce levels of SO_2 that exceed the tolerable threshold set out by the municipality's guidelines (Diab *et al.*, 2002; Areington *et al*., 2015). The financial resources invested into monitoring air pollution levels within the EMA are therefore necessary but not entirely viable, nor effective (Conti and Cecchetti 2001; Diab and Motha, 2007; Moodley *et al.*, 2011; Naiker *et al.*, 2012). This highlights the need for alternative, more feasible methods of monitoring air pollution, such as the use of bioindicators within the EMA.

The formulation and evaluation of air quality monitoring mitigation strategies must be based on sound, accessible and scientifically relevant data. As alluded to above, one way of generating such data involves using biological organisms as indicators of air pollution (Mičieta and Murín, 1998; Moraes *et al.*, 2002; Madejón *et al.*, 2004). These bioindicators can be used to identify and measure the effects of human-induced stresses on the environment (Conti and Cecchetti 2001). Novak *et al.* (2003) argued that it is

best to use an indigenous species as a bioindicator, but irrespective of their origin of the species the bioindicators must be able to withstand high pollution levels, have a wide geographical distribution, be abundant, easily accessible and must be negatively impacted by pollution levels in the surrounding environment (Conti and Cecchetti 2001). Many trees, lichens and mosses are actively used as bioindicators of pollution within the Northern Hemisphere (Santamaría and Martín, 1997; Conti and Cecchetti 2001; Madejón *et al.*, 2004). The use of plants and trees as bioindicators of air pollution has been reported in many countries (Mičieta and Murín, 1998; Moraes *et al.*, 2002; Madejón *et al.*, 2004). In South Africa, researchers have previously suggested lichens (Olowoyo *et al.*, 2011), tree bark (Mandiwana *et al.*, 2006), river crabs (Schuwerack *et al.*, 2001) and ants (Majer *et al.*, 2007) as useful bioindicators. Numerous studies (e.g. Conti and Cecchetti, 2001; Moraes *et al.*, 2002; Novak *et al.*, 2003; Tiwari *et al.*, 2006; Tripathi and Gautam, 2007; Bermudez and Pignata, 2011; Rai *et al.*, 2011) highlight the benefits of using bioindicators for monitoring air quality which include, provision of quick and accessible information on pollutant levels that allow for appropriate and timeous management interventions. Though not as popular in the Southern Hemisphere as it is in the North, tree leaves can serve as effective bioindicators (Lau and Luk 2000, Madejón *et al.*, 2004; Hijano *et al.*, 2005). This motivated the present study on the utility of *B. discolor* as a bioindicator of air pollution within selected industrial areas within the EMA, which is one of the most rapidly developing parts of the province of KwaZulu-Natal, South Africa. This study adds to the growing body of knowledge on the use of plants as bioindicators of air pollution.

1.4. Research aims and objectives

The broad aim of the present study was to assess the utility of *B. discolor* as a bioindicator of air pollution within selected industrial areas in the eThekwini Municipality, KwaZulu-Natal, South Africa.

This involved comparing the efficacy of selected leaf biochemical, physiological and morphological stress biomarkers in reflecting *B. discolor* exposure to different levels of atmospheric SO_2. For this purpose, biomarkers were measured during all four seasons for trees located at three industrial (treatment) sites and an *ex situ*, greenhouse-based, control (unpolluted) site. In each case, biomarker levels were related to [SO_2].

Specific objectives included the following:

i. To measure and compare biomarkers related to oxidative stress (biochemical biomarkers) across trees at industrial (treatment) sites and the control.
ii. To measure and compare biomarkers related to photosynthetic capacity (physiological biomarkers) across trees at industrial (treatment) sites and the control.
iii. To measure and compare biomarkers related to light harvesting capacity (a morphological biomarker) across trees at industrial (treatment) sites and the control.
iv. To assess the effects of cardinal direction and season of sampling on SO_2 biomarker relationships for leaves from trees growing at industrial (treatment) sites and the control.
v. Comparing relationships between the individual biomarkers and [SO_2] across leaves from trees growing at industrial (treatment) sites and the control to identify the biomarkers that are most suitable for measuring the effects of SO_2 exposure in *B. discolor* leaves.

1.5. Methodological approach

All biomarker measurements were performed on *B. discolor* leaves based on previous biomonitoring studies which have shown leaves to be very effective at reflecting the effects of various air pollutants; this includes: SO_2 (Lau and Luk, 2000; Hijano *et al.*, 2005; Rai *et al.*, 2011), O_3 (Novak *et al.*, 2003; Jochner *et al.*, 2015) and particulate matter (PM) (Lau and Luk, 2000; Jochner *et al.*, 2015). The biomarkers used in this study were measured for trees growing at three industrial sites at which ground-level SO_2 levels were measured. Based on the findings of Areington *et al.* (2015), biomarkers were also measured for *ex situ* control trees which were grown within a greenhouse prior to and during the study.

This study compared the utility of leaf biochemical (intracellular superoxide [$\cdot O_2^-$] and hydrogen peroxide [H_2O_2], total aqueous antioxidant [TAA] activity, superoxide dismutase [SOD], catalase [CAT], lipid peroxidation [LPO] and electrolyte leakage), physiological (leaf chlorophyll fluorescence and chlorophyll content) and

morphological (leaf area [LA]) stress biomarkers in reflecting the effects of SO_2 pollution. The selection of the biomarkers were also based on the findings of Areington *et al.* (2015) who showed that leaf H_2O_2, electrolyte leakage, chlorophyll content and leaf area (LA) in *B. discolor* were affected by industrial air pollution. Other studies have also shown various parameters related to leaf oxidative metabolism (for example, superoxide dismutase [Tripathi and Gautam, 2007; Bermudez and Piagnata, 2011], catalase [Tripathi and Gautam, 2007; Bermudez and Piagnata, 2011], lipid peroxidation [Conti and Cecchetti, 2001; Bermudez and Piagnata, 2011], electrolyte leakage [Conti and Cecchetti, 2001]), physiology (e.g., chlorophyll fluorescence [Conti and Cecchetti, 2001; Flowers *et al.*, 2007], chlorophyll content [Assadi *et al.*, 2011]) and morphology (e.g. leaf area [Assadi *et al.*, 2011; Rai *et al.*, 2011]) to be useful stress biomarkers. Leaves were sampled in all four seasons and from different cardinal directions on the trees based on suggestions that the effects of air pollution can vary within seasons (Novak *et al.*, 2003; Hijano *et al.*, 2005) and/or with changes in wind direction (Rai *et al.*, 2011).

1.6. Study sites

This study was conducted within the SDB, which occupies approximately 96 km^2, along the eastern seaboard of KwaZulu-Natal in South Africa (Batterman *et al.*, 2008). The EMA is home to a number of industrial hubs, most notably the South Durban Basin (SDB) which is dominated by petroleum-based industries that give rise to numerous pollutants (Diab and Motha, 2007; Batterman *et al.*, 2008; Buthelezi and Davies, 2015). The selection of the three treatment sites was based on the location of eThekwini Municipality-controlled air quality monitoring stations within the SDB (**Fig. 1.1**). Also, consideration was given to whether the air quality monitoring station could provide ground-level [SO_2] data for the duration of the study (April 2014–September 2015). A further pre-requisite was the presence of sufficient *B. discolor* trees growing within 1000 m of these monitoring stations. The following three air quality monitoring stations met the requirements: Prospecton (30˚ 0' 10.44"S; 30˚ 55' 43.64"E); Ganges (29˚56' 54.60"S; 30˚57' 52.63"E), Southern Works (29˚57' 25.20" S; 30˚58' 23.77"E), KwaZulu-Natal, South Africa.

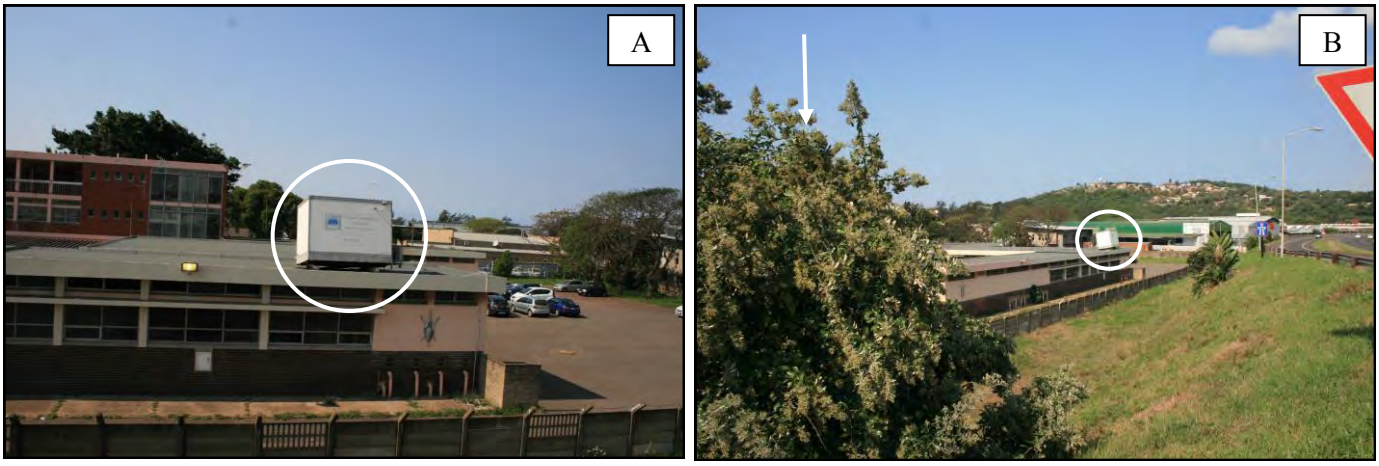

Figure 1.1 Air quality monitoring station (**A**, encircled) at one of the treatment sites (Prospecton) at which *B. discolor* trees (**B**, indicated by arrow) were sampled (Photos by: Minoli Appalasamy and Candyce Areington).

The control trees were grown in a greenhouse located on the Westville campus of the University of KwaZulu-Natal (29˚49'3.76"S; 30˚56'23.56"E), which is in the EMA but *ca.*19 km away from the SDB.

1.7. Study species

This study was conducted on the leaves of *Brachylaena discolor* DC. (**Fig. 1.2 A**), commonly known as silver oak, which is a member of the Asteraceae family (Cilliers, 1993; Pooley, 1993; Boon, 2010). *Brachylaena discolor* is a multi-stemmed small tree, almost shrub-like with a very irregular growth form and grooved stems (Cilliers, 1993; Boon, 2010). Leaves are bicoloured with a green adaxial and white abaxial surface (Boon, 2010) (**Fig. 1.2 B)**. *Brachylaena discolor* flowers from July to September and fruits until November (Pooley, 1993; Boon, 2010). This indigenous species was selected based on its widespread distribution along the KwaZulu-Natal coast line (**Fig. 1.3**), and also based on reports that it is sensitive to industrial air pollution and known to survive in polluted areas (Cilliers, 1993; Pooley, 1993; Boon, 2010; Areington *et al.,* 2015) (**Fig. 1.2 C**). *Brachylaena discolor* trees sampled in this study were taxonomically identified based on the descriptions of Cilliers (1993), Pooley (1993) and Boon, (2010).

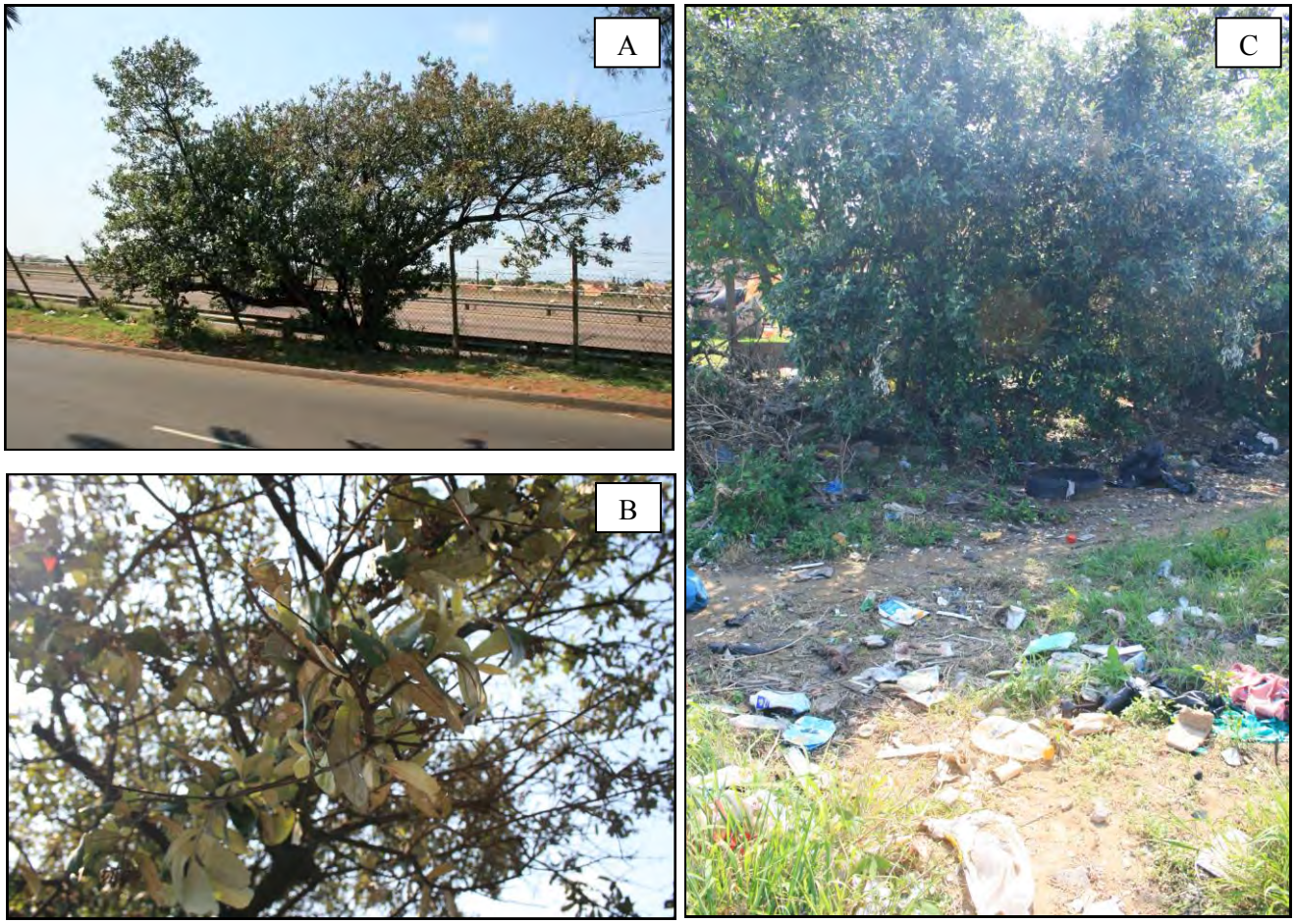

Figure. 1.2 *B. discolor* (**A**) at Ganges treatment site; *B. discolor* leaves (**B**) showing white abaxial surface; *B. discolor* (**B**) at Southern Works study site surviving in polluted area. (Photos by: Minoli Appalasamy and Candyce Areington).

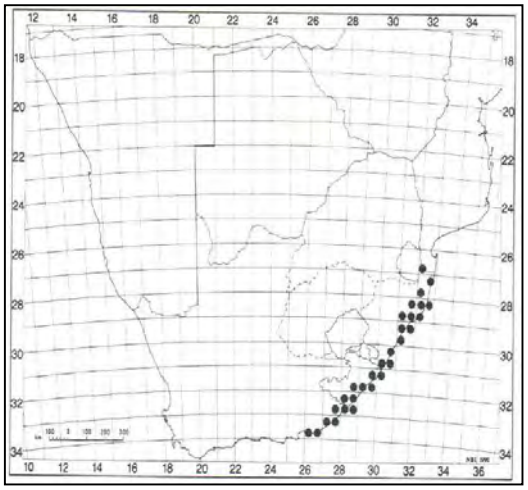

Figure. 1.3 Distribution of *Brachylaena discolor* along the eastern coastline of South Africa (Source: Cilliers, 1993, pp. 182)

1.8. Structure of dissertation

This dissertation comprises six chapters. The present chapter (Chapter 1) identifies the problems that motivated this study, frames the study rationale and approach, states its aim and objectives and briefly describes the methodological approach, study sites and species. Chapter 2 reviews the relevant literature on air pollution with special focus on its effects on plants and the use of bioindicators in order to contextualise the study's design, methodology and conclusions. Chapter 3 details the sampling framework and methods used for all biomarker measurements and the data processing, and analyses. Chapter 4 present the results obtained (using graphs and tables) and describes the major trends/differences observed in relation to the statistical analyses conducted. Chapter 5 discusses the key findings of the study in relation to the broader literature on the effects of air pollution on plants. Finally, Chapter 6 provides the conclusions of the study and offers recommendations for future research based on the challenges/ difficulties encountered.

1.9. Conclusion

This study which builds on earlier research on the use of *B. discolor* as a bioindicator of air pollution (conducted by Areington *et al.*, 2015) could assist the EMA in establishing this species as a bioindicator of air pollution within one of South Africa's most polluted industrial areas. The range of biomarkers assessed in the study, also allow for the identification of suitable markers for measuring the effects of air pollution in tree leaves. The study's findings highlight the value of using trees as bioindicators of air pollution within rapidly developing cities such as eThekwini and contribute to the growing body of knowledge on the use of plants as bioindicators of environmental change/disturbance.

CHAPTER 2 REVIEW OF LITERATURE

2.1. Air pollution

Air pollution refers to changes in the concentration of atmospheric gases to an extent that can be detrimental to environmental and human health (Taylor *et al.*, 1994; Hijano *et al.*, 2005; Assadi *et al.*, 2011; Rai *et al.*, 2011). Air pollution is a result of many anthropogenic activities, specifically the burning of fossil fuels for energy and transportation (Taylor *et al.*, 1994; Bytherowicz *et al.*, 2007; Ramanathan and Feng, 2009). During the combustion of fossil fuels air pollutants are emitted into the atmosphere and once in the atmosphere, these pollutants can chemically interact and combine with one another to form secondary pollutants such as particulate matter (PM) and O_3 (Ramanathan and Feng, 2009; Henneman *et al.*, 2016). The main pollutant gases are: carbon dioxide (CO_2), carbon monoxide (CO), nitrogen oxides (NO_x), sulphur oxides (SO_x) and ozone (O_3) (Taylor *et al.*, 1994; Ramanathan and Feng, 2009; Assadi *et al.*, 2011; Rai *et al.*, 2011). Air pollution is a transboundary event with no particular area being immune to it (Ramanathan and Feng, 2009). Furthermore, where the effects are being experienced may not necessarily be where the air pollution was emitted (Diab *et al.*, 2002; Ramanathan and Feng, 2009; Rai *et al.*, 2011). An example of this was discussed by Ramanathan and Feng (2009), where air pollution emitted in Europe could travel to Africa in less than a week due to the air flow and air transportation routes.

2.2. Linking air pollution to climate change

Air pollution and climate change are more interconnected than originally assumed (Bytherowicz *et al.*, 2007). One link made between climate change and air pollution is that both arise from common sources (Swart e*t al.*, 2004). The element/compound chemistry that makes up the atmosphere is altered both chemically and physically when pollutants are released into the atmosphere (Mickley *et al.*, 2004; Swart *et al.*, 2004; Ramanathan and Feng, 2009). This altered atmosphere is what has been affecting current climatic conditions and will continue to do so, unless appropriate action such as a drastic reduction in the emission of greenhouse gases by developed and developing countries are undertaken (Mickley *et al.*, 2004; Swart *et al.*, 2004; Ramanathan and Feng, 2009).

The various pollutants and their concentrations play a role within the atmosphere when it comes to reflecting and/or absorbing incoming/outgoing UV radiation (Swart *et al.*, 2004; Ramanathan and Feng, 2009). Some pollutants (PM, O_3 and SO_2) can absorb and reflect incoming radiation promoting a phenomenon known as global cooling (Swart *et al.*, 2004; Ramanathan and Feng, 2009), while others (such as CO_2) trap outgoing UV radiation resulting in a phenomenon known as global warming, which is the current situation facing the planet (Swart *et al.*, 2004; Bytherowicz et al., 2007; Ramanathan and Feng, 2009). These pollutants have negative impacts on the climate and weather patterns as well as on the hydrological cycle (from evaporation rates to precipitation) (Swart *et al.*, 2004; Ramananthan and Feng, 2009).

Even though air pollution is one of the established causes of climate change, policy makers as well as a few scientists still view the two as separate, unrelated events (Swart *et al.*, 2004; Bytherowicz *et al.*, 2007). Currently, policies for air pollution are short-term while those for climate change are long-term; they also fail to recognise the link between the two (Swart *et al.*, 2004). So, if climate change and air pollution are interlinked and affect the environment at both local and regional scales, then, mitigation policies should incorporate these linkages (Swart *et al.*, 2004).

2.3. Air pollution in South Africa

Air pollution poses a major concern globally, but is especially heightened in developing countries such as South Africa (Emberson *et al.*, 2001; Matooane and Diab, 2001; Rai *et al.*, 2011; Henneman *et al.*, 2016). More specifically, industrial areas of South Africa have the highest pollution concern (Emberson *et al.*, 2001; Matooane and Diab, 2001; Josipovic *et al.*, 2010; Naiker *et al.*, 2012; Buthelezi and Davies, 2015; Henneman *et al.*, 2016). Due to the high dependence on industries for economic growth, developing countries such as South Africa often overlook the damage these industries inflict on natural/indigenous vegetation, unless it is impacting on agricultural vegetation (Emberson *et al.*, 2001; Matooane and Diab, 2001; Josipovic *et al.*, 2010; Naiker *et al.*, 2012; Henneman *et al.*, 2016). The pollutants that are associated with industrial activities that are of concern for South Africa are SO_2, NO_2, CO and O_3 (Buthelezi and Davies, 2015). In the present study we examined the effects of SO_2 pollution on an indigenous tree species (viz. *Brachylaena discolor*) growing within a highly polluted

industrial hub in the eThekwini Municipal Area (EMA, South Africa), specifically the South Durban Basin (SDB).

2.4. Sulphur dioxide levels in South Africa

Sulphur dioxide has been chosen as the reference pollutant for this study because SO_2 has been identified as a key pollutant in South Africa; it is the most predominant pollutant emitted within industrial areas across the country, and has damaging effects on vegetation (Emberson *et al.*, 2001; Matooane and Diab, 2001; Diab *et al.*, 2002; Hijano *et al.*, 2005; Diab and Motha, 2007; Assadi *et al.*, 2001; Buthelezi and Davies 2015). Sulphur dioxide is a problem in many developing countries including South Africa (Emberson *et al.*, 2001; Rai *et al.*, 2011). Sulphur dioxide is also the precursor to other harmful pollutants such as PM and acid rain which have serious ecological consequences (Ramanathan and Feng, 2009; Josipovic *et al.*, 2010; Ramllal *et al.*, 2015).

Air pollution, especially in terms of SO_2, within the SDB (the area of interest in this study), is of major concern within South Africa (Matooane and Diab, 2001). The SO_2 levels within the SDB have been well documented over the years and previous studies have suggested it to be the major pollutant released by the petrochemical and chemical industries (Matooane and Diab, 2001; Diab *et al.*, 2002; Diab and Motha, 2007; Rai *et al.*, 2011). The focus on SDB is due to the fact that it is infamous for its high air pollution levels and has been deemed a "hotspot" for industrial air pollution (Matoooane and Diab, 2001; Diab and Motha, 2007; Buthelezi and Davies, 2015).

2.5. Air pollution monitoring

To avoid unnecessary damage to plants, animals and humans that may be caused by air pollution (Matooane and Diab, 2003; Ramanathan and Feng, 2009) monitoring air quality has become invaluable (Emberson *et al.*, 2001; Josipovic *et al.*, 2010). Monitoring air pollution efficiently allows for more effective air pollution mitigation strategies (Josipovic *et al.*, 2010). Josipovic *et al.* (2010) highlighted the need for studies such as the present one, in saying that not much effort has gone into evaluating the effects of air pollution on indigenous vegetation within South Africa. Air pollution needs to be accurately monitored in order to completely understand these effects and strategies need to be accurately informed so that the appropriate policies are developed

in order to prevent unnecessary damage to the environment (Moodley *et al.*, 2011). This monitoring of air pollution can be achieved by means of technology and/or biological methods (Conti and Cecchetti, 2001).

2.5.1. Air pollution monitoring technology

There are two main technological methods used in air pollution monitoring, analytical (active) and passive techniques (Conti and Cecchetti, 2001; Bogdal *et al.*, 2013; Moodley *et al.*, 2011; Snyder *et al.*, 2013). Most instrumental samplers of air pollution are pollutant specific and generally monitor only one pollutant at a time (Josipovic *et al.*, 2010; Snyder *et al.*, 2013). In order for such samplers to be effective individuals making use of this technology also require extensive knowledge on how to access, process and interpret the data (Moodley *et al.*, 2011; Bogdal *et al.*, 2013; Snyder *et al.*, 2013).

Passive samplers involve the molecular diffusion of the gases across a filter, which is specific to each pollutant species (Moodley *et al.*, 2011; Josipovic *et al.*, 2010; Snyder *et al.*, 2013). They are more cost-effective than the conventional methods currently being used (Bogdal *et al.*, 2013; Snyder *et al.*, 2013). They are portable, unlike the analytical methods of monitoring, and offer more opportunity for air pollution data to be shared with the public (Moodley *et al.*, 2011; Bogdal *et al.*, 2013; Snyder *et al.*, 2013). However, passive samplers cannot be left in the field to record data continuously over a prolonged period as there are limits to their operational time (which are product dependent) (Snyder *et al.*, 2013). Passive samplers are also not reliable over large distances and therefore multiple samplers would be required, increasing the cost (Snyder *et al.*, 2013). Those authors also insist that a large number of passive samplers measuring simultaneously would increase the reliability of the data being received. Passive samplers are still under rigorous scrutiny with respect to the level of accuracy when compared to analytical samplers (Josipovic *et al.*, 2010; Snyder *et al.*, 2013). Josipovic *et al.* (2010) reports the use of passive sampling in their study where these samplers were distributed across the Mpumalanga Highveld industrial area. However, the administrative responsibilities involved in a study of such magnitude are enormous (Josipovic *et al.*, 2010).

The most conventional method of monitoring air pollution is by means of analytical monitoring techniques (Conti and Cecchetti, 2001). Analytical methods do however, require long periods of continuous data to attain accurate air pollution data for a specific region (Conti and Cecchetti, 2001; Diab and Motha, 2007; Moodley *et al.*, 2011). They are also expensive to install, require extensive maintenance and man power to ensure that the equipment is accurate (Conti and Ceccehetti, 2001; Emberson *et al.*, 2001; 2003; Moodley *et al.*, 2011; Naiker *et al.*, 2012). Analytical techniques are stationary and require a continuous power source; this makes them vulnerable to data gaps and equipment malfunction (Moodley *et al.*, 2011; Snyder *et al.*, 2013). Due to the extensive knowledge needed to operate/interpret and cost associated with maintenance, the data received have often been limited to the party that install the monitoring station, which in the present study is the air quality monitoring stations owned by the eThekwini Municipality (Diab *et al.*, 2002; Moodley *et al.*, 2011; Snyder *et al.*, 2013).

A number of studies have used air pollution data generated using analytical techniques. Tripathi and Gautam (2007) for example, made use of analytical methods for monitoring air quality at various sites in India; the bioindicator stations were used to measure SO_2, NO_x and suspended PM. South African examples include Diab *et al.* (2002) and Diab and Motha (2007) who obtained data from some of the eThekwini Municipality air quality monitoring stations used in this study. Due to political and socio-economic circumstances within the EMA, air quality monitoring and enforcement of air pollution mitigation policies have not been prioritised over the years (Naiker *et al.*, 2012; Buthelezi and Davies, 2015). This once again highlights the need for biological monitoring techniques within municipalities, such as eThekwini, as they offer a cheaper and more comprehensive alternative for monitoring air pollution (Conti and Cecchetti, 2001).

2.5.2. Biological monitoring of air pollution

Nature offers a quick, more feasible option for monitoring air pollution in the form of biological organisms known as bioindicators (Conti and Cecchetti, 2001; Moraes *et al.*, 2002; Madejón *et al.*, 2004; Hijano *et al.*, 2005). Bioindicators can be used to evaluate the detrimental effects of anthropogenically caused environmental stresses, such as air pollution, on abiotic and biotic components in a specific ecosystem (Conti and

Cecchetti, 2001; Hijano *et al.*, 2005; Bermudez and Pignata, 2011). In order for an organism to qualify as a bioindicator it must fulfil certain requirements (Conti and Cecchetti, 2001). Some of those criteria include whether the potential bioindicator is an indigenous species, is able to withstand high pollution levels, has a wide geographical distribution, is abundant, easily accessed and is negatively impacted, without succumbing, as a result of the pollution levels in the surrounding atmosphere (Conti and Cecchetti, 2001; Manning *et al.*, 2002; Moraes *et al*., 2002; Novak *et al.*, 2003). Native *in situ* bioindicators allow for continuous monitoring over prolonged periods of time (Mičieta and Murín, 1998; Conti and Cecchetti, 2001; Novak *et al.*, 2003). Monitoring air quality with bioindicators can prevent irreversible damage to ecosystems, aid with appropriate policy making, guard against global/local pollution standards being breached, complementing existing air quality monitoring technology, and ultimately alleviate the financial strain that air quality monitoring technology places on developing countries (Conti and Cecchetti, 2001; Naumann *et al.*, 2007; Tripathi and Gautam, 2007; Moodley *et al.*, 2011; Naiker *et al.*, 2012; Ismail *et al.*, 2014). Bioindicators are also beneficial since they react to the cumulative effect of air pollution on the ecosystem rather than just individual pollutants as is the case with monitoring technologies (Conti and Cecchetti, 2001; Hijano *et al.*, 2005; Ismail *et al.*, 2014). Additionally, bioindicators belong to the biological system under air pollution threat and only biological organisms have the ability to reflect the tolerance limits of that specific environment (Conti and Cecchetti, 2001).

A review of the literature illustrates the successful use of many bioindicators of air pollution. For example, a number of studies have shown the utility of lichens in monitoring air pollution (Conti and Cecchetti, 2001; Hijano *et al.*, 2005). Furthermore, Moraes *et al.* (2002) successfully established tree species, viz. *Pisidium guajava* L. and *Pisidium cattlyanum* Sabine., as bioindicators of air pollution within Brazil. The authors placed tree saplings around various petrochemical and other industrial sites, with known air pollution levels, and evaluated the response of the saplings to the levels of air pollution. Successful attempts to establish trees as bioindicators of air pollution include *Tillandsia capillaries* Ruín & Pav, *Tillandsia recurvata* L., and *Tillandsia tricholepis* Baker. in Argentina (Bermudez and Pignata, 2011), *Mangifera indica* Linn., *Cassia fistula* Linn., and *Eucalyptus* hybrid in India (Tripathi and Gautam, 2007), *Pinus*

sylvestris L., *Pinus nigra* Arn. and *Pinus mugo* L. in Slovakia (Mičieta and Murín, 1998), and *Bauhinia blakeana* Dunn. in Hong Kong (Lau and Luk, 2007).

While research on bioindicators is extensive in other parts of the world there is little, to no, knowledge on their applicability within South Africa (Josipovic *et al.*, 2010). These authors emphasised the need for more systematic and detailed research on indigenous vegetation in South Africa to examine whether the threshold for air pollution is being breached. While there are examples of bioindicators identified in South Africa, these are few and highly diverse. For example, ants were used as bioindicators in Richards Bay to monitor the heavy metals deposited around industrial areas (Majer and de Kock, 1992; Majer *et al*., 2007). *Potamonautes warreni* (Calman, 1918), a river crab was also used as a bioindicator of heavy metals in the Mooi River in the North West Province, South Africa (Schuwerack *et al.*, 2001). Olowoyo *et al.* (2011) established lichens as a bioindicator for trace elements in Tshwane Municipality, South Africa; while tree bark of *Acacia karroo* Hayne was used as a bioindicator of hexavalent chromium (Cr VI) in the North West Province, South Africa (Mandiwana *et al.*, 2006).

There is, however, a critical need for more widespread and easily available bioindicators due to the lack of legislation and knowledge on threshold for South African vegetation to air pollution (Josipovic *et al.*, 2010).

2.6. Air pollution effects on plants

To date, the focus has been largely on the effects of air pollution on human health and very few studies have focused on its effects on local vegetation (Emberson *et al.*, 2001; Matooane and Diab, 2003). Air pollution can have detrimental effects on plants growing in different habitats (Emberson *et al.*, 2001; Tiwari *et al.*, 2006; Ramanathan and Feng, 2009; Rai *et al.*, 2011; Jochner *et al.*, 2015). These effects cannot be ignored since plants are an important part of ecosystems and humans are dependent on them (Assadi *et al.*, 2011; Rai *et al.*, 2011). The effects of air pollution on plants are still not entirely known and the way a plant responds to air pollution is dependent on the combination of pollutants acting together, the concentration of each pollutant and the duration of exposure (Novak *et al.*, 2003; Hijano *et al.*, 2005; Assadi *et al.*, 2011; Rai *et al.*, 2011; Li and Yi, 2012). The response of plants to air pollution also varies across species (Hijano *et al.*, 2005; Gillespie *et al.*, 2011; Assadi *et al.*, 2011; Minibayeva *et al.,* 2009).

Exposure to air pollution for extended periods of time or short periods of very high levels of pollution can result in plant death (Hijano *et al.*, 2005; Rai *et al.*, 2011). Therefore, understanding the mechanisms of plant responses to stresses, such as air pollution in this study, is essential for the development of suitable conservation and climate change mitigation strategies (Chapin 1991; Taylor *et al.*, 1994; Naumann *et al.*, 2007; Rai *et al.*, 2011; Jochner *et al.*, 2015). Plant responses to stress, in particular air pollution stress, can be biochemical, physiological and/or morphological (Naumann *et al.*, 2007; Tripathi and Gautam, 2007; Assadi *et al.*, 2011; Rai *et al.*, 2011; Jochner *et al.*, 2015).

Some general responses of plants to air pollution stress include: damage to photosynthetic apparatus and mechanisms, which influences growth and development of the plant, premature senescence, programmed cell death, altered metabolic activity, gene expression, and eventually plant death (Naumann *et al.*, 2007; Rai *et al.*, 2011; Seyyednejad and Koochak, 2011; Li and Yi, 2012; Ahmad *et al.*, 2014; Ismail *et al.*, 2014). Air pollution could also cause morphological damage such as chlorosis and necrosis (Rai *et al.*, 2011; Seyyednejad and Koochak, 2011; Li and Yi, 2012; Jochner *et al.*, 2015) and alter leaf area (Burton *et al.*, 1991; Conti and Cecchetti, 2001; Tiwari *et al.*, 2006).

2.6.1. Biochemical effects

Air pollution affects plants at the biochemical level when the air pollutants are taken up through the stomata of the leaves (**Fig. 2.1 A**) which results in the formation of intra or extracellular reactive oxygen species (ROS) (Arora *et al.*, 2002; Gill and Tuteja, 2010; Rai *et al.*, 2011). The production of ROS is a natural consequence of various metabolic processes in all organisms (Vranová *et al.*, 2002; Minibayeva *et al.*, 2009; Gill and Tuteja, 2010; Li and Yi, 2012). In fact, ROS has been implicated in signalling for other necessary metabolic activities such as germination, plant growth, development and programmed cell death; ROS can even cause the expression of stress-tolerant genes (Arora *et al.*, 2002; Vranová *et al.*, 2002; Minibayeva *et al.*, 2009; Gill and Tuteja, 2010; Li and Yi, 2012). Abiotic and biotic stresses disrupt the normal metabolic processes within organisms (Mittler *et al.*, 2004; Ahmad *et al.*, 2014). Organisms in general, and plants in particular, can control/manage natural ROS production and

understanding this relationship is vital in characterising plant biochemical responses to a stress (Mittler *et al.*, 2004; Suzuki and Mittler, 2006). These biochemical responses are also usually among the first to occur under stressful conditions (Tripathi and Gautam, 2007).

2.6.1.1. Reactive oxygen species

Biological redox reactions involving oxygen are a natural occurrence within cells of all biotic organisms (Arora *et al.*, 2002; Vranová *et al.*, 2002; Gill and Tuteja, 2010). Reactive oxygen species consist of free radicals, molecules and ions that evolve from oxygen (O_2) (Sharma *et al.*, 2012; Ahmad *et al.*, 2014). The unique properties of the O_2 molecule are that it possesses two valence electrons (Gill and Tuteja, 2010). This makes O_2 more inclined to absorb energy that reverses the spin on the valance electron to form singlet oxygen or to accept unpaired electrons/protons (to form all other ROS) (Gill and Tuteja, 2010; Sharma *et al.*, 2012). These excess electrons are donated by the electron transport chain (ETC), which is responsible for the creation of the necessary energy (adenosine triphosphate [ATP]) required for cell metabolism such as photosynthesis in plants (Gill and Tuteja, 2010). The ETC within the mechanics of the photosynthetic process allows for the formation of ROS such as singlet oxygen ($^1O_2$), hydroxyl radical ($\cdot OH$), superoxide anion radical ($\cdot O_2^-$) and hydrogen peroxide molecule (H_2O_2) (**Fig 2.1 C**) (Arora *et al.*, 2002; Vranová *et al.*, 2002; Gill and Tuteja, 2010; Foyer and Shigeoka, 2011). The sites where most ROS production occurs in plants are the mitochondria (the power station of the plants) and chloroplasts (Mittler *et al.*, 2004; Gill and Tuteja, 2010; Suzuki *et al.*, 2012; Ahmad *et al.*, 2014). For example, excess electrons are released in the chloroplasts, and become highly reactive; due to this the electrons are more inclined to donate themselves to atmospheric O_2 by the electron carrier ferredoxin (Fd_{red}) and thus creating $\cdot O_2^-$ (**Fig. 2.1 B**) (Arora *et al.*, 2002; Sharma *et al.*, 2012).

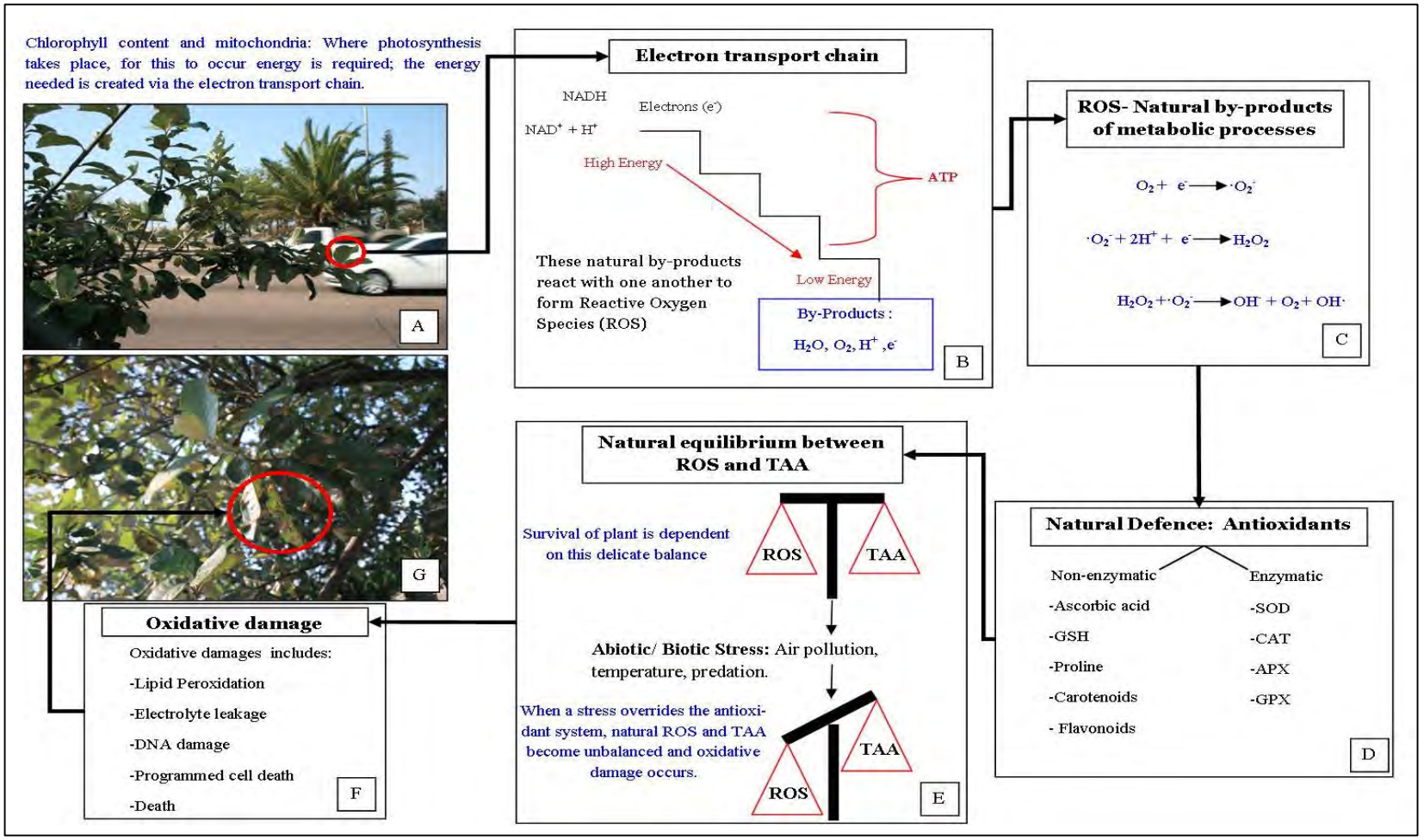

Figure 2.1 Flow diagram illustrating pro- and anti- oxidative metabolism in plants: (**A**) Leaf from a *B. discolor* tree at a sampling site, exposed to the air pollution, (**B**) Energy transformation processes and by-products produced via the electron transport chain, (**C**) Some of the reactions that lead to ROS formation, (**D**) The antioxidant system, (**E**) The delicate balance between the antioxidant system and ROS production in the context of stress, (**F** and **G**) the possible damage that can occur as a result of oxidative stress (Photo **A** and **G**: Minoli Appalasamy and Candyce Areington) (adapted from: Arora *et al.*, 2002; Vranová *et al.*, 2002; Gill and Tuteja, 2010).

Superoxide is the first ROS to be formed and is the precursor to other more harmful species (**Fig 2.1 C**) (Arora *et al.*, 2002; Fleck *et al.*, 2003; Gill and Tuteja, 2010; Sharma *et al.*, 2012). Superoxide has a relatively short life span (usually micro seconds) within the tissue and is the least toxic ROS, $\cdot O_2^-$ is generally the precursor to other more long lived and toxic ROS (Vranová *et al.*, 2002; Tripathi and Gautam, 2007; Ahmad *et al.*, 2014). The formation of other ROS is commonly catalysed by the means of an enzyme or metal (Sharma *et al.*, 2012). For example, the dismutase of $\cdot O_2^-$ (by means of superoxide dismutase [SOD]) leads to the formation of H_2O_2 (Vranová *et al.*, 2002; Fleck *et al.*, 2003; Gill and Tuteja, 2010; Sharma *et al.*, 2012; Ahmad *et al.*, 2014). Unlike $\cdot O_2^-$, H_2O_2 is more toxic and long lived; making mobility across membranes easier and hence the potential range of damage larger than that of any other ROS (Fleck *et al.*, 2003; Ahmad *et al.*, 2009; Gill and Tuteja, 2010; Sharma *et al.*, 2012). The fact that H_2O_2 does not have any unpaired electrons is what makes this a more stable molecule that is easy to move around the organism (Sharma *et al.*, 2012). Hydrogen peroxide is the precursor to one of the most toxic ROS, the hydroxyl radical ($\cdot OH$), which is produced through a Fenton's reaction when H_2O_2 reacts with Fe^{2+} and/or through the Haber-Weiss reaction where H_2O_2 and $\cdot O_2^-$ react with each another (Bolwell and Wojtaszek, 1997; Fleck *et al.*, 2003; Ahmad *et al.*, 2009; Gill and Tuteja, 2010; Sharma *et al.*, 2012).

Leaves are in direct contact with the atmosphere, and hence, can be negatively influenced by air pollution (**Fig 2.1 A**) (Lau and Luk, 2000). Rai *et al.* (2011) explained that water within the apoplastic region of the leaf dissolves atmospheric SO_2, which is taken up by the leaves through the stomata and once within the cytoplasm of leaf cells it is converted into sulphurous acid. This, in turn, is initiated by light and forms sulphite (SO_3^{2-}) and bisulfite (HSO_3^-) which further decomposes into sulphates (SO_4^{2-}) (Arora *et al.*, 2002; Hijano *et al.*, 2005; Tripathi and Gautam, 2007; Rai *et al.*, 2011; Li and Yi, 2012). Sulphur dioxide breaks down into anions, which is facilitated by the ETC and can then evolve into various toxic species of itself (e.g. SO_3^{2-}, SO_3^-, HSO_3^-) with by-products such as $\cdot OH$, $\cdot O_2^-$ and H_2O_2 (Arora *et al.*, 2002; Tripathi and Gautam, 2007; Rai *et al.*, 2011) (**Fig 2.1 C**). Studies have shown that air pollution stress can lead to excessive production of many of these ROS within the plant tissues (Arora *et al.*, 2002;

Tiwari *et al.*, 2006; Tripathi and Gautam, 2007; Li and Yi, 2012), including leaving (Lau and Luk, 2000; Areington *et al.*, 2015).

2.6.1.2. ROS induced damage

All organisms have the ability to counter the formation of ROS; however, excessive and uncontrolled production of ROS can undermine the survival of an organism (Minibayeva *et al.*, 2009; Rai *et al.*, 2011; Li and Yi, 2012). Abiotic or biotic stresses such as air pollution (increase in [SO_2]) for example, can result in a 'burst' of ROS (Arora *et al.*, 2002; Gill and Tuteja, 2010; Sharma *et al.*, 2012). Should ROS be left unquenched, it could cause oxidative damage, that could severely hinder cellular functioning (Seyyednejad and Koochak, 2011; Gill and Tuteja, 2010). Irreparable damage to nucleic acids, proteins and lipids could also be consequential to excess ROS production and this can lead to programmed cell death and ultimately the death of the organism (Gill and Tuteja, 2010; Seyyednejad and Koochak, 2011; Li and Yi, 2012) (**Fig. 2.1 F** and **G**).

The polyunsaturated fatty acids (PUFAs) in the cell membranes of organisms are particularly susceptible to attack by ROS (specifically ·OH), which in excess can lead to irreversible damage to membranes (Valavanidis *et al.*, 2006; Ahmad *et al.*, 2009; Sharma *et al.*, 2012). Reactive oxygen species can break the double bond between the carbon atoms or sever the ester link between glycerol and fatty acids (Sharma *et al.*, 2012). This compromises the PUFAs by breaking the chains which lead to the breakdown of cell membranes and eventual loss of cell membrane integrity (Sharma *et al.*, 2012). Peroxidation of cell membrane lipids is a common and most damaging consequence of oxidative stress in most organisms (Gill and Tuteja, 2010; Seyyednejad and Koochak, 2011; Li and Yi, 2012; Sharma *et al.*, 2012). Lipid peroxidation (LPO) consists of three distinct stages: initiation, propagation and termination (being controlled by the antioxidant systems [discussed later]) (Gill and Tuteja, 2010; Sharma *et al.*, 2012). The initiation stage involves the transfer of a hydrogen atom from a PUFA (specifically the unsaturated fatty acyl chain) to, generally, ·OH radicals which then forms a lipid alkyl radical (R·) (Gill and Tuteja, 2010; Sharma *et al.*, 2012). The propagation stage involves the uptake of O_2 which leads to a lipid peroxy radical (ROO·), which then through a further transfer of hydrogen atoms from an adjacent

PUFA results in other secondary reactive species such as: lipid alkoxyl radicals, aldehydes (e.g. malonyldialdehyde [MDA]), alkanes, and lipid expoxides (Gill and Tuteja, 2010; Sharma *et al.*, 2012). These reactive species are then responsible for the breakdown of the PUFA chains which causes membrane damage which could cause leakage of electrolytes and an abnormal increase in membrane fluidity and permeability (Gill and Tuteja, 2010; Sharma *et al.*, 2012).

Reactive oxygen species can also modify the components that makeup proteins (e.g. amino acids) (Gill and Tuteja, 2010; Sharma *et al.*, 2012). They do this through protein oxidation which is essentially irreversible. Reactive oxygen species will generally attack proteins that contain sulphur (Gill and Tuteja, 2010; Sharma *et al.*, 2012). These modifications can lead to DNA damage (Gill and Tuteja, 2010; Sharma *et al.*, 2012). The ·OH radical is generally known to attack purine, pyrimidine bases and deoxyribose; while singlet oxygen ($^1O_2$) attacks guanine (Gill and Tuteja, 2010; Sharma *et al.*, 2012). These lesions can ultimately lead to the deletion of bases, cross links, strand breaks or base modification, all of which can affect the physiology and morphology of the organism (Gill and Tuteja, 2010; Sharma *et al.*, 2012).

2.6.1.2. Natural defence mechanisms

Due to the natural occurrence of oxidative metabolism within organisms, there are natural/house-keeping defence mechanisms in place to aid in the detoxification of harmful and/or excessive ROS into less toxic ROS or other chemicals (Arora *et al.*, 2002; Vranová *et al.*, 2002; Ahmad *et al.*, 2008; Gill and Tuteja, 2010; Varjovi *et al.*, 2015). A plant's survival is dependent on the balance between ROS production and the antioxidant system (Arora *et al.*, 2002; Mittler *et al.*, 2004; Ahmad *et al.*, 2008; Ahmad *et al.*, 2014; Varjovi *et al.*, 2015) (**Fig. 2.1 E**). The antioxidant system that is responsible for ensuring the quenching and detoxification of ROS includes enzymatic and non-enzymatic antioxidants (Vranová *et al.*, 2002; Ahmad *et al.*, 2008; Gill and Tuteja, 2010; Ahmad *et al.*, 2014; Varjovi *et al.*, 2015). The main enzymatic antioxidant systems includes: superoxide distmutase (SOD), catalase (CAT), ascorbate peroxidise (APX), glutathione peroxidise (GPX) and glutathione S-transferase (GST) (Mittler *et al.*, 2004; Gill and Tuteja, 2010; Ahmad *et al.*, 2014; Varjovi *et al.*, 2015) (**Fig 2.1 D**). Non-enzymatic antioxidants which were not examined in this study include: ascorbic

acid (vitamin C), glutathione (GSH), proline (Pro), α-tocopherols (vitamin E), carotenoids (Car) and flavonoids (Gill and Tuteja, 2010; Ahmad *et al.*, 2014). These antioxidants can be located at different antioxidant-specific locations within the cells (Varjovi *et al.*, 2015).

Superoxide dismutase is central to defending the organism against oxidative stress; for example, SOD dismutases $\cdot O_2^-$ by converting it to H_2O_2 (which is then quenched further by CAT) and O_2, hence avoiding the formation of ·OH (Ahmad *et al.*, 2008; Sharma *et al.*, 2012; Ahmad *et al.*, 2014). Superoxide dismutase has been suggested to be a very important antioxidant against excessive ROS (Arora *et al.*, 2002; Tripathi and Gautam, 2007; Gill and Tuteja, 2010; Li and Yi, 2012; Sharma *et al.*, 2012; Ahmad *et al.*, 2014). The increased SOD activity during a stress condition can result in an increase in the stress tolerance of the organism (Ahmad *et al.*, 2014). Catalase is the enzyme that can be specific for quenching H_2O_2 by converting it to O_2 and H_2O (Arora *et al.*, 2002; Fleck *et al.*, 2003; Gill and Tuteja, 2010; Ahmad *et al.*, 2008; Sharma *et al.*, 2012; Ahmad *et al.*, 2014). Catalase is also said to be the most active enzyme among the enzymatic antioxidants, having the highest turnover rate making it irreplaceable to an organism under stress conditions (Ahmad *et al.*, 2008; Arora *et al.*, 2002; Gill and Tuteja, 2010; Sharma *et al.*, 2012). This is largely because CAT is generally found across all parts of the cell (Varjovi *et al.*, 2015). According to Sharma *et al.* (2012), both SOD and CAT activity in plants increase with an increase in abiotic/biotic stress.

Ascorbic acid, a non-enzymatic antioxidant, has been extensively studied (Ahmad *et al.*, 2008; Gill and Tuteja, 2010; Sharma *et al.*, 2012; Ahmad *et al.*, 2014). Ascorbic acid is an overall detoxifier for excessive ROS due to its ability to donate electrons to help minimise damage to/and protect membranes, but ascorbic acid has also been known to quench H_2O_2 (Ahmad *et al.*, 2008; Gill and Tuteja, 2010; Sharma *et al.*, 2012; Ahmad *et al.*, 2014). Glutathione is responsible for the protection of many cellular components (e.g. the protection of nucleic acids, proteins and membrane activity); being a general scavenger it has the ability to quench: $^1O_2$, H_2O_2 and ·OH (Ahmad *et al.*, 2008; Gill and Tuteja, 2010; Sharma *et al.*, 2012; Ahmad *et al.*, 2014). Alpha (α)-tocopherols acts as a protective shield for lipids against lipid radicals and hence protects the cells from lipid

peroxidation (Ahmad *et al.*, 2008; Gill and Tuteja, 2010; Sharma *et al.*, 2012; Ahmad *et al.*, 2014).

2.6.2. Physiological effects

2.6.2.1 Chlorophyll fluorescence

Light is necessary for photosynthesis to occur and chlorophyll is responsible for harvesting the light energy (Murchie and Lawson, 2013). However, all the light that is absorbed by plants cannot all be effectively utilised; therefore plants have strategies that safely dispose of the excess light (Naumann *et al.*, 2007). These mechanisms include the re-emittance of light by means of heat, fluorescence or non-photochemicals (such as the xanthophyll cycle) (Naumann *et al.*, 2007). Chlorophyll fluorescence, in essence, is the measurement of light that is re-emitted by photosystem II (PSII) (Murchie and Lawson, 2013).

Chlorophyll fluorescence is directly linked to the plant's ability to photosynthesise; hence this biomarker has the potential to monitor the overall health of the plant (Murchie and Lawson, 2013). When measuring chlorophyll fluorescence, there are various parameters that can be considered, for example, F_o represents the minimum value for chlorophyll fluorescence (when PSII has been exposed to light); F_m is the maximum value for chlorophyll fluorescence (when PSII has not been exposed to light) (Naumann *et al.*, 2007; Murchie and Lawson, 2013; Ismail *et al.*, 2014) and F_v is the difference between F_o and F_m. This gives rise to the commonly used indicator of stress in plants, viz. F_v/F_m, which is a measure of the maximum quantum yield of PSII under dark adaptation conditions (Naumann *et al.*, 2007; Murchie and Lawson, 2013; Ismail *et al.*, 2014). Chlorophyll fluorescence has been used as a biomarker of various plants' stress (e.g. *Myrica cerifera* L. and *Phragmites australis* Cav. to salt and drought stress [Naumann *et al*., 2007]; and *Pisum sativum* L. to O_3 stress [Ismail *et al.*, 2014]). Murchie and Lawson (2013) state that healthy leaves will experience a F_v/F_m value of *ca.* 0.83 and this value will decrease if the plant is exposed to a stress (Naumann *et al.*, 2007; Murchie and Lawson, 2013; Ismail *et al.*, 2014). It is also important to note that chlorophyll fluorescence can indicate stress before physical manifestations or even before chlorophyll content is altered (Naumann *et al.*, 2007; Ismail *et al.*, 2014).

2.6.2.2. Chlorophyll content

Chlorophyll is the main pigment in chloroplasts and is responsible for the absorption of light, which aids in the production of the energy plants require for survival (Assadi *et al.*, 2011; Tanee and Albert, 2013). Chlorophyll absorbs light to initiate a process of conversion of water and oxygen to carbohydrates that are necessary for plant growth and survival (Tanee and Albert, 2013). Assadi *et al.* (2011) stated that the chlorophyll content provides valuable information on the overall health of the plant, especially in terms of its photosynthetic capabilities. An example would be the study of Rai *et al.* (2011) who showed that high [SO_2] would lead to the breakdown of chlorophyll within leaves. Both an increase or a decrease in chlorophyll content as a result of air pollution have been reported; however, this is dependent on the species observed, as well as the combination of pollutants involved (Hijano *et al.*, 2005; Tiwari *et al.*, 2006; Tripathi and Gautam, 2007; Assadi *et al.*, 2011; Tanee and Albert, 2013; Areington *et al.*, 2015).

2.6.3. Morphological effects

2.6.3.1. Leaf area

Leaves house the photosynthesis machinery and therefore a change in leaf area (LA) affects these processes (Burton *et al.*, 1991). Gas exchange, light absorption, evapotranspiration and photosynthesis are dependent on leaf area (Burton *et al.*, 1991). Leaf area has been reported to be negatively affected by SO_2 levels and air pollution in general (Burton *et al.*, 1991; Conti and Cecchetti, 2001; Tiwari *et al.*, 2006; Assadi *et al.*, 2011; Rai *et al.*, 2011). A plant may reduce its leaf area as a defence mechanism, in order to limit the amount of surface that is being exposed to air pollution (Assadi *et al.*, 2011; Jochner *et al.*, 2015). This parameter can reflect long term effects of air pollution on the plants and provides evidence as to whether or not the plant is coping with the stress (Assadi *et al.*, 2011; Jochner *et al.*, 2015).

2.7. Biomarkers

A bioindicator can respond morphologically, physiologically and biochemically to an environmental stress (Tripathi and Gautam, 2007; Assadi *et al.*, 2011; Bermudez and Pignata, 2011; Jochner *et al.*, 2015). These responses represent biomarkers and by measuring various biomarker responses to air pollution stress and the air quality of a specific ecosystem can be monitored (Tripathi and Gautam, 2007; Bermudez and

Pignata, 2011). Oxidative metabolism has been considered to be a reliable biomarker for air pollution stress by many authors (Tripathi and Gautam, 2007; Ismail *et al.*, 2014). When any organism is placed under stress, the biochemical responses are the first to be initiated (Tripathi and Gautum, 2007; Rai *et al.*, 2011). Biochemical biomarkers can, therefore, act as an early form of stress detection (Tripathi and Gautam, 2007; Rai *et al.*, 2011; Ismail *et al.*, 2014). By determining whether thresholds for air pollution have been reached within a specific ecosystem before physiological and morphological manifestations occur thereby increases the chance of survival for the ecosystem and shortens the recovery time (Hijano *et al.*, 2005; Naumann *et al.*, 2007; Tripathi and Gautam, 2007; Rai *et al.*, 2011; Seyyednejad and Koochak, 2011; Ismail *et al.*, 2014).

A biomarker should be easily measurable and produce specific trends unique to the stress that is being monitored as to not be confused with other environmental processes/stresses (Bermudez and Pignata, 2011). The biomarkers selected in this study were based on published reports of their value in reflecting/responding to a wide range of environmental stresses. The ROS that will be examined in this study, viz. $\cdot O_2^-$ and H_2O_2, have been used in previous studies on plant responses to air pollution (Tripathi and Gautum, 2007). Bermudez and Pignata (2011) explained that although it is widely known that the antioxidant system is a defence mechanism for excess stress, it has rarely been used as a biomarker. With previous studies showing the value of this system as a potential biomarker (Moraes *et al.*, 2002; Bremudez and Pignata, 2011), it was examined here. The antioxidants measured include: TAA, SOD and CAT. Electrolyte leakage is another biomarker that has been shown to be widely reported as a useful biomarker of air pollution (Conti and Cecchetti, 2001; Bremudez and Pignata, 2011). Lipid peroxidation, an indicator of excess ROS mediated oxidative damage has also been used as a biomarker in many previous studies (Conti and Cecchetti, 2001; Tiwari *et al.*, 2006; Li and Yi, 2012; Sharma *et al.*, 2012) and was examined here.

The physiological biomarkers used included chlorophyll fluorescence and chlorophyll content (Tiwari *et al.*, 2006; Assadi *et al.*, 2011; Murchie and Lawson, 2013; Tanee and Albert, 2013). Chlorophyll fluorescence is especially valuable due to its non-destructive way of assessing plant health (Murchie and Lawson, 2013; Ismail *et al.*, 2014). Physiological changes/responses generally occur after biochemical and before

morphological responses, thus making it a useful biomarker of air pollution as well (Naumann *et al.*, 2007; Assadi *et al.*, 2011; Ismail *et al.*, 2014).

Morphological biomarkers such as LA, which was also measured in this study, have been used in many studies with great sucess (Novak *et al.*, 2003; Tiwari *et al.*, 2006). Chlorosis and necrosis were not examined in this study (Moraes *et al.*, 2002; Novak *et al.*, 2003; Hijano *et al.*, 2005; Bermudez and Pignata, 2011; Jochner *et al.*, 2015). Though chlorosis and necrosis are quick and easy to determine they usually manifest when the leaves are at a point of no return (Tripathi and Gautam, 2007; Assadi *et al.*, 2011; Seyyednejad and Koochak, 2011) and are not easily quantifiable.

The organ of choice for this study was the leaf. Leaves are in direct contact with the atmosphere and are thus the most exposed to air pollution (Madejón *et al.*, 2004; Rai *et al.*, 2011; Seyyednejad and Koochak, 2011) (**Fig. 2.1. A**). Leaves have stomata which allow for gaseous exchange in order for photosynthesis to occur, making the leaf vulnerable to the infiltration of harmful pollutants (Rai *et al.*, 2011). In order to establish a bioindicator, the correct biomarkers that are sensitive enough to the air pollution that are best reflected within the species being examined need to be first determined. Moraes *et al.* (2002) emphasised the value of examining biochemical, physiological and morphological biomarkers when establishing trees as bioindicators for air pollution. A similar approach was undertaken which compared the responses of various biochemical, physiological and morphological biomarkers of plant stress to SO_2 pollution in *B. discolor* tree leaves.

CHAPTER 3 MATERIALS AND METHODS

3.1. Site selection

Selection of the industrial (treatment) sites used in this study was based on two factors: (a) the presence of an air quality monitoring station that measured ground-level SO_2 levels and, (b) the presence of a minimum of four mature *B. discolor* trees, within 1000 m of the monitoring station.

The following three study sites met the requirements mentioned above (co-ordinates of monitoring station given in parenthesis): Prospecton (30˚ 0' 10.44"S; 30˚ 55' 43.64"E), Ganges (29˚56' 54.60"S; 30˚57' 52.63"E) and Southern Works (29˚57' 25.20" S; 30˚58' 23.77"E). As shown in **Fig. 3.1**, all three treatment sites were located within the SDB, which forms part of the EMA, in KwaZulu-Natal, South Africa. Geographic co-ordinates of the individual trees sampled are given in **Table A** (see Appendix). The control trees were housed for one month before and for the duration of the study in a greenhouse (after Areington *et al.*, 2015) on the Westville Campus, University of KwaZulu-Natal (29˚ 49'3.76"S; 30˚56'23.56"E), located within the EMA, *ca.* 19 km from the SDB.

3.2. Air pollution data

The air pollution, more specifically SO_2 data used in this study, was measured by eThekwini Municipality-owned monitoring stations at each of the three treatment sites. Ground-level SO_2 concentrations were measured hourly for the duration of the study. The SO_2 detector installed at these monitoring stations used a fluorescent analyser (Monitor lab 9850B, 2003, Europe/Scotland). Sulphur dioxide was selected as an indicator of air pollution in this study since it is the only common pollutant measured across all three monitoring stations selected; however, it should be noted that a number of other pollutants have been associated with industries in the SBD (e.g. CO, O_3, NO_2 [Buthelezi and Davies, 2015]). The data received was processed to remove all erroneous values prior to any statistical analyses.

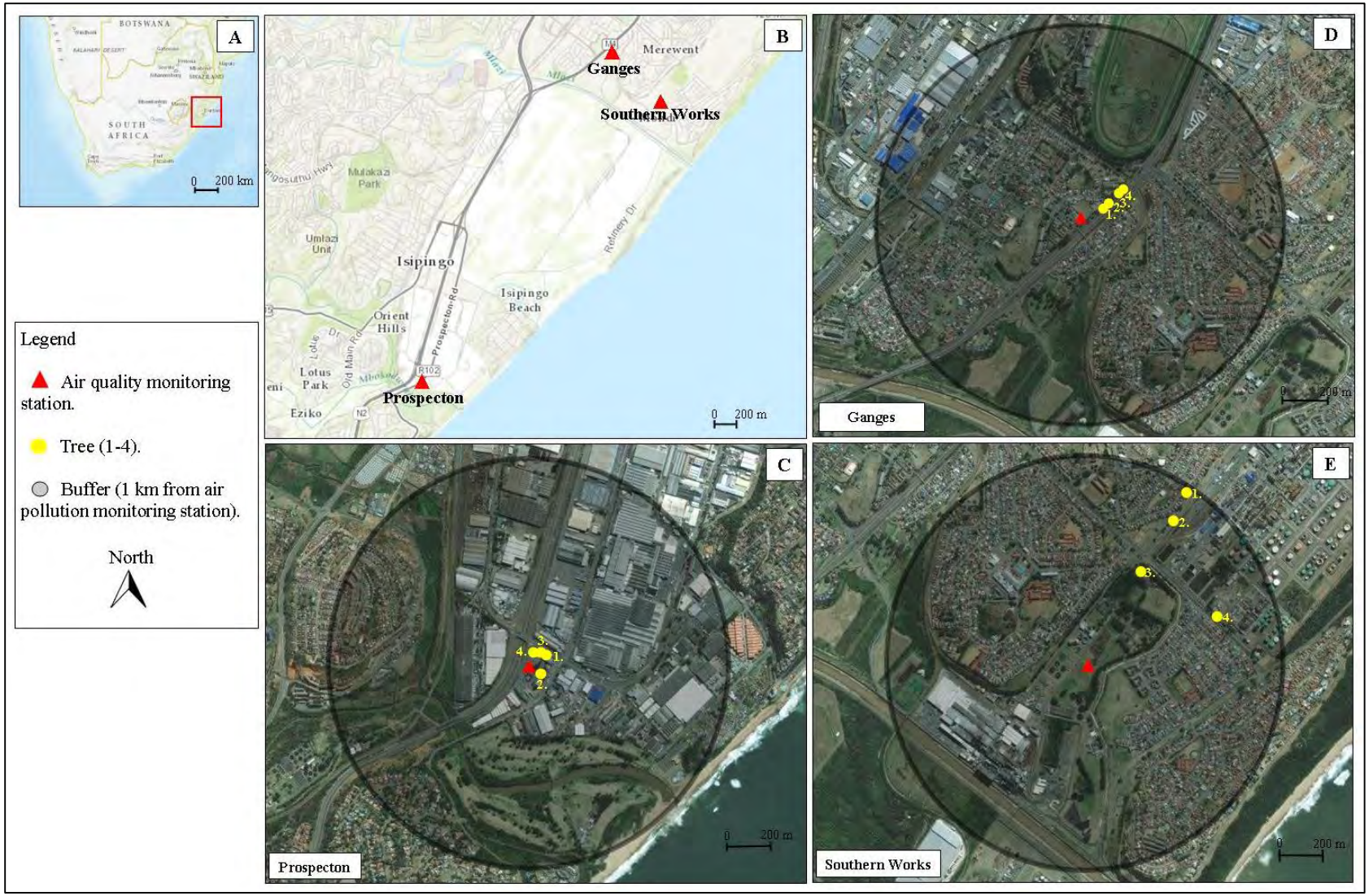

Figure 3.1 Location of eThekwini Municipal Area within South Africa, (**A**) and study sites within the South Durban Basin (**B**). Locations of the monitoring station and the four trees (1-4) sampled within a 1 km buffer zone at each study site (Prospecton [**C**], Ganges [**D**] and Southern Works [**E**]) (Source for Base Maps and aerial photos: NatGeo Mapmaker Interactive; adapted by Candyce Areington).

Due to the limited availability of the equipment, SO_2 levels were measured within the greenhouse in which the control trees were housed and at three random points (*n*=8), within 1 km of the greenhouse (on the same university campus) only during autumn (March-May, 2014). Measurements were carried out using a portable gas analyser (PG-350E, HORIBA, Ltd, UK). The SO_2 detector operates with a cross-flow modulation using a cross flow modulation non-dispersive infrared (NDIR) absorption method (according to the European Standard: DIN EN 15267-3, DIN EN 14181).

3.3. Plant material and sampling regime

Leaves were used for measurement of biomarker responses to SO_2 (after Lau and Luk, 2001; Tripathi and Gautam, 2007; Suzuki *et al.*, 2009 and Areington *et al.*, 2015). Trees at the treatment and control sites were always sampled >24 h after a rain event, with no tree being sampled more than once on any particular day. Sampling was carried out in each of the four seasons: autumn (March-May, 2014), winter (June–August, 2014), spring (September–November, 2014) and summer (December–February, 2014-2015), since plant biomarker responses to air pollution can differ across seasons (Novak *et al.*, 2003; Hijano *et al.*, 2005). To accommodate for the potential effects of wind direction (Rai *et al.*, 2011), leaves from each of the four trees were collected from all four cardinal directions (north, east, south and west) at each sampling event. The four trees at each site were sampled until a sample size *n*=24 was achieved for each parameter, for each season.

On each sampling day four 30-40 cm branches (one from each cardinal direction) were detached from the sampled tree and placed into water. The branches were transported back to the lab where the leaves were plucked and gently rinsed in deionised water (d.H_2O), to remove particulate matter before being processed for a range of bioassays. In order to avoid the confounding effects of leaf age (Rai *et al.*, 2011), the third leaf from the top of each branch (excluding immature leaves that were yet to shed its white tomentulose, which commonly coats the adaxial surface in young *B. discolor* leaves [authors observations; Appendix **B**]) was used for all bioassays.

3.4. Ex situ control

Based on recommendations made by Areington *et al.* (2015) with regards to the use of a greenhouse-based control in a study of this nature, the control trees were for one month

before (to allow acclimation) and for the duration of the study were grown in a greenhouse that exhibited no measureable levels of SO_2. Furthermore, to avoid other stresses the control trees were watered (*ca.* 500 ml) thrice a week and treated with nutrients (0.1 g Dr. Fisher's Multifeed® Classic [Grovida Horticultural Products CC, Durban, South Africa] in 100 ml d.H_2O) once a month.

The greenhouse was constructed of clear 5 mm thick Naxel polycarbonate sheeting (Mazey Plastics, South Africa) which has a light transmittance of ±90%. Nevertheless, light intensity, measured using a portable photosynthesis system (Li-6400, LI-COR, Lincoln, NE, USA) at midday on four clear sunny days; was slightly lower in the greenhouse than at the treatment sites. In order to investigate whether this difference in light intensity had any confounding effects on the results obtained, the light-dependent biomarkers measured in this study, viz. chlorophyll content and leaf area were also measured (in autumn and spring) for four trees located within 1 km of the greenhouse in which the control trees were housed. These data were in turn related to the SO_2 measurements carried out within 1 km of the greenhouse (describe above) in order to validate the trends observed for chlorophyll content and leaf area data collected at the treatment and control sites.

3.5. Biochemical parameters

All fine chemicals used in the biochemical assays described below were supplied by Sigma-Aldrich (Germany), unless otherwise stated. Additionally, all centrifugation of < 5,000 rpm, whenever needed was carried out using a Eppendorf™ 5 ml tube centrifuge (Eppendorf Centrifuge 5702, Hamburg, Germany): while all centrifugation of > 10,000 rpm was carried out using a Eppendorf™ cooling centrifuge (Microcentrifuge 5415 R, Hamburg, Germany). Spectrophotometric measurements were carried out using a Shimadzu-UV Vis spectrophotometer (Model UV-2600, Shimadzu, Japan). All biochemical assays were carried out on control and treatment leaves (n=24, for each season).

3.5.1. Intracellular superoxide

Estimation of leaf intracellular superoxide production was carried out as per Elstner and Heupel (1976). The fresh leaves were weighed and then ground using liquid nitrogen (LN) pre-chilled mortar and pestle with 100 mg of insoluble PVP

(polyvinylpyrrolidone) and 4 ml of ice cold phosphate buffer (65 mM, pH 7.8). The homogenate was centrifuged at 4,400 rpm for 30 min. One ml of this supernatant was mixed with 1 ml of 10 mM hydroxylamine HCl (1.7 mg hydroxylamine HCl dissolved in 25 ml of 65 mM phosphate buffer, pH 7.8) and incubated for 30 min in the dark. After incubation, 0.5 ml of the above mixture was mixed with 0.5 ml of 17 mM sulphanilamide (29.27 mg sulphanilamide in 10 ml of phosphate buffer, pH 7.8) and 0.5 ml of 7 mM 2-naphthylamine (prepared by dissolving 20.04 mg 2-naphthylamine in 400 µl of 100% ethanol and then brought to a final volume of 20 ml using 65 mM phosphate buffer, pH 7.8). Finally 30 µl of 5 N HCl was added and the solution was left for 30 min in the dark. This mixture was centrifuged at 13,000 rpm for 5 min at 4°C and the absorbance of the supernatant was measured at 530 nm using a spectrophotometer. A standard curve was constructed using sodium nitrite ($NaNO_2$) at concentrations 0.1 µM to 50 µM and was used to estimate superoxide concentration which was expressed in nmol g^{-1} on a fresh weight (FW) basis.

3.5.2. Intracellular hydrogen peroxide

Leaf intracellular hydrogen peroxide production was measured according to Jana and Choudhuri (1981) and modified by Hung *et al.* (2008). Using 4 ml of phosphate buffer (50 mM, pH 6.5), which contained 1 mM hydroxylamine; fresh leaves were ground with LN using a pre-chilled mortar and pestle with 100 mg of insoluble PVP. The homogenate was centrifuged at 4,400 rpm for 30 min. The supernatant was removed and mixed with 0.1% titanium (III) chloride in 20% sulphuric acid. After incubation in the dark for 15 min, the mixture was centrifuged at 4,400 rpm for 30 min. The absorbance of the supernatant was read at 410 nm using a spectrophotometer. The levels of H_2O_2 within the leaves were calculated using the extinction co-efficient 0.28 µmol g^{-1} and expressed as µmol H_2O_2 g^{-1} FW.

3.5.3. Total aqueous antioxidants

Brachylaena discolor leaves were measured for total aqueous activity (TAA) using the using 2,2'-azino-bis-3-ethylbbenzthiazoline-6-sulphonic acid (ABTS) assay (after Re *et al.*, 1999; as described by Berjak *et al.*, 2011). Fresh leaves were weighed and ground with LN in a pre-chilled mortar and pestle along with 100 mg of insoluble PVP. The ground tissue was then extracted using 4 ml of a 50 mM potassium phosphate buffer,

pH 7.0 (containing 1 mM $CaCl_2$, 1 mM KCl and 1 mM EDTA) then placed in 5 ml tubes. The extract was then centrifuged at 4,400 rpm and then the extract transferred into chilled 2 ml Eppendorf® tubes. The extract was briefly vortexed (Heidolph® Reax 2000, Gemini BV, the Netherlands) every 5 min for 15 min and then centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was then collected and held on ice for the assay.

Approximately 12-16 h prior to the assay, the ABTS solution (7 mM ABTS and 2.45 mM potassium persulphate in 1 ml of d.H_2O) was prepared. In order to ensure an initial absorbance of 0.68-0.72 at 734 nm, the ABTS was diluted with a 0.1 M phosphate buffer saline (PBS; pH 7.4) to generate the working solution. Five µl of the antioxidant extract was then added to 1 ml of the working solution and the absorbance measured at 0 and 120 seconds using a spectrophotometer at 734 nm. Using 0.1-1.5 M Trolox™ (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) dissolved in PBS, a standard curve was constructed and used to calculate TAA activity which was expressed as µmol Trolox equivalent g^{-1} FW.

3.5.4. Superoxide dismutase

Leaf superoxide dismutase (SOD) activity was measured according to Beauchamp and Fridovich (1971) as discussed by Varghese *et al*. (2011). Fresh leaves were processed as described for TAA extraction, up until the stage where the supernatant was incubated on ice. Immediately thereafter, the supernatant was transferred to the dark and 50 µl of the extract was mixed in a cuvette with 1715.4 µl of 50 mM sodium phosphate (pH 7.8), 23.4 µl (17 µM) riboflavin, 200 µl (0.01 M) methionine and 11.2 µl (0.056 mM) nitroblue tetrazolium (NBT). The cuvette was inverted and then read, at 0 min at 560 nm using a spectrophotometer. The cuvette was then placed in a container lined with aluminium foil and a 55-W fluorescent light, to activate of the reaction. After 10 min under the light the absorbance was read again at 560 nm. The auto-oxidation of NBT was used to calculate the enzymic activity of SOD. One unit of SOD was equivalent to 50% inhibition of photoreduction of NBT by the enzyme. SOD activity was expressed as units of SOD g^{-1} FW.

3.5.5. Catalase activity

The Catalase (CAT) activity was measured as per Claiborne (1985). Fresh leaves were processed as described for TAA extraction, up until the stage where the supernatant was incubated on ice. A decline in absorbance due to the breakdown of H_2O_2 was measured by mixing 250 µl of the tissue extract with 1750 µl of 0.05 M phosphate buffer (pH 7.0) and 1000 µl of 0.019 M H_2O_2 (30%) to give a total volume of 3000 µl (kept in the dark). The decline in absorbance of the supernatant was measured by reading it at 240 nm at 0 sec and 180 sec, using a spectrophotometer. CAT activity was expressed as µmol CAT min^{-1} g^{-1} FW.

3.5.6. Lipid peroxidation

Fresh leaves were ground with LN in a pre-chilled mortar and pestle with 5 ml of 20% trichloroacetic acid (TCA) in 0.5% thiobarbituric acid (TBA), as described by Heath and Packer (1968). The homogenate was then boiled in a water bath for 30 min at 95°C after which the homogenate was immediately cooled on ice for 10 min. The homogenate was then centrifuged for 45 min at 4,400 rpm. The absorbance of the supernatant was measured at three wavelengths: 440 nm, 532 nm for malondialdehyde (MDA) estimation and 600 nm for turbidity estimation. To ensure that there was no interference of sugar in the estimation, the absorbance at 440 nm was used in the formula as per Du and Bramlage (1992) to calculate the MDA (Refer to Appendix, Formula **C.**). The calculations for lipid peroxidation were expressed as µmol g^{-1} FW.

3.5.7. Electrolyte Leakage

Fresh leaves were cut into 1 cm^2 segments weighing *ca.* 0.1 g and used to measure electrolyte leakage according to Santamaría and Martín (1997). These leaf segments were placed into test tubes filled with 20 ml d.H_2O. The tubes were then incubated in a water bath held at 30°C for 2 h, after which 1.5 ml of leachate (from each tube) was pipetted into two cells (per sample) of the conductivity plate. Electrolyte leakage was then measured using an electrical conductivity meter (CM 100-2 Conductivity Meter, Reid and Associates, South Africa). The two pseudoreplicated readings for each sample, expressed as Sm^{-1} g^{-1} FW, were averaged prior to any further analyses.

3.6. Physiological parameters

3.6.1. Leaf chlorophyll fluorescence

Leaf maximum quantum efficiency of PSII (F_v/F_m) was measured using a portable pulse amplitude modulated fluorometer (Li-6400XT, LI-COR, Lincoln, NE, USA). The branches that were collected from the treatment sites were placed in the dark for 40 min, to allow for all electrons to drain from the photosystems (Kitajima and Butler 1975; Moradi and Ismail, 2007). One measurement per leaf was taken on the lamina, midway between the base and the apex of the third leaf from the top.

3.6.2. Leaf chlorophyll content

Leaf chlorophyll content was measured using a hand-held chlorophyll meter SPAD (Minolta SPAD-502, Minolta Camera Co. Ltd.). The SPAD was then used to take three measurements: one at the apex, one to the right and one to the left of the midrib as shown in **Fig 3.2** (after Coste *et al.*, 2010). The pseudoreplicates were averaged for each and chlorophyll content was expressed in terms of SPAD units. As discussed earlier, in order to validate the trends observed, leaves (n=24) of *B. discolor* trees located 1 km from the greenhouse were measured for chlorophyll content in autumn and spring.

Figure 3.2 Three points (indicated with circles) on a *B. discolor* leaf where the chlorophyll content was measured using a hand-held chlorophyll meter. (Photo: Minoli Appalasamy and Candyce Areington)

3.7. Morphological parameter

3.7.1 Leaf area

Leaf area (LA) was measured in cm^2 using a leaf area meter (CID, Inc., CI-202 Area Meter; Lincoln, Nebraska, USA) as per Tiwari *et al.* (2006). As discussed earlier, in order to validate the trends observed, leaves (n=24) of *B. discolor* from trees located with 1 km of the greenhouse were also measured for leaf area in autumn and spring.

3.8. Qualitative data

A series of driven transects were carried out each site in order to characterise the potential air pollution sources and land-use practices that could have impacted on the trees sampled. Photographic images were used to capture information on the location of the sampled trees relative to different land-use types, e.g. industrial, residential, commercial, green spaces and roads.

3.9. Statistics

All statistical analyses were performed (at level of 0.05 level of significance) using PASW 23 statistic version 23 (SPSS Inc., Chicago, Illinois, USA). All biomarkers data were tested for normality using the Shapiro-Wilk test, while the Kolmogorov-Smirnov test was used to test the air pollution data for normality. Non-parametric data was subject to one of two transformations: log or square root. Where biomarker data was not normally distributed, a non-parametric Analysis of Variance (ANOVA) was run on untransformed ranked data. For normally distributed data, a two-way ANOVA was used to test for significant differences, across the cardinal directions within sites and seasons; within sites across seasons; across sites within seasons; and finally across sites with annual data (i.e. biomarker data for different seasons pooled). A Kruskal-Wallis test/ANOVA was used to test for seasonal differences in [SO_2] across sites within season and for annual data (i.e. SO_2 data for different seasons pooled). The Pearson's correlation test was used to test for relationships between individual biomarkers and seasonal [SO_2] (if the assumptions for normality, even after transformations, were not met a non-parametric Spearman's correlation test was used).

CHAPTER 4 RESULTS

4.1. Introduction

This chapter presents the primary and secondary data generated in this study. It also reports on the results of the statistical analyses used to compare data for individual biomarkers across the three industrial (treatment) sites and the control. Statistical relationships between individual biomarkers and seasonal atmospheric [SO_2] at the different sites are also described, in order to assess the suitability of the various biomarkers. The light intensity was slightly significantly different between the greenhouse control (1938.65±100.28 µmol of photons m^{-2} s^{-1}) and the treatment sites (2027.65± 124.25 µmol of photons m^{-2} s^{-1}) (n=20, $p<0.05$, T-test). This then led to the additional chlorophyll content and leaf area measurements data for *B. discolor* trees located outside (but within 1 km) of the greenhouse in which the controls were housed. These data are also presented in order to validate the trends reported for these two light dependent biomarkers.

Initial analyses of data for all biomarkers revealed no significant difference ($p>0.05$, ANOVA) across leaves from different cardinal directions within sites; so data for the different cardinal directions were pooled for each site in all subsequent analyses. For these analyses biomarker data was compared within sites across seasons, across sites within seasons and across sites with biomarker data for different seasons pooled (henceforth referred to as 'annual data'). Biomarker data across sites were also related to seasonal SO_2 levels via correlation analyses.

4.2. Air pollution

Within sites, SO_2 levels were significantly different across seasons, except for Southern Works where levels were comparable in summer, autumn and spring but significantly lower in winter (**Table 4.1**). When compared within sites, across seasons, SO_2 levels at Prospecton and Ganges were highest in winter, followed by autumn, spring and summer. The highest SO_2 levels were recorded at Ganges (in winter) and the lowest at Prospecton (in summer). Annual average atmospheric SO_2 levels were significantly higher at Southern Works and lowest at Prospecton. The levels of SO_2 at the control site (within a greenhouse) were below the detectable limits of the instrument. The SO_2 levels measured during autumn at three random points exterior to, but within 1 km of

the greenhouse (*ex situ*) control site (in the same university campus) were lower (2.73±0.31 ppb) than the treatment sites but higher than the control (statistical analysis was not possible due to n=8 for the control validation site as opposed to n>3500 for the treatment sites).

Table 4.1 Ground level SO_2 concentrations (ppb) measured at the three industrial sites investigated. Values of ground-level SO_2 levels represent means±SD (n ranged from 172 to 1752 for seasonal data and from 3632 to 4827 for annual data), measured at the three treatment sites for the seasons of 2014. Values labeled with upper case letters indicate significant differences in annual [SO_2] (i.e. seasonal data pooled) across sites ($p<0.001$; ANOVA), while values labeled with lower case letters are significantly different when compared across the different site×season combinations ($p<0.001$, ANOVA).

Study Sites	**Seasonal [SO_2] (ppb)**			
(Annual [SO_2], ppb)	**Summer**	**Autumn**	**Winter**	**Spring**
Prospecton (4.39^{C}±3.92)	2.61^{g} ±1.91	4.03^{d}±3.32	6.58^{c}±5.07	3.97^{de}±3.36
Ganges (5.10^{B}±4.73)	3.31^{ef}±2.48	6.54^{bc}±5.04	9.52^{a}±6.67	3.79^{d}±2.87
Southern Works (6.71^{A}±5.47)	6.92^{c}±5.76	7.69^{c}±5.63	3.24^{f}±2.64	6.09^{c}±5.08

4.3. Biochemical biomarkers

4.3.1. Intracellular superoxide

High levels of variation of $\cdot O_2^-$ levels (indicated by the high standard deviations) within treatment sites during summer, winter and spring made statistical comparisons largely irrelevant and negated the need to carry out measurements for this biomarker in autumn (**Fig. 4.1**). Nevertheless, $\cdot O_2^-$ levels differed significantly across seasons, within sites for the control and Prospecton; with the lowest values recorded during summer. The $\cdot O_2^-$ levels at the treatments sites were higher than the control across all three seasons; however, these differences were only significant for Ganges and Southern Works during summer. There were no significant differences within seasons, across the treatment sites. Comparisons of annual data showed no significant difference with respect to $\cdot O_2^-$ levels across all treatment sites. However, $\cdot O_2^-$ levels at Ganges were observed to be

significantly higher than the control. Annual $\cdot O_2^-$ levels therefore did not reflect annual [SO_2] across sites very well (**Table 4.1**). Despite the high levels of variation observed for the treatment sites, $\cdot O_2^-$ levels were significantly positively correlated, in terms of, seasonal [SO_2] and leaf $\cdot O_2^-$ levels ($p=0.041$; $r=0.596$, Pearson's correlation).

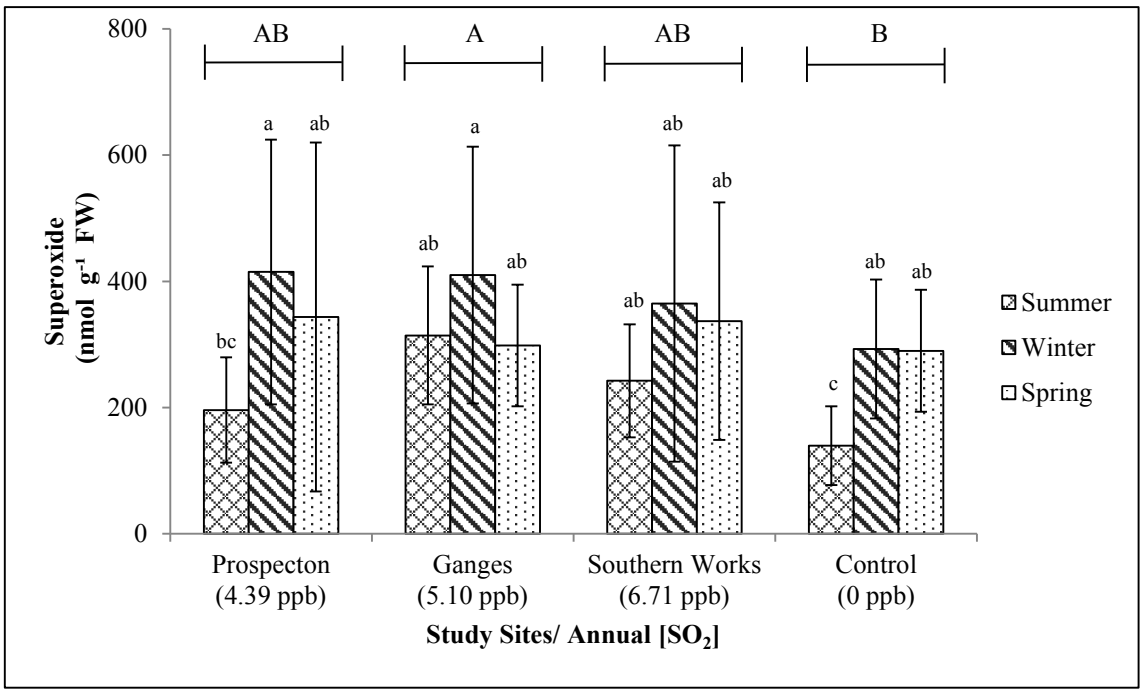

Figure 4.1 Intracellular superoxide ($\cdot O_2^-$) levels in *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD ($n=24$). Upper case letters indicate significant differences across sites when data for different seasons were pooled ($p<0.001$, ANOVA). Lower case letters indicate significant differences across different site×seasons combinations ($p<0.001$, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each site.

4.3.2. Intracellular hydrogen peroxide

Although leaf H_2O_2 levels differed significantly within sites, across seasons, there were no apparent trends in this regard (**Fig. 4.2**). Within seasons, H_2O_2 levels were higher at the treatment sites when compared to the control; these differences were significant across all treatments, during all seasons, except for the H_2O_2 levels at Prospecton during autumn. Levels of H_2O_2 during summer were comparable between Ganges and Southern Works but significantly lower at Prospecton. During summer, levels of H_2O_2

were comparable across the treatment sites in autumn. The levels of H_2O_2 appeared lower at Prospecton during winter; however, this was not significant between Southern Works and Prospecton, though. During spring, H_2O_2 levels across treatment sites were significantly highest at Prospecton and lowest at Southern Works, whereas annual H_2O_2 levels were significantly highest at Ganges, lowest in the control and comparable between Southern Works and Prospecton. Annual H_2O_2 levels therefore reflect differences in $[SO_2]$ between the control and the treatment sites but this did not apply to differences in annual $[SO_2]$ across the treatment sites (**Table 4.1**). Seasonal $[SO_2]$ was not significantly correlated with seasonal H_2O_2 production ($p=0.058$, $r=0.483$, Pearson's correlation).

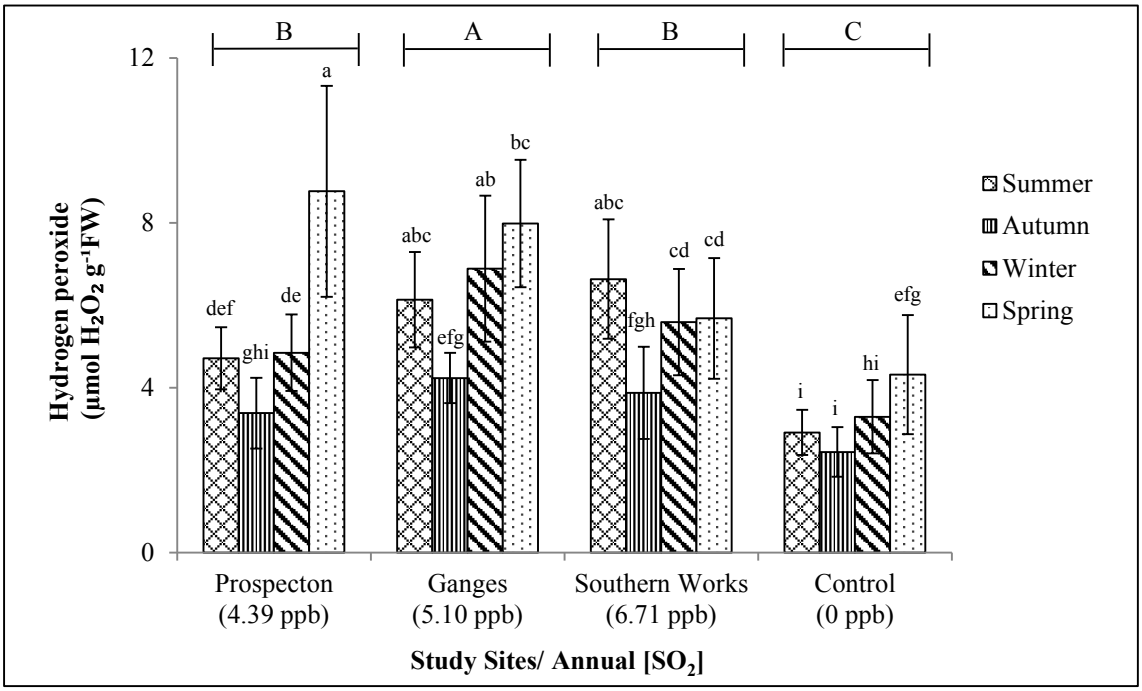

Figure 4.2 Intracellular hydrogen peroxide (H_2O_2) levels in *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD ($n=24$). Upper case letters indicate significant differences across sites when data for different seasons were pooled ($p<0.001$, ANOVA). Lower case indicate significant differences across site×season combination ($p<0.001$, ANOVA). Annual $[SO_2]$ in ppb is given in parenthesis for each site.

4.3.3. Total aqueous antioxidants

Leaf total aqueous antioxidant (TAA) activity was significantly higher in winter and comparable across summer, autumn and spring, except with Prospecton spring which was significantly higher than summer and autumn, within treatment sites (**Fig. 4.3**).

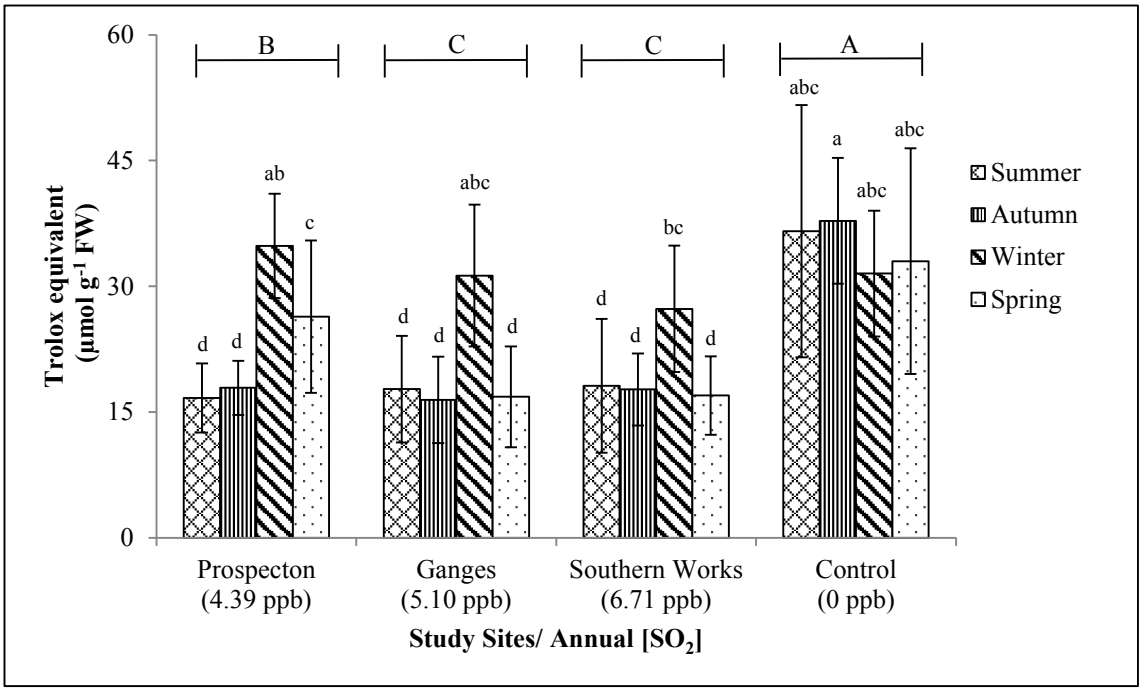

Figure 4.3 Total aqueous antioxidant (TAA) activity in *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD (*n*=24). Upper case letters indicate significant differences across sites when data for different seasons were pooled ($p<0.001$, ANOVA). Lower case indicate significant differences across different site×season combinations ($p<0.001$, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each site.

The TAA activity was comparable across seasons for the control. With the exception of winter for all sites and Prospecton in spring (in which TAA activity was comparable across the treatment sites and the control), TAA activity in the control was significantly higher than the treatments. Total aqueous antioxidant activity was comparable across the treatment sites within summer, autumn and winter, whereas TAA activity during spring was significantly higher at Prospecton when compared to Ganges and Southern Works. Annual TAA data were significantly highest in the control, lower at Prospecton and lowest at Southern Works and Ganges. Annual TAA levels could therefore reflect

differences in [SO_2] to the control and across treatment sites (**Table 4.1**). Seasonal TAA activity was not significantly correlated with seasonal SO_2 levels (p=0.147, *rs*= –0.379, Spearman's rank correlation).

4.3.5. Superoxide dismutase

Leaf superoxide dismutase (SOD) activity showed no significant differences across the seasons within sites (except for winter at Southern Works, which was significantly higher than spring and in winter at the control, which was significantly lower than the other seasons) (**Fig. 4.4**).

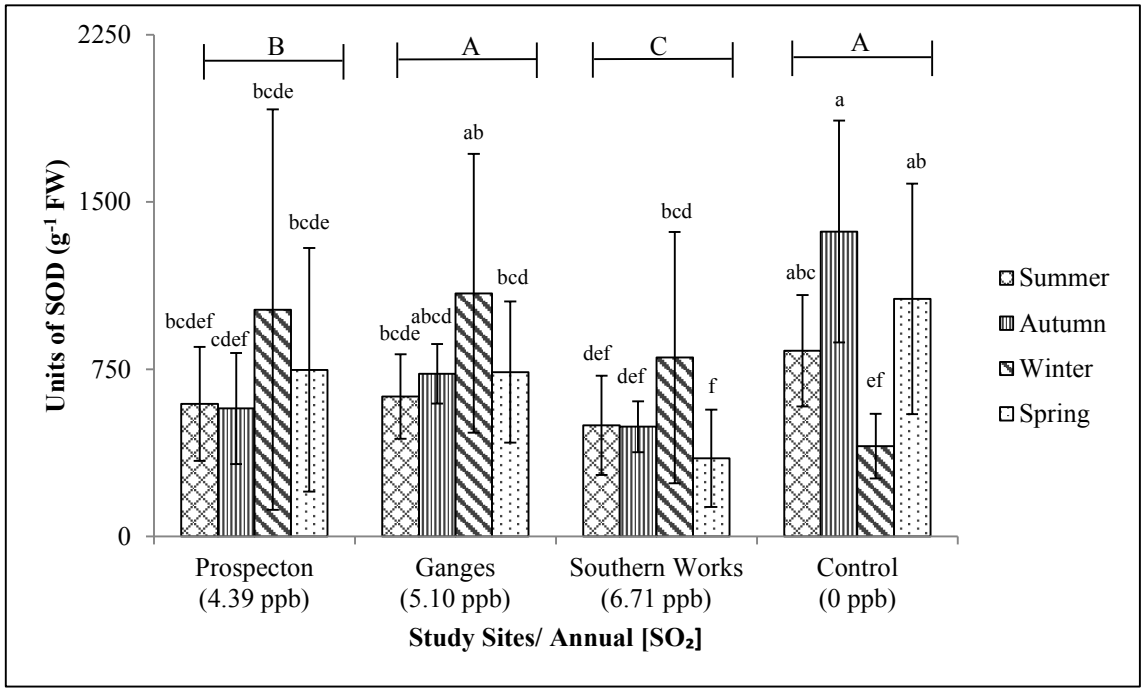

Figure 4.4 Superoxide dismutase (SOD) activity within *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD (*n*=24). Upper case letters indicate significant differences across sites when data for different seasons were pooled (p<0.001, ANOVA). Lower case letters indicate significant differences across different site×seasons combinations (p<0.001, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each site.

Within seasons, SOD activity in leaves during winter was significantly higher across all treatment sites than the control, while during summer and spring, SOD activities at the treatment sites were relatively lower than the control but these differences were not

always significant. Superoxide dismutase activity at Southern Works was significantly lower than the control during summer, autumn and spring. At Prospecton also, SOD activity during autumn was significantly lower than the control. Across the treatment sites, SOD was comparable within seasons, apart from Southern Works during spring which was significantly lower than Prospecton and Ganges. When annual data was compared, Ganges and the control had the highest SOD activity followed (in decreasing order) by Prospecton and Southern Works ($p<0.001$). Annual SOD activities could therefore not discriminate between the control and the treatment sites, nor could it reflect the differences in annual [SO_2] across the treatment sites (**Table 4.1**). There was no significant correlation between seasonal SOD activity and seasonal SO_2 levels ($p=0.475$, $r=0.192$, Pearson's correlation).

4.3.5. Catalase activity

Within the treatment sites leaf catalase (CAT) activitirs across the seasons were lower during summer (significant in all cases except for spring and summer at Southern Works) (**Fig. 4.5**). Within the control, CAT activity was significantly highest in autumn. Catalase activity in the control was significantly higher than Ganges during summer and Southern Works during autumn; the control was significantly lower than all the treatment sites during winter and lower than Prospecton and Ganges in spring. When the treatment sites were compared within seasons, CAT activity was comparable in summer but tended to be relatively (but not always significantly) lower at Southern Works in the remaining seasons. Comparisons of annual data revealed CAT activity at Prospecton to be significantly higher than the control and Southern Works; lower at Southern Works than at Ganges and comparable between Southern Works and the control. Southern Works exhibited the lowest annual CAT activity and Prospecton the highest, across treatment sites. These data suggest that annual CAT activities did not reflect differences in [SO_2] between the control and the treatment sites very well but did indicated that there were differences reflected in annual [SO_2] across the treatment sites (**Table 4.1**). Seasonal SO_2 levels were not significantly correlated with seasonal CAT activities ($p=0.503$, $r=0.181$, Pearson's correlation).

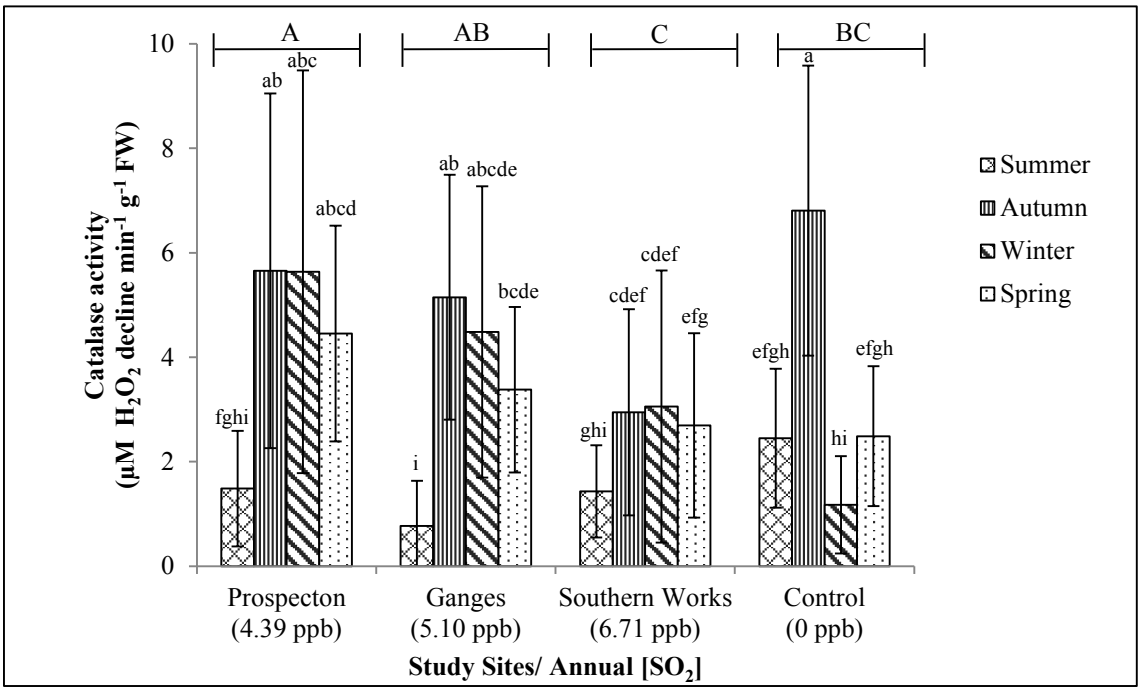

Figure 4.5 Catalase activity (CAT) within *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD (*n*=24). Upper case letters indicate significant differences across sites when data for different seasons were pooled ($p<0.001$, ANOVA). Lower case letters labeled different indicate significant differences across different site×season combinations ($p<0.001$, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each site.

4.3.6. Lipid peroxidation

Leaf LPO levels exhibited no consistent trends when compared across seasons within sites. At Prospecton LPO levels during winter and spring were significantly higher than summer and autumn, at Ganges summer and winter levels were significantly higher than autumn and spring, while at Southern Works winter levels were significantly higher than spring (**Fig. 4.6**). In the leaves from the control trees, LPO levels were comparable across seasons; however, these levels were lower than all treatment sites when compared within seasons. This was significant for most seasons except for spring at Southern Works. When LPO levels were compared within seasons, across treatment sites no consistent trends were evident. For example, during summer, LPO levels at Ganges were significantly higher than the other sites and during autumn levels at Ganges were significantly higher than at Southern Works. During winter, the levels of

LPO at Southern Works were significantly lower than the other treatment sites, and during spring Prospecton levels were significantly higher than the other sites. Comparisons of annual data revealed lipid peroxidation levels to be significantly highest at Ganges followed (in decreasing order) by Prospecton, Southern Works and the control ($p<0.001$). Annual lipid peroxidation levels could therefore reflect differences in [SO_2] between the control and the treatment sites but this did not apply to differences in annual [SO_2] across the treatment sites (**Table 4.1**). Seasonal lipid peroxidation levels were significantly, positively correlated with seasonal SO_2 levels ($p=0.015$, $r=0.593$; Pearson's correlation).

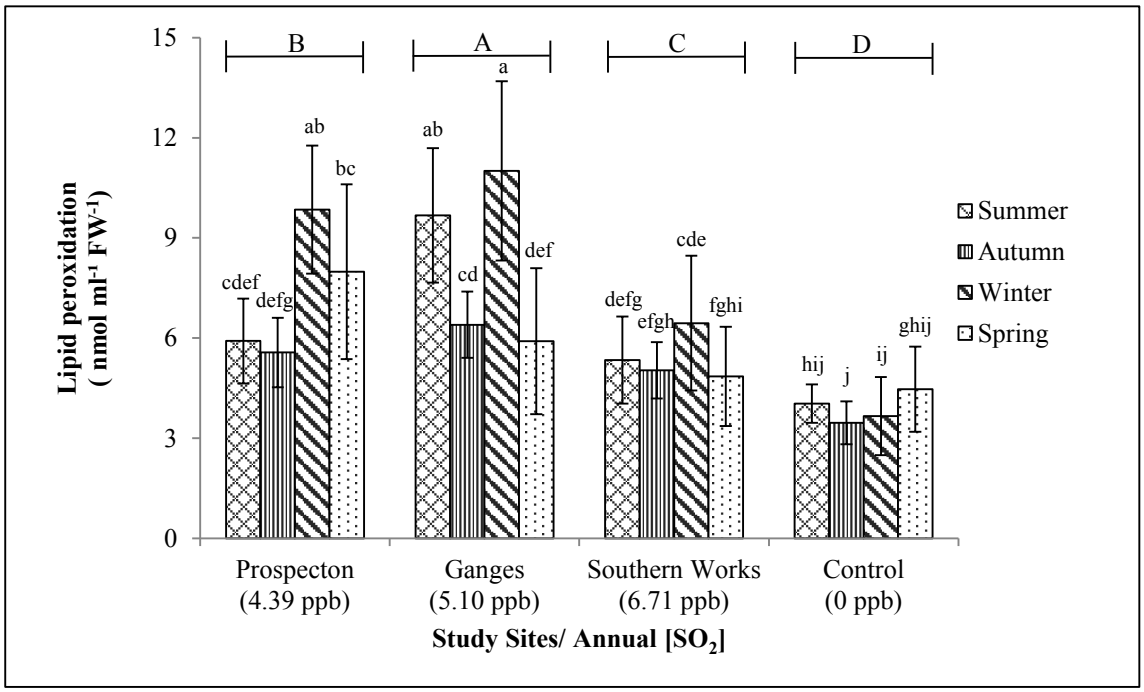

Figure 4.6 Lipid peroxidation levels in *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD ($n=24$). Upper case letters indicate significant differences across sites when data for different seasons were pooled ($p<0.001$, ANOVA). Lower case letters indicate significant differences across different site×season combinations ($p<0.001$, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each sites.

4.3.7. Electrolyte leakage

Leaf electrolyte leakage values were significantly higher during winter, at all sites (treatment and control), when compared across seasons within a site (**Fig. 4.7**).

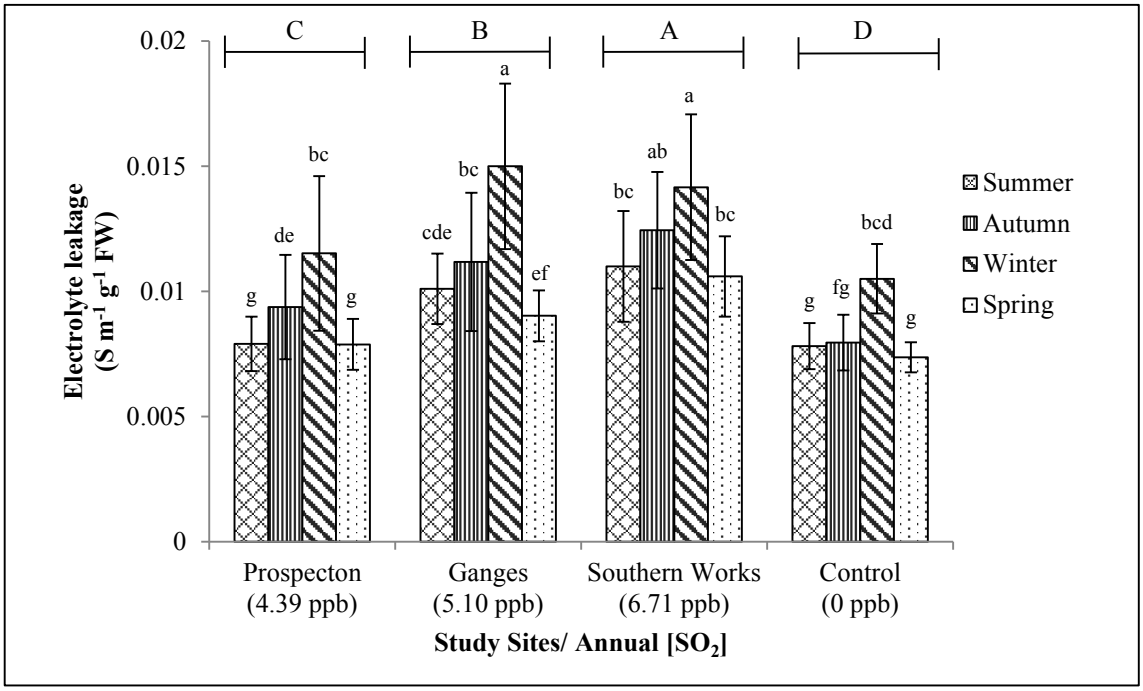

Figure 4.7 Electrolyte leakage of *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD (*n*=24). Upper case letters indicate significant differences across sites when data for different seasons were pooled ($p<0.001$, ANOVA). Lower case letters indicate significant differences across different site×season combinations ($p<0.001$, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each site.

Additionally, electrolyte leakage values for autumn at Prospecton were significantly higher than summer and spring; whereas at Ganges, electrolyte leakage during spring was significantly lower than autumn. In the control leaves, during winter, these values were significantly higher than during the other seasons. When the treatment sites were compared to the control within seasons, control electrolyte leakage values were significantly lower than Ganges and Southern Works for all seasons but significantly lower than Prospecton only during autumn. Annually, leakage levels were found to be highest at Southern Works, followed (in decreasing order) by Ganges, Prospecton and the control. Annual electrolyte leakage levels could therefore reflect differences in [SO_2] between the control and the treatment sites and differences in annual [SO_2] across the treatment sites (**Table 4.1**). Seasonal [SO_2] were also significantly, positively

correlated with seasonal electrolyte leakage levels (p=0.002, *r*=0.702, Pearson's correlation).

4.4. Physiological biomarkers

4.4.1. Leaf chlorophyll fluorescence

Chlorophyll fluorescence (specifically F_v/F_m) was analyzed across the seasons within sites; values were comparable between Prospecton and the control for all seasons, while summer and spring values at Ganges were significantly higher than autumn and winter (**Fig 4.8**).

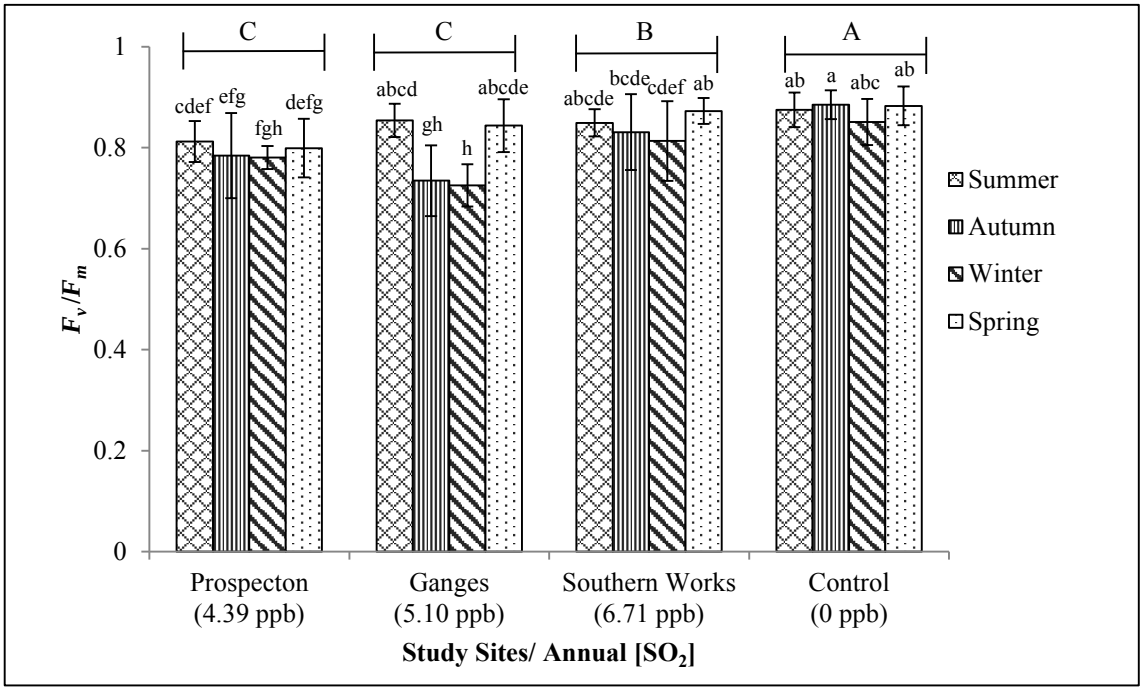

Figure 4.8 Chlorophyll fluorescence (LCF) in *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD (*n*=24). Upper case letters indicate significant differences across sites when data for different seasons were pooled (p<0.001, ANOVA). Lower case letters indicate significant differences across different site×season combinations (p<0.001, ANOVA). Annual [SO_2] in ppb is given in paraenthesis for each site.

At Southern Works only spring values were significantly higher than winter, while values for all other seasons were comparable. Within seasons, values for the control

were higher than the treatment sites for all seasons (significant for all treatment sites in autumn, Prospecton in summer, winter and spring and only Ganges in winter). When compared within seasons across treatment sites, chlorophyll fluorescence values were comparable across sites in summer; values at Ganges were significantly lower than at Southern Works in autumn and winter, whilst values at Prospecton were significantly lower than at Southern Works in spring. Annual chlorophyll fluorescence values in the control were significantly higher than all other treatment sites while values at Prospecton and Ganges were significantly lower than at Southern Works. Annual chlorophyll fluorescence could therefore reflect differences in [SO_2] between the control and treatment sites, but this did not apply to differences in annual [SO_2] across the treatment sites (**Table 4.1**). There was also a significant and negative correlation between seasonal [SO_2] and seasonal chlorophyll fluorescence ($p=0.006$, $r= -0.656$, Pearson's correlation).

4.4.2. Chlorophyll content

When chlorophyll content was compared across seasons, within sites, there were no clear trends (**Fig. 4.9**). Within individual sites, chlorophyll content at Prospecton during winter were significantly higher than summer and spring and autumn content was significantly higher than summer. At Ganges, chlorophyll content during autumn and spring were comparable, but significantly lower than summer and winter (which were comparable). At Southern Works chlorophyll content during summer were significantly lower than the other seasons. In the control chlorophyll content for autumn were significantly higher than the other seasons (which were comparable). Chlorophyll content values at Ganges during summer and autumn were significantly higher and lower, respectively than the controls, whilst in winter and spring values at Ganges were comparable to the control. Within seasons, across treatment sites, chlorophyll content during autumn and spring at Ganges were significantly lower than the other sites; winter values at Ganges were significantly lower than at Prospecton and summer values at Southern Works were significantly lower than at the other sites. When annual data was analysed chlorophyll content at Prospecton and Southern Works were significantly higher than Ganges and the control. With the exception of Ganges, annual chlorophyll content could only reflect differences in [SO_2] between the control, Prospecton and Southern Works, however this did not apply to differences in annual [SO_2] across the

treatment sites (**Table 4.1**). Seasonal chlorophyll content and seasonal [SO_2] were not significantly correlated (p=0.276, *r*=0.290, Pearson's correlation).

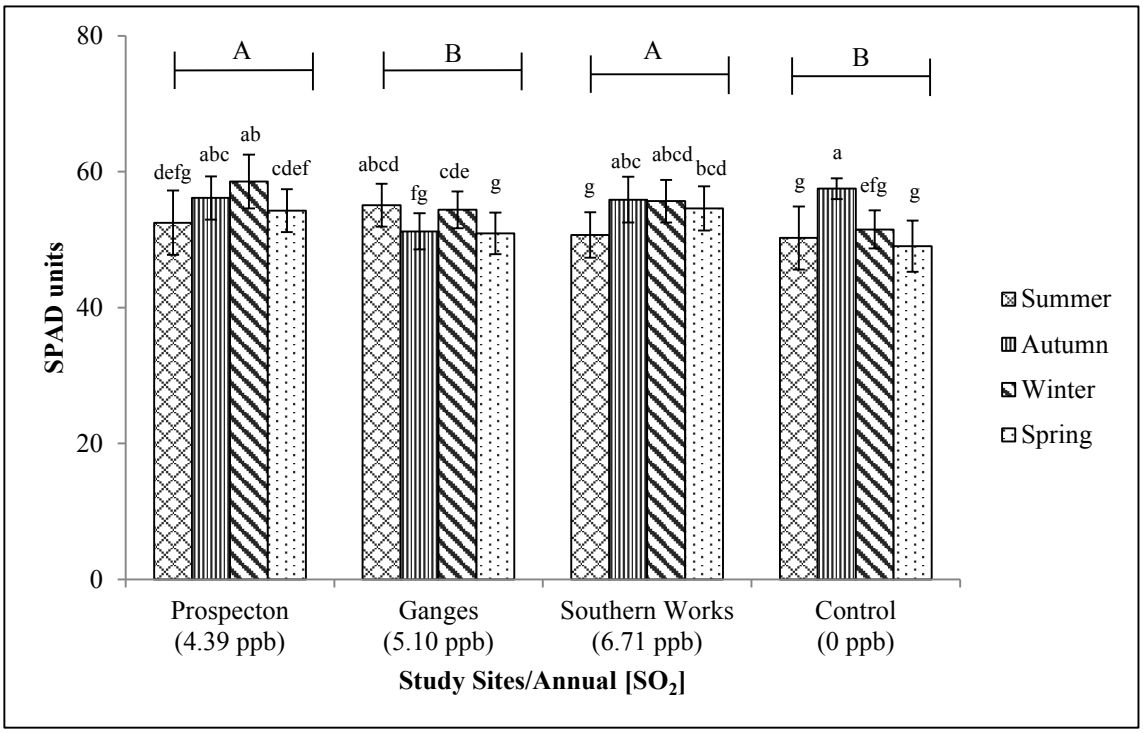

Figure 4.9 Chlorophyll content of *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD (*n*=24). Upper case letters indicate significant differences across sites when data for different seasons were pooled (p<0.001, ANOVA). Lower case letters indicate significant differences across different site×season combinations (p<0.001, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each site.

Chlorophyll content values for trees growing in the university campus site within 1 km of the greenhouse (where the control trees were housed) that were in autumn exposed to [SO_2]of 2.73±0.31 ppb as 50.62±2.25 SPAD units. During autumn the chlorophyll content for the 'campus site' was comparable to Ganges but significantly higher than Prospecton, Southern Works and the control. During spring the chlorophyll content for the 'campus site' was 51.17±3.52 SPAD units, which was comparable to the control and Ganges but were higher than Prospecton and Southern Works, but only significantly so at Southern Works (p<0.001, ANOVA). Correlations between chlorophyll content and

[SO_2] (for autumn) for the control, treatments sites and the 'campus site' were not significant for autumn (p=0.671, *r*= –0.261, Pearson's correlation).

4.5. Morphological biomarkers

4.5.1. Leaf area

When LA was compared across the different seasons within sites, no clear trend could be established (**Fig 4.10**).

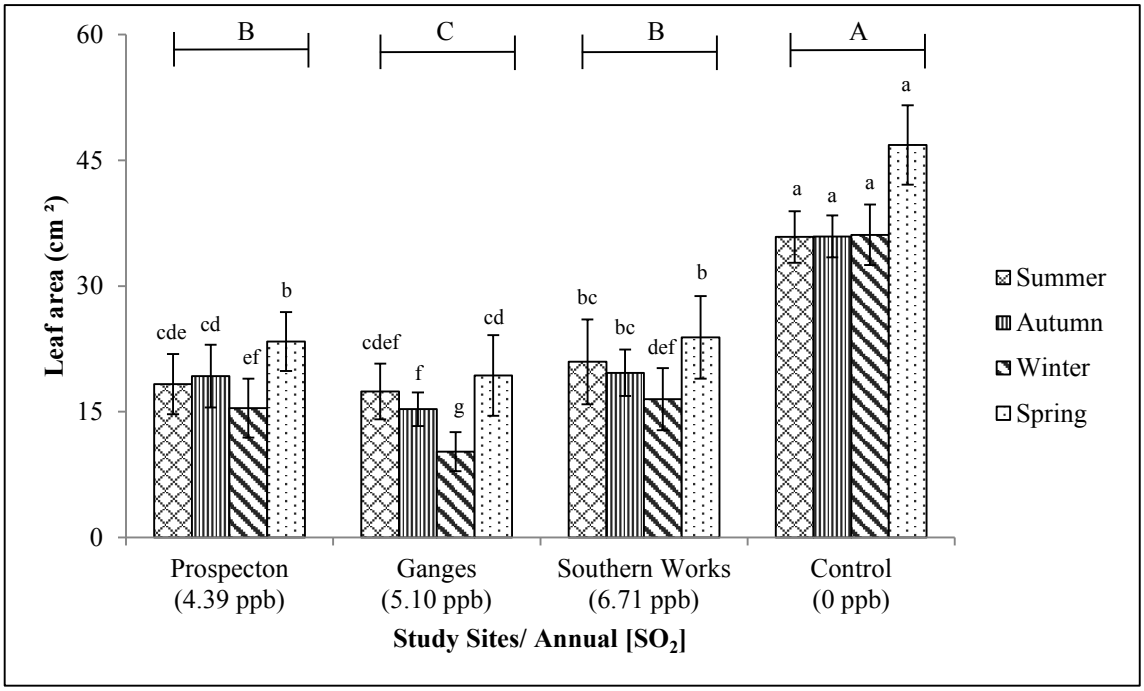

Figure 4.10 Leaf area of *B. discolor* leaves at the treatment sites and the control. Columns represent mean±SD (*n*=24). Upper case letters indicate significant differences across sites when data for different seasons were pooled (p<0.001, ANOVA). Lower case letters indicate significant differences across different site×season combinations (p<0.001, ANOVA). Annual [SO_2] in ppb is given in parenthesis for each site.

At Prospecton, leaf area was significantly highest during spring and significantly lowest in winter. At Ganges, leaf area was relatively higher in summer and spring and lower in winter (but these differences were not always significant). At Southern Works, during winter LA values were significantly lower than the other seasons, while LA was comparable across seasons in the control. Leaf area in the control was significantly higher than all treatment sites within all seasons. When LA was compared within

seasons across treatment sites values for Prospecton and Southern Works were comparable, while values at Ganges were relatively lower (significant for autumn and spring). Annual LA was significantly highest in the control and significantly lowest at Ganges. Annual LA could therefore reflect differences in [SO_2] between the control and treatment sites, but this did not apply to differences in annual [SO_2] across the treatment sites (**Table 4.1**) There was also a significantly strong negative correlation between seasonal LA and seasonal [SO_2] ($p<0.001$, $r= –0.816$). The 'campus site' trees which were exposed to [SO_2] of 2.73±0.31 ppb in autumn yielded LA values (23.98±5.62 cm^2) that followed the seasonal trends of LA and [SO_2] (**Fig. 4.10** and **Table 4.1**). Spring LA (29.50±5.24 cm^2) for the 'campus site' followed the trend established in autumn, with control which was significantly higher across all treatment sites, followed in decreasing value by campus (which was comparable to all other sites, except Ganges), LA was significantly lowest at Ganges (for both spring and autumn, $p<0.001$, ANOVA). Correlations between leaf area and [SO_2] (for autumn) for the control, treatment sites and the 'campus site' were not significant for autumn ($p=0.054$, $r= –0.872$, Pearson's correlation).

4.6 Location of and land-use practices at treatment sites

Systematic observations of the geographical location of the treatment sites relative to the industrial hub of the SDB and land-use practices at each site revealed that trees could have been variably impacted upon by air pollution sources other than the petroleum industries that dominate the SDB. More specifically, the trees at the Prospecton site, situated on the outskirts of the SDB (**Fig. 3.1**), were located within a business park with relatively low levels of light motor vehicle traffic [associated with the carbon monoxide (CO) emissions] with no major industries associated with air pollution (**Fig. 4.11 A** and **B**). The trees at the Ganges site were located alongside a major highway [*viz.* Southern/Northern highway (M4)] and a secondary road (*viz.* off of Himalayas Road) prone to high levels of light and heavy motor vehicle traffic industrial (**Fig. 4.11 C** and **D**). The Ganges site was also situated more inland than Prospecton and more central of the SDB's industrial hub, which was dominated by petroleum-based industries (**Fig. 3.1**). The trees at Southern Works site were located within residential areas, subjected to low light motor vehicle traffic levels (**Fig. 4.11 E** and **F**); however,

though this site was located closer to the coast-line than the Ganges site, it was still within close proximity to the SDB's industrial hubs.

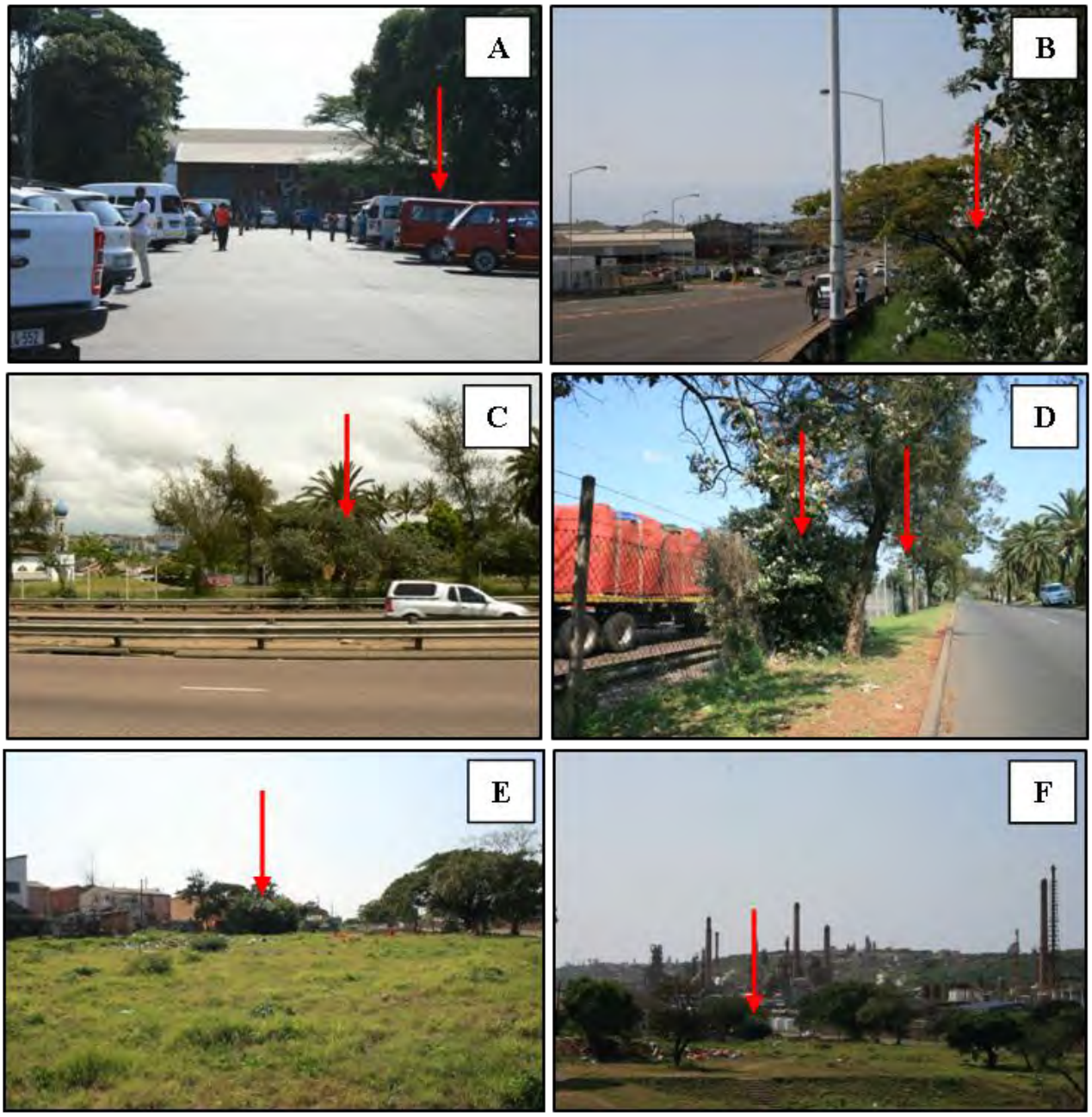

Figure 4.11 Summary of qualitative observations of land-use practices at the treatment sites: Prospecton: (**A** & **B**); Ganges (**C** & **D**); Southern Works (**E** & **F**). Red arrow indicates location of *B. discolor* trees sampled at each site (Photos by: Minoli Appalasamy and Candyce Areington).

4.7. Conclusion

Average SO_2 levels differed across seasons within sites, across sites within seasons and annually across the three treatment sites (**Table 4.1**). Values for all biomarkers differed across seasons within sites but these differences were more often significant between the treatment sites and the control, than across the treatment sites themselves. Seasonally, SO_2 levels were higher at Prospecton and Ganges in winter; this was reflected by most biomarkers (except: H_2O_2, CAT, chlorophyll content).

Annual values for a number of biomarkers differed significantly across the treatment sites and the control (viz. H_2O_2, TAA, lipid peroxidation, electrolyte leakage, LCF and LA). The correlation analyses revealed seasonal [SO_2] to be significantly correlated with seasonal $\cdot O_2^-$ levels, lipid peroxidation, electrolyte leakage, LCF and LA averages. Biomarkers that reflected differences in annual [SO_2] across the treatment sites (i.e. that suggest Southern Works has the highest [SO_2]) include TAA and electrolyte leakage (whilst electrolyte leakage being the only significantly positively correlated).

CHAPTER 5 DISCUSSION

Although air pollution is a serious threat to environmental and human health within many parts of the eThekwini Municipal Area (EMA), the monitoring regime/methods currently employed by the eThekwini Municipality are insufficient for long-term and multiple pollutant monitoring (Diab *et al.*, 2002; Carmichael *et al.*, 2003). Long-term and multiple pollutant monitoring is essential for the design of effective air pollution mitigation strategies (Conti and Cecchetti, 2001; Carmichael *et al.*, 2003; Assadi *et al.*, 2011). Bioindicators such as plants offer a more feasible means of monitoring air quality than monitoring instruments/stations, especially within developing countries (Novak *et al.*, 2003; Tiwari *et al.*, 2006; Tripathi and Gautam 2007; Rai *et al.*, 2011). Their use does, however, demand a fundamental understanding of the biochemical, physiological and morphological response of the plant(s) to air pollution in order to select the most suitable biomarker(s) to be used (Emberson *et al.*, 2001; Tiwari *et al.*, 2006; Tripathi and Gautam 2007; Assadi *et al.*, 2011; Rai *et al.*, 2011). This formed the focus of the present study which was conducted on the leaves of an indigenous tree, *B. discolor*, growing at three industrial sites within the highly industrialised South Durban Basin (SDB).

Sulphur dioxide which was selected as the reference (indicator) pollutant for this study, is one of the most damaging pollutants to natural and agricultural vegetation, and a major pollutant within the SDB (Emberson *et al.*, 2001; Diab *et al.*, 2002; Matooane and Diab, 2003; Diab and Motha, 2007; Rai *et al.*, 2011). The decision to use SO_2 was based on the fact that this is the only pollutant monitored across all air quality monitoring stations within the EMA. Annual average SO_2 levels ranged from 4.39–6.71 ppb across the three treatment sites investigated (**Table 4.1**). Josipovic *et al.* (2010) placed the threshold range of [SO_2] for natural and agricultural crops between 3.8–11.4 ppb, where irreversible damage may be inflicted. This reinforces suggestions by Matooane and Diab (2001) that the SDB has already reached its carrying capacity in terms of SO_2. Like other industrialised areas in South Africa (e.g. Elandsfontein which exhibited a [SO_2] of *ca.* 6.99 ppb [Carmichael *et al.*, 2003]), the [SO_2] across the industrial sites investigated here also appear to be relatively high by global standards (Carmichael *et al.*, 2003) and well within the range reported to be detrimental to plants (Josipovic *et al.*, 2010).

Air pollution is a transboundary event and the location of the pollution source may not necessarily overlap with the site at which this pollution inflicts the most damage (Ramanathan and Feng, 2009). This is largely due to the dispersal of air pollution, which is influenced by air pressure systems, prevailing winds, wind speeds and seasonality (Scott and Diab 2000, Diab *et al.*, 2002; Batterman *et al.*, 2008). Leaves sampled from different cardinal directions on the trees sampled in this study were, however, comparable in terms of all the biomarkers assessed (data not shown) and this is why data for different cardinal directions were pooled for all subsequent analyses. There were, however, distinct differences in SO_2 levels across seasons within sites, with the highest [SO_2] being recorded at Ganges in winter (**Table 4.1**). Both Ganges and Prospecton recorded their highest SO_2 readings in winter, which is in accordance with previous reports that SO_2 levels are generally higher within the SDB in winter (Scott and Diab, 2000; Matooane and Diab, 2001; Diab *et al.*, 2002; Batterman *et al.*, 2008). Winter brings about high pressure systems (colder weather), which can lead to the retention of air pollution within the SDB by reducing atmospheric mixing and dispersal of air pollution (Scott and Diab, 2000; Diab *et al.*, 2002; Batterman *et al.*, 2008). Furthermore, this high pressure system together with berg winds (a characteristic of winter) bring all the inland air pollution towards the coast (Scott and Diab, 2000; Diab *et al.*, 2002; Batterman *et al.*, 2008). However, SO_2 levels at Southern Works, which is located closer to the coastline than the other two treatment sites, were significantly lower in winter (**Table 4.1**). This may be due to the prevailing north-easterly (NE) and south-westerly (SW) winds along the coastline which are likely to move air pollution away from Southern Works (Scott and Diab, 2000; Diab *et al.*, 2002; Batterman *et al.*, 2008).

Topography also influences pollution (Scott and Diab, 2000; Moraes *et al.*, 2002); for example, the unique shallow basin shape of the SDB may prevent air flow which would hinder pollutant dispersal (Batterman *et al.*, 2008). The Prospecton station is located at the southern end of the basin, while the Ganges station is located more centrally and further inland, and the Southern Works station is located close to the coastline (Diab *et al.*, 2002) (**Fig. 3.1**). The topography of the SDB, provide the perfect directing system for the prevailing NE and SW winds (Batterman *et al.*, 2008) which do have an impact on the monitoring stations (Scott and Diab, 2000; Diab *et al.*, 2002). This may have also

contributed to the differences in annual average SO_2 levels across sites: highest at Southern Works followed by Ganges and then Prospecton (**Table 4.1**).

Plant responses to a stress are often interactive and therefore should not be considered in isolation (Tripathi and Gautam, 2007; Assadi *et al.*, 2011). These responses can be biochemical, physiological and/or morphological (Tripathi and Gautam, 2007; Assadi *et al.*, 2011), but the first response to any stress, including pollution, is usually biochemical (Tripathi and Gautam, 2007; Rai *et al.*, 2011), in the form of uncontrolled reactive oxygen species (ROS) production (Gill and Tuteja, 2010; Bermudez and Pignata, 2011). Superoxide is, usually, the first free radical to be produced under stress conditions (Gill and Tuteja, 2010; Bermudez and Pignata, 2011). Li and Yi (2012) showed an increase in $\cdot O_2^-$ levels in the shoots of *Arabidopsis thaliana* L. when exposed to high SO_2 levels. In the present study, seasonal [SO_2] was significantly positively correlated with $\cdot O_2^-$ levels; however, the high variability (evidenced by the high SD) in $\cdot O_2^-$ values within sites (treatment and control) led to a lack of significant differences between the treatment sites and the control (**Fig. 4.1**). Furthermore, when $\cdot O_2^-$ values for winter, spring and summer were pooled they did not reflect annual differences in [SO_2] across the treatment sites. For these reasons, $\cdot O_2^-$ did not represent a suitable biomarker in this study. Areington *et al.* (2015) when measuring $\cdot O_2^-$ production in *B. discolor* leaves from trees growing at various distances from an oil refinery within the SDB also found leaf $\cdot O_2^-$ levels to be unsuitable for predicting air pollution levels. Superoxide is the precursor to more toxic ROS molecules making it an important indicator of stress in plants but the lack of sensitivity of the $\cdot O_2^-$ assay used here and elsewhere (Areington *et al.*, 2015) may be based on the extremely short lifespan and high reactivity of $\cdot O_2^-$ in plant tissues (Arora *et al.*, 2002; Minibayeva *et al.*, 2009; Gill and Tuteja, 2010).

Hydrogen peroxide is relatively more toxic and long-lived than $\cdot O_2^-$ in plant tissue (Scandalios *et al.*, 1997; Gill and Tuteja, 2010) and in this study its levels in SO_2 exposed leaves (at all three treatment sites) were significantly higher than the control leaves (**Fig. 4.2**). Tissues of a number of plant species have been shown to exhibit higher H_2O_2 levels upon exposure to a wide range of stressors: physical wounding (Minibayeva *et al.*, 2009), heavy metals (Tewari *et al.*, 2013; Srivastava *et al.*, 2014), chilling (Prasad *et al.*, 1994), heat stress (Volkov *et al.*, 2006) and air pollution (Li and

Yi, 2012) etc. More specifically, Li and Yi (2012) showed an increase in H_2O_2 production levels within the shoots of *Arabidopsis thaliana* when exposed to an increase in [SO_2]. Although it is evident from literature that excess levels of SO_2 can lead to increased H_2O_2 production in plants (Gill and Tuteja, 2010; Rai *et al.*, 2011), at the time of this report there were no published reports on an SO_2 exposed increase in H_2O_2 in tree leaves of any other tree species other than the species investigated here (Areington *et al.*, 2015). Seasonal H_2O_2 levels were, however, not significantly correlated with seasonal [SO_2] and annual H_2O_2 averages did not reflect differences in annual average [SO_2] across the treatment sites. Hydrogen peroxide's lack of sensitivity therefore suggests that it may not represent an ideal biomarker of SO_2 pollution in *B. discolor* leaves. However, *B. discolor* leaves were able to discriminate, significantly so, between the treatment and control, suggesting further research with other pollutants to asses this parameter as potential biomarker for air pollution. Areington *et al.* (2015) showed H_2O_2 levels in *B. discolor* leaves to be significantly negatively correlated with distance from a point source of petroleum-based air pollution. However, it should be noted that although those authors used the same assay, they did not sample across seasons and worked with a considerable smaller sample size.

The production of $\cdot O_2^-$ and H_2O_2 and other ROS is a natural consequence of metabolic activity and organisms have a natural defence system to quench these ROS if their levels are within certain limits (Arora *et al.*, 2002; Mittler *et al.*, 2004; Tiwari *et al.*, 2006; Valavanidis *et al.*, 2006; Gill and Tuteja, 2010; Bermudez and Pignata, 2011). In the present study, total antioxidant activity (TAA), which does not differentiate between enzymatic and non-enzymatic antioxidants, in SO_2 exposed leaves (at all three treatment sites) was significantly lower than the control leaves (**Fig. 4.3**). This suggests that SO_2 exposure may have compromised the ability of *B. discolor* leaves to quench ROS. Studies have shown plant tissues such as roots to increase their TAA in response to stress-induced ROS production (e.g. Ramlall *et al.*, 2015); which involved root exposure to acid rain. However, at the time of this study, there were no published reports on tree leaf TAA responses to SO_2 exposure except for the report on the same species *B. discolor* by Areington *et al.* (2015). Even though TAA could be used to discriminate between SO_2-exposed and control leaves in this study, seasonal TAA was not significantly correlated with seasonal [SO_2] and annual average TAA values did not

reflect the annual differences in annual average [SO_2] across the treatment sites. This supports previous findings (Areington *et al.*, 2015) that TAA may not be an ideal biomarker of SO_2 pollution in *B. discolor* leaves; but further research would be required to establish TAA as a biomarker of air pollution.

Superoxide dismutase, an enzymatic antioxidant, is directly responsible for quenching $\cdot O_2^-$, whilst CAT is directly responsible for quenching H_2O_2 (Li and Yi, 2012). Both an increase and decrease in SOD and CAT activity is possible in stressed plants, depending on the stressor, the stress intensity, duration and the plant organ (Tripathi and Gautam, 2007; Bermudez and Pignata, 2011; Li and Yi, 2012). For example: Bermudez and Pignata (2011) reported an increase in both SOD and CAT in *Tillandisia recurvata* leaves exposed to various air pollution sources; Li and Yi (2012) reported a decrease in CAT activity and an increase in SOD in *Arabidopsis* shoots exposed to high levels of SO_2; while Tripathi and Gautam (2007) showed an increase in SOD activity in leaves of *Mangifera indica* and a *Eucalyptus* hybrid exposed to high levels of air pollution. The SOD and CAT activities in SO_2 exposed *B. discolor* leaves in this study were not able to clearly discriminate between those in control leaves (**Fig. 4.4** and **4.5**, respectively). Furthermore, seasonal CAT and SOD activities were not significantly correlated with seasonal [SO_2], while only CAT activity was able to reflect the difference between annual [SO_2] across the treatment sites. The delicate balance between oxidative stress and oxidative damage is controlled by the plant's antioxidant systems (Mittler *et al.*, 2004; Valavanidids *et al.*, 2006; Gill and Tuteja, 2010), but like TAA, CAT and SOD activity also do not appear to be suitable biomarkers of SO_2 pollution in *B. discolor*. Lin and Kao (2000) also showed that *Oryza sativa* leaves exhibited no significant change in CAT when exposed to a sodium chloride (NaCl; salt) stress. A lack of a response by antioxidants is still not a clear indication that the plants are not coping with a stress though (Bermudez and Pignata, 2011). Since the antioxidant system is involved in both signaling and acting as a defence system and also that responses are often species-specific, interpreting the results of antioxidant studies may be far too complex (Vranová *et al.*, 2002; Tripathi and Gautam, 2007; Minibayeva *et al.*, 2009; Gill and Tuteja, 2010) (and probably beyond the scope) for bioindicator studies such as the present one.

When ROS becomes excessive and overrides the antioxidant capacities of the plant, oxidative damage occurs (Arora *et al.*, 2002; Mittler *et al.*, 2004; Tiwari *et al.*, 2006; Valavanidis *et al.*, 2006; Gill and Tuteja, 2010; Bermudez and Pignata, 2011). This damage usually encompasses lipid peroxidation which if quantified can serve as an indication of membrane integrity (Conti and Cecchetti, 2001; Valavanidis *et al.*, 2006; Li and Yi 2012; Tewari *et al.*, 2013; Srivastava *et al.*, 2014). In the present study lipid peroxidation levels in SO_2-exposed leaves were significantly higher than those in the control (**Fig. 4.6**). Lipid peroxidation has been shown to increase in plants exposed to various stresses: heavy metals (*Morus alba* leaves [Tewari *et al.*, 2013]), chilling (Keshavkant and Naithani, 2001), air pollution (lichens [Conti and Cecchetti, 2001]), specifically SO_2 (carrot seedlings [Tiwari *et al.*, 2006]; *Arabidopsis* shoots [Li and Yi, 2012]). Bermudez and Pignata (2011) also found an increase in lipid peroxidation in *Tillandsia recurvata* leaves in response to increased [SO_2]. Although annual lipid peroxidation average did not reflect annual [SO_2] averages across the treatment sites, seasonal lipid peroxidation levels were significantly positively correlated with seasonal [SO_2]. Given the previous reports on the utility of lipid peroxidation as a biomarker of plant stressors (including air pollution) and the results described above for this parameter may represent a suitable biomarker of SO_2 pollution in *B. discolor* leaves.

Electrolyte leakage is an indirect way of measuring the oxidative damage, particularly lipid peroxidation in plant tissues (Santamaría and Matrín, 1997; Conti and Cecchetti, 2001). In the present study (**Fig. 4.7**), SO_2-exposed leaves (at all three treatment sites) had a significantly higher electrolyte leakage than the control. Tree bark from *Quercus ilex* L. (Santamaría and Matrín, 1997), lichens (Conti and Cecchetti, 2001) and the leaves of *Tillandsia tricholepis* (Bermudez and Pignata, 2011) have all exhibited a stress-induced increase in electrolyte leakage. In the present study, seasonal electrolyte leakage was significantly positively correlated with seasonal [SO_2], and annual electrolyte leakage averages reflected annual average [SO_2] across the treatment sites. Areington *et al.* (2015) also found electrolyte leakage to be significantly negatively correlated with distance from an air pollution source in *B. discolor* leaves. Electrolyte leakage may therefore represent an ideal biomarker of SO_2 pollution in *B. discolor* leaves. While lipid peroxidation, measures the actual damage to lipids within the cell membranes due to excess ROS, electrolyte leakage measures the electrolytes that leak

out of cell as a consequence of this membrane damage (Valavanidis *et al.*, 2006; Bermudez and Pignata, 2011). These parameters were not significantly correlated ($p=0.059$, $r=0.481$; Pearson's correlation) in this study but given their physiological link these two biomarkers should be measured in combination, in studies of this nature.

A plant's ability to utilize light; often measured in terms of chlorophyll fluorescence can serve as a reliable indication of its overall health (Naumann *et al.*, 2007; Murchie and Lawson, 2013). In this regard F_v/F_m (maximum quantum yield) has been reported to decline in plants under conditions of increased pollution (i.e. O_3 [Guidi *et al.*, 1997; Tiwari *et al.*, 2006; Flowers *et al.*, 2007], and NO_2 and SO_2 [Tiwari *et al.*, 2006]). In the present study F_v/F_m (**Fig. 4.8**) was significantly lower in *B. discolor* leaves exposed to SO_2 pollution (at all three sites), which is indicative of some level of photoinhibition (Naumann *et al.*, 2007). Seasonal chlorophyll fluorescence was significantly negatively correlated with seasonal [SO_2]; however, annual chlorophyll fluorescence averages did not reflect the annual average [SO_2] across the treatment sites. So whilst there are indications that this biomarker may be able to reflect the effects of SO_2 pollution there is a need for further investigation.

Chlorophyll is necessary for photosynthesis and photosynthesis which is essential for plant growth and survival (Hijano *et al.*, 2005; Assadi *et al.*, 2011). Both an increase (as previously seen in the species investigated here) and a decrease in chlorophyll content has been reported for a range of abiotic and biotic stressors including air pollution (Hijano *et al.*, 2005; Tiwari *et al.*, 2006; Tripathi and Gautam, 2007; Assadi *et al.*, 2011; Tanee and Albert, 2013; Areington *et al.*, 2015). While a decrease in chlorophyll may suggest the plant's inability to cope with the air pollution stress, an increase can be induced by a pollution-induced reduction in leaf area (Burton *et al.*, 1991; Tanee and Albert, 2013; Areington *et al.*, 2015). In the present study, however, chlorophyll content appeared to be unaffected by SO_2 exposure (**Fig. 4.9**) with seasonal average chlorophyll content being unrelated to seasonal average [SO_2]. This is in contrast with results obtained by Areington *et al.* (2015) for *B. discolor* chlorophyll content and which was significantly positively correlated with distance from the air pollution source. However, it must be mentioned that whilst Areington *et al.* (2015) assayed chlorophyll content via a biochemical method that used photometric measurements of solvent-extracted

chlorophyll (Arnon, 1949), in the present study chlorophyll content was measured using a non-destructive, inexpensive hand-held SPAD meter (Coste *et al.*, 2010).

The last responses of a plant to a stress are usually morphological in nature; leaf area for example, has been widely used as a biomarker for a range of stressors (Burton *et al.*, 1991; Dineva, 2004; Tiwari *et al.*, 2006; Assadi *et al.*, 2011; Tewari *et al.*, 2013). In the present study leaf area of SO_2 exposed leaves of *B. discolor* exhibited a significantly lower leaf area than control leaves (**Fig. 4.10**). Seasonal leaf area was also significantly negatively correlated with seasonal [SO_2]; however, annual leaf area across the treatment sites did not reflect annual [SO_2]. Leaf area has been suggested to be a reliable biomarker of air pollution, with many species showing a decrease in leaf area in response air pollution (Burton *et al.*, 1991; Tiwari *et al.*, 2006; Dineva, 2004; Assadi *et al.*, 2011). The findings of Areington *et al.* (2015), which also showed a decrease in leaf area in *B. discolor* trees closest to the pollution source, together with the data obtained here suggest leaf area may represent a useful biomarker of SO_2 pollution in this species.

Light is a crucial part for photosynthesis and light intensity can alter the plant photosynthetic capabilities (Bolhár-Nordenkampf *et al.*, 1989; Hijano *et al.*, 2005; Assadi *et al.*, 2011), morphological/anatomical features and biochemical activity (Hijano *et al.*, 2005; Assadi *et al.*, 2011). Light intensity differed slightly between the treatment sites and inside the greenhouse in which the control trees were housed. This provided impetus for the validation light measurement comparisons study which was performed in autumn and spring on *B. discolor* trees growing on the same university campus at which the greenhouse-based control trees were located. The results validated the trends observed for chlorophyll content and leaf area in this study and also highlighted the need to consider potentially confounding factors such as inter-site differences in photosynthetically active radiation when interpreting data for biomarkers such as leaf area and chlorophyll content.

It is also important to note that the air pollution stress imposed on plants within the SDB is not solely a consequence of SO_2 and can also differ in terms of severity across the SDB (Buthelezi and Davies, 2015). Qualitative observations of land-use practices at the treatment sites in this study support this statement. *Brachylaena discolor* trees at the Ganges treatment site for example, were located near a major highway (the

Southern/Northern highway, M4) and off a secondary road (Himalayas Road), which would have exposed them to higher levels of carbon monoxide and other vehicular pollutants than trees at the other treatment sites (**Fig. 3.1** and **Fig. 4.11**). High levels of light and heavy motor vehicle traffic have also been suggested to contribute towards air pollution within the SDB, particularly in terms of carbon monoxide (CO) (Diab *et al.*, 2002; Rai *et al.*, 2011). Heavy traffic during industrial working hours can lead to CO plumes (Diab *et al.*, 2002) that may explain why trees at Ganges for example, appeared to be more stressed (in terms of $\cdot O_2^-$, H_2O_2, SOD, lipid peroxidation, leaf chlorophyll fluorescence and LA) than those at Southern Works, even though annual SO_2 levels were highest at Southern Works. The inland location of Ganges (as opposed to Southern Works which is located on the coast-line and influenced by a NE or SW winds) could have also exacerbated the effects of pollution at Ganges relative to the other sites. The effects on all forms of pollution and their synergetic effects were beyond the scope of the present study. However, findings of this study emphasise the importance of considering topography and weather when interpreting effects of air pollution on specific biomarkers and suggests that biomarkers should ideally be related to more than one pollutant (i.e. proxy of pollution).

In summary, all biomarkers appear to have been negatively affected by air pollution relative to the control but the degree of these effects varied across biomarkers and within biomarkers sites and seasons. Seasonal averages for five of the 10 biomarkers compared, viz. $\cdot O_2^-$, lipid peroxidation, electrolyte leakage, chlorophyll fluorescence and LA, were significantly correlated with [SO_2]. However, only electrolyte leakage was sensitive enough to reflect differences in SO_2 levels across the treatment sites. Electrolyte leakage would therefore be the most suitable biomarkers of SO_2 pollution for establishing *B. discolor* as a bioindicator of air pollution within the SDB.

The establishment of *B. discolor* and trees in general as bioindicators of air pollution within industrialised areas in South Africa can help alleviate some of the present limitations to active (instrumental) air quality monitoring experienced by numerous municipalities across the country.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

6.1. Major findings

This study aimed to compare the effects of varying levels of SO_2 pollution on a range of biochemical, physiological and morphological stress biomarkers in *B. discolor* leaves. This was done with the intention of identifying biomarkers that can be used to establish *B. discolor* leaves as a bioindicator of air pollution within the SDB.

As in other studies (Hijano *et al.*, 2005; Tripathi and Gautam, 2007; Li and Yi, 2012) [SO_2] was used as a proxy for air pollution at three industrial sites (viz., Prospecton, Ganges and Southern Works) within the SDB in the present study. Analysis of SO_2 data received from air quality monitoring stations at each of these sites revealed SO_2 levels within the SDB to be relatively high in terms of global standards (Matooane and Diab, 2001; Diab *et al.*, 2002; Josipovic *et al.*, 2010), with Southern Works exhibiting the highest SO_2 levels in the general study area. These data, however suggests that [SO_2] can vary greatly both temporally and spatially within the SDB, as reported by other authors (Scott and Diab, 2000; Diab *et al.*, 2002; Batterman *et al.*, 2008).

The suitability of the biomarkers investigated in this study was based on whether: (i) the biomarker could discriminate between SO_2 exposed and unexposed leaves, (ii) the biomarkers were significantly correlated with seasonal [SO_2], and (iii) the biomarker could reflect exposure to different levels of SO_2. In this regard, six of the ten biomarkers (viz., H_2O_2, TAA, lipid peroxidation, electrolyte leakage, leaf chlorophyll fluorescence, and leaf area) were able to discriminate between SO_2 exposed and unexposed leaves. It should be noted that these included biochemical, physiological and morphological parameters, which emphasises the need to consider a wide range of parameters in order to gauge the effects of a stress on plants and the environment in general (Moraes *et al.*, 2002; Tiwari *et al.*, 2006; Tripathi and Gautam, 2007; Assadi *et al.*, 2011). Of the six biomarkers mentioned above, only four (viz. lipid peroxidation, electrolyte leakage, leaf chlorophyll fluorescence, and leaf area) were significantly correlated with seasonal [SO_2]. Seasonal $\cdot O_2^-$ was significantly correlated with seasonal [SO_2] but it was not able to discriminate between SO_2 exposed and unexposed leaves. Electrolyte leakage was, however, the only biomarker sensitive enough to reflect differences in [SO_2] across the

treatment sites. The results also suggest that biomarker responses were strongly influenced by seasonal variations in [SO_2] which may also have been influenced by site topography.

6.2 Challenges and short comings

The lack of data for pollutants other than SO_2 represented a major challenge during the study. Furthermore, where SO_2 data were available there were a number of either missing or erroneous data points. This limited the data available for this study to just three of the 12 air quality monitoring stations (Ganges, Southern Works and Prospecton) in eThekwini. Diab and Motha (2007) have also acknowledged gaps in the data received from eThekwini Municipality operated monitoring stations. This validates the need to establish bioindicators that can provide complementary monitoring mechanisms of air quality within the EMA and particularly for areas like the SDB.

The qualitative data collected indicated that there are a diverse range of pollution sources and hence pollutants across the three sites were investigated. Selecting SO_2 as the sole proxy for air pollution was therefore not ideal since pollutants act cumulatively on the environment (Novak *et al.*, 2003; Assadi *et al.*, 2011; Rai *et al.*, 2011; Li and Yi 2012; Buthelezi and Davis, 2015). This may also explain why trees at Ganges which did not exhibit the highest SO_2 levels often exhibited higher levels of stress (in terms of biomarkers such as $\cdot O_2^-$, H_2O_2, LPO and LA, at least) than those at Southern Works, at which SO_2 levels were highest.

6.3 Recommendations for future research

The SO_2, qualitative and biomarker data presented in this study provide ample motivation for the need to establish bioindicators of air pollution in areas like the SDB, to complement and support current air quality monitoring techniques (Tripathi and Gautam, 2007). According to Conti and Cecchetti (2001) bioindicators act as an alarm of human induced stress on ecosystems, and in rapidly developing cities like eThekwini bioindicators are likely to become increasingly important. Knowing, the responses of bioindicators to specific pollutants, will also allow for the quick and appropriate mitigation strategies (Hijano *et al.*, 2005; Tripathi and Gautam, 2007). On this note, the

biomarkers shown to be useful in reflecting the effects of [SO_2] in this study should be ideally be related with other pollutants to further validate their use.

Based on the results obtained here, future research on the establishment of leaves of other tree species as bioindicators must seek to include/compare a range of biomarkers (biochemical, physiological and morphological) and consider physiological interactions between/among them as well. For example, the activity of non-photochemical quenching (NPQ) strategies are severely compromised in plants exposed to stress (Ismail *et al.*, 2014), and could therefore be measured in combination with chlorophyll fluorescence in studies of this nature. Another biomarker for future studies to consider is the hydroxyl radical (·OH). This radical is only formed if SOD and CAT are unable to handle stress levels (Sharma *et al.*, 2012) and may prove more useful than superoxide as a biomarker since plants do not have a ·OH specific antioxidant making it potentially more harmful (Sharma *et al.*, 2012). It is also recommended that before chlorophyll content is disregarded as a biomarker for *B. discolor,* a comparison between biochemically determined chlorophyll content and SPAD units be performed to evaluate how sensitive the SPAD instrument is to changes in chlorophyll content (Yamamoto *et al.*, 2002; Coste *et al.*, 2010).

Finally, the results of the present study provide ample motivation for the establishment of leaves of *B. discolor* as bioindicators of air pollution within the EMA. Further refinement may be needed in terms of the exact combination of leaf biomarkers to be used but for now it is evident that in *B. discolor*, leaf LPO, electrolyte leakage, chlorophyll fluorescence and LA can provide valuable information on air pollution levels within industrial areas such as the SDB.

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APPENDIX

A

Table A GPS co-ordinates of the four *B. discolor* trees sampled at each treatment sites.

	Tree 1	**Tree 2**	**Tree 3**	**Tree 4**
Prospecton	30˚0'8.8"S 30˚55'46.6"E	30˚0'11.8"S 30˚55'45.9"E	30˚0'8.0"S 30˚55'45.1"E	30˚0'8.0"S 30˚55'44.2"E
Ganges	29˚56'50.5"S 30˚57'58.7"E	29˚56'51.8"S 30˚57'57.3"E	29˚56'50.3"S 30˚57'59.2"E	29˚56'48.9"S 30˚58'00.9"E
Southern Works	29˚56'58.4"S 30˚58'43.1"E	29˚57'02.4"S 30˚58'39.7"E	29˚57'10.0"S 30˚58'33.6"E	29˚57'28.1"S 30˚58'58.7"E

B

Figure **B** Immature *B. discolor* leaf (left), a leaf in the process of shedding the tomentulose (middle) and a mature leaf (right) that was suitable for testing various parameters used in this study (scale bar = 1 cm).

C Du and Bramlage (1992) equation to correct for interference of sugars MDA

1. $A = (Abs_{532} - Abs_{600})$
2. $B = (Abs_{440} \times 0.0571)$
3. $MDA\ total\ \ (n\ mol\ ml^{-1}) = \frac{A-B}{157000\ (M^{-1}cm^{-1})} \times 10^{6}$