CHAPTER 1

INTRODUCTION

All life is dependent on photosynthesis. Were this process to cease any other could not adequately replace it. Such is the rate at which non-photosynthetic organisms consume plant material and each other so that their stock of food would rapidly be depleted and the higher forms of life would become distinct (Fogg, 1968).

The term photosynthesis literally means building up or assemble by light. Absorbed light energy drives a series of photosynthetic reactions that, ultimately, lead to the formation of new organic carbons (Prézelin & Nelson, 1990). Not all the visible light that is absorbed is used for photosynthesis, just as not all the light absorbing molecules within a plant cell function in photosynthesis. However, photosynthesis remains a very fragile mechanism in the cell and can be easily altered by various environmental factors (Prézelin & Nelson, 1990). When light strikes a plant, variable fractions of the photosynthetically available radiation (PAR) are reflected off the leaf surface, transmitted through the plant or absorbed by molecular components within the plant. Although environmental stresses can have a major effect on the photosynthetic productivity of crops, stress-induced depressions in crop growth and yield can often be primarily associated with an inability of the plant to develop a fully functional photosynthetic apparatus (Baker & Ort, 1992). Exposure of leaves to ozone can result in the depression of photosynthesis. This has been attributed to decreases in carboxylation efficiency, the rate of regeneration of ribulose 1,5-bisphosphate and stomatal conductance (Guderian *et al*., 1985).

Ozone was first recognised as a phototoxic air pollutant in 1958. It originated from reactions between constituents of photochemical smog. Industrial pollution, originally a rare natural hazard for plants, has increased to a crisis point over the past decade. Pollution stresses are largely chemical and are the result of either direct poisoning by toxic materials or the effects of secondary toxic substances created in the air or plant (Bidwell, 1974).

Ozone is mainly found in two regions of the earth's atmosphere. Most ozone (about 90%) resides in a layer between approximately 10 and 50 kilometres above the earth's surface, in the region of the atmosphere called the stratosphere (Manning & Feder, 1980). Paradoxically, ozone plays a beneficial role in the stratosphere by absorbing most of the biologically damaging ultraviolet sunlight specifically UV-B, allowing only a small quantity to reach the earth's surface (Allen *et al*., 1998). It shields terrestrial life, but conversely, in the lower atmosphere (troposphere) it causes oxidative stress in cells. While ozone is formed naturally, the recent increases in the troposphere are due to increased industrialisation (Ashmore & Marshall, 1999). Low-lying ozone is a key component of smog, a familiar problem in the cities around the world and increasingly higher than usual concentrations of surface-level ozone are being observed in rural areas as well. (Middelton *et al*., 1950).

Ozone is not emitted as such, but its concentrations are correlated with industrialisation and automobile traffic (Grobbelaar & Mohn, 2002). It is also considered to be the most important air pollutant in the lowest strata of the troposphere over central Europe and North America (Heagle, 1989). It is toxic to humans, vegetation and animals and is responsible for smog formation. It can be regarded as a secondary pollutant formed as a result of the reaction between gas emissions and sunlight. Considering its highly reactive nature, ozone is unlikely to penetrate leaf tissue and reach the chloroplast. The primary site of actions is likely to be the plasmalemma, with the resulting modifications to membrane structure and function producing changes in the ionic and solute relations of cellular compartments, which then could perturb photosynthetic metabolism (Nie *et al*., 1993).

Over the course of several decades, the research community has addressed the effects if elevated levels of tropospheric ozone on agricultural crops. Findings of negative impacts on crop production have raised public concern first in the United States and later in Europe. More recently, the concern about this issue has been raised in other parts of the world as well (Fuhrer & Booker, 2003). Thus, when we look at the future needs for research on ozone effects, we should keep the global dimension of the problem in mind. The impact of elevated ozone concentrations on plants has negative implications, particularly in relation to production and

sustainability. Above a certain threshold level, ozone inhibits plant growth and development, which means that production to a greater or lesser extent is, inhibited (Heck *et al.,* 1988; Reiling & Davison, 1994). Ozone interventions can vary significantly over short periods of time and it is crucial to determine how rapidly crops react to ozone stress. It is also important to find out to what extent the stress impairment might be reversible, once the ozone concentrations decrease (Reiling & Davison, 1994).

Unlike animals, plants cannot defend themselves against microbial attack by producing circulating antibody proteins or specialised cells. Instead, they resist pathogen infection through physical and chemical defences that may be either performed (cuticle and cell wall) or induced after pathogen penetration. Induced defences include production of ROS, cell wall strengthening phytoalexin biosynthesis and accumulation of defence related protein such as pathogenesis-related (PR) proteins (Rivera *et al*., 2002). The accumulation of PR proteins upon infection with pathogens is well-documented (Van Loon, 1997). There are suggestions that stress related reactions are universal being independent of the stressor, either biotic or abiotic.

The chemical, physiological and morphological changes in leaves caused by ozone can also alter plant sensitivity to other stresses (Schraudner *et al*., 1992). Suggested plant responses to O_3 are: induction of PR-proteins, accumulation of phenolic compounds and increases of volatile compounds (Piffanelli *et al*., 1999). Amongst these are the hydrolytic enzymes β-1,3-glucanases, which are capable of hydrolysing the β-1,3-glucans found in the cell walls of several genera of fungi (Farkas, 1979). Induction of β-1,3-glucanase has been demonstrated in many plant pathogen interactions and they are thought to play several roles in plant defence. Firstly, they can degrade the cell wall of the pathogen or disrupt its deposition, contributing to pathogen death (Mauch *et al*., 1988), and secondly they can release cell wall fragments that act as elicitors of active defence response (Yoshikawa *et al*., 1993).

The adverse effect that ozone has on plants depends on the dose (i.e. concentrations) of ozone and the time plants are exposed (Stintzi *et al*., 1993). At the

planet's surface, ozone comes into direct contact with life forms and displays its destructive side. Because ozone reacts strongly with other molecules, high levels are toxic to organisms and cause severe damage in plant tissue. Ironically plants, by emitting volatile organic compounds (the fuel of photochemical oxidation) and nitric oxide (the catalyst of ozone formation), also contribute to ozone built-up in the troposphere (Saitanis & Karandinos, 2001). Vegetation, due to its emissions of reactive hydrocarbons, is thus a major contributor to the production of ozone. High atmospheric concentrations of ozone are produced as a result of a complex series of reactions, which involves emissions of nitrogen oxides and certain hydrocarbons (Ashmore & Marshall, 1999).

Abiotic and biotic stresses have received much attention by researchers over the years. They found it necessary to analyse the impact of e.g. ozone stress in combination with other abiotic and biotic stresses (Reiling & Davison, 1994). Many studies like those of McKee (1994) have confirmed some of the harmful effects of ozone on crop production, forest growth and human health.

Hydrocarbons are substances consisting of carbon and hydrogen atoms only. They need ozone and other elements such as sunlight, carbon monoxide (CO), and nitrous oxides (NOx), to form. These reactive hydrocarbons (RH) are available from many sources such as; automobiles, trees, industrial smog, etc. Reactions leading to ozone formation are favoured by high temperatures and light intensities, and it is characteristically a pollutant of hot summer days (Harris & Bishop, 2001). Although the impact of ozone on agriculture in North America and Western Europe has received considerable attention, there has been little recognition of it present potential impact in the developing countries of Asia, Africa, South, and Central America and South Afirca. However, it remains vitally important to investigate the impact of ozone, especially on crop plants, in these countries, because they all rely heavily on annual increases in food production to meet the requirements of the ever-growing population. If production was reduced due to ozone pollution, the economic and social implications would be near disastrous (Ashmore & Marshall, 1999). Currently in South Africa O_3 concentrations can fluctuate between 50–300ppb. While in Europe it is not uncommon for O_3 concentrations to reach 400ppb and more during

summer months for short periods, causing necrotic lesions of leaf surfaces of susceptible plants (Grobbelaar & Mohn, 2002).

There is some evidence (Kohut *et al*., 1987) that brief moderate to high doses of O_3 are more harmful to plants than long-term exposure to low doses. O_3 appears to affect plant health in two ways, i.e. low doses over a long time, which mainly affects physiological processes and metabolism without causing visible injuries, while high doses over short periods cause visible injuries. In both instances net photosynthesis is reduced, thereby, resulting in crop losses (Heagle, 1989).

The importance of understanding the dynamics and impact on crop production would provide the basis for corrective measures to be taken. Tools are required to identify the stressed from the unstressed plants, at an early stage, in order to assess the overall magnitude of the impact of ozone on crop yields. Analysis of chlorophyll fluorescence combined with certain plant physiological determinants may become such a tool.

1.1 PURPOSE OF THE STUDY

The overall purpose of this study was to investigate the impact of the abiotic stress factor, ozone, on the selected crop plant, *Zea mays*, by using chlorophyll fluorescence and selected physiological indicators. The focus fell on O_3 as an important constituent of photochemical air pollution and its effects on plants. The impact of O_3 on agriculture is more subjective because it is bases upon extrapolation taken from experimental exposures. During this study the objective was to obtain a holistic understanding of ozone stress impairment on crops and possibly to identify critical concentrations when damage becomes non-reversible and detrimental for natural ecosystems, food production and the environment as a whole.

1.1.1 ISSUES ADRESSED

There are three, natural responses when a new problem has been identified; namely cause, cure and prevention. Various approaches have been used to assess plant response to stress, including visual observation, biochemical and biophysical responses. In most cases the damage caused had already reached irreversible proportions before the defence responses could be detected. During this study we aimed to assess the response of *Zea mays* to ozone stress using chlorophyll fluorescence techniques. To complement this fluorescence analyses, specific physiological responses of *Zea mays* towards O_3 were also investigated and analysed.

CHAPTER 2

LITERATURE STUDY

In general, photosynthesis is regarded as the process by which plants synthesise organic compounds from inorganic raw materials in the presence of sunlight. All forms of life on this planet require energy for growth and maintenance. Thus, the ultimate source of all metabolic energy on our planet is the sun and photosynthesis is essential for maintaining all forms of life on earth (Hall & Rao, 1972).

Plants are often exposed to unusual conditions, which forces them to acclimate and adapt. Figure 2.1 depicts schematic representation of a state-change as a consequence of an applied stressor, also illustrating the different terms used in analysing stress. These are; *Stressor*; every factor that provokes 'stress'; *Stress*: every established condition which forces a system away form its thermodynamic 'optimal state'; Optimal state of a biological system: the state at which the system is in full 'harmony' with its environment; *Harmony* of a biological system with its environment: the achieved situation in which the system does not need to change any activity or conformation; and Strain: any physical or chemical change caused by stress (Strasser & Tsimilli-Michael, 2001). Biological stress is any change in environmental conditions, which may reduce or adversely change a plant's growth or development (its normal functions). When environmental conditions are such that a plant responds maximally it can grow optimally and the plant is unstressed. This implies that when the plant is grown under conditions that are less than optimum, it might be stressed.

2. AIR POLLUTION

2.1 OZONE

Air pollution is not a localised problem and this is especially true of ozone. Rapid increases in industrialisation, and other human activities, during the twentieth century have contributed significantly to toxic gaseous pollutants in the troposphere, which pose a significant threat to the survival and productivity of native and cultivated ecosystems (Rao *et al*., 2000).

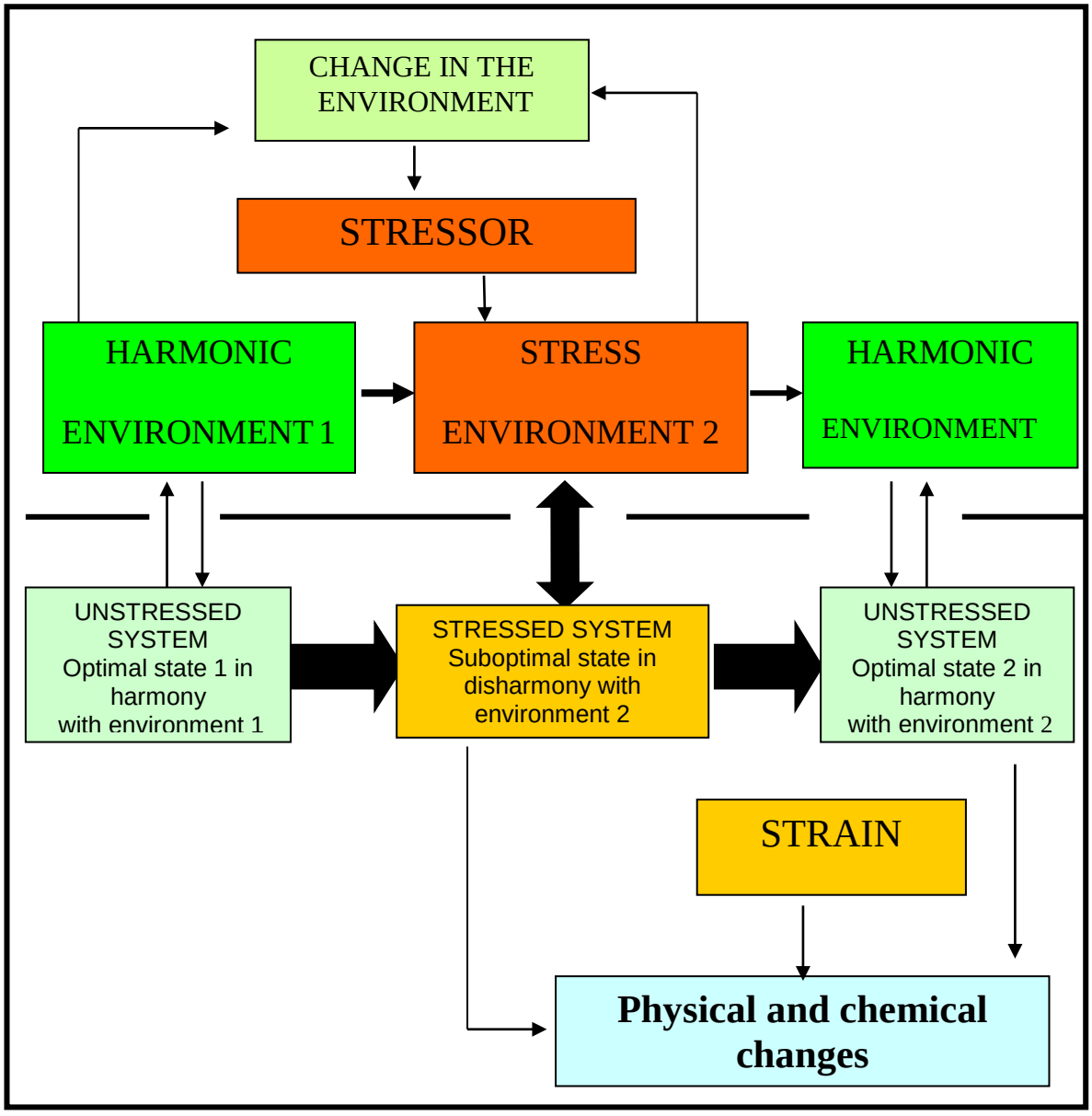

Figure 2.1: A schematic presentation of a state-change as a consequence of an applied stressor (Strasser and Tsimilli-Michael, 2001)

Above certain concentration levels several types of air pollutants may have negative impacts on plants. Of these gaseous pollutants, ozone is regarded as one of the most important in the cause of crop damage (Heath, 1994; Aunan *et al*., 2000). Krupa & Kickert (1989) have remarked that ozone has caused more damage to both natural and cultivated crop plants in industrialised countries than any other air

pollutant. O_3 plays a controlling role in the oxidation capacity of the troposphere. Besides being an oxidant itself, O_3 is a major precursor for all known oxidising agents in the troposphere, most notably for the hydroxyl radical ($OH^{\bullet}$) (Ehhalt, 2001).

2.1.1 Issues related to long-term effects of ozone

Yield and quality:

Long-term effects of ozone on annual crop production result form the cumulative impact of ozone taken up over the course of a single growing season. In many developed countries, domestic agriculture production levels are sufficiently high that changing consumer preferences may become more important in driving research in the coming years. In developing countries the improvement of the nutritional value of crops is an issue for the food industries and studies on the possible impact of ozone on nutritional aspects need to be considered (Fuhrer & Booker, 2003).

In the case of perennial crops (e.g. maize), relevant long-term effects of ozone may develop over several years. Forage quality may be changed because of ozone effects on leaf chemistry. This could be a direct effect on secondary metabolism, or a change in plant development. As discussed in later sections, long-term ozone exposure can lead to increased levels of phenolic acids, flavoniods and related compounds, that may negatively affect enzyme systems.

2.1.2 Issues related to ozone action

Ozone is a three-atom allotrope of oxygen that reacts with plants in (1) solid phase (e.g. with the cuticular components of plant leaves), (2) gas phase (e.g. hydrocarbons emitted by plants) and (3) liquid phase. This induces the dissolution of O_3 in aqueous media followed by reacting with lipids, proteins and other cellular components (Rao *et al.,* 2000). The solid and liquid phase reactions are the most important in plants. However, most studies have focused on the reactions of O_3 in the liquid phase, as dissociation of O_3 in the leaf's extracellular spaces has the greatest affect on plants (Mudd, 1997).

Ozone, in the troposphere, is formed when nitrogen dioxide (NO_2) is converted to nitric oxide (NO^-) when exposed to sunlight. With $h\nu$ (radiation energy) as common denominator, CO, CH_4 and RH also react with O_2 to form water (H_2O) and O_3 (Figure 2.2). The liberated oxygen atom reacts with an oxygen molecule (O_2) to form O_3. In the absence of competing or scavenging molecules, the reaction reverses to produce a state of equilibrium between O_3, NO_2 and NO^-. However, when organic molecules, largely the volatile organic hydrocarbons (VOCs), are present they react with NO^-, stopping the back reaction so that O_3 accumulates. Other molecules, notably hydroxyl radicals ($OH^\bullet$) and its precursors, are also important (Treshow & Anderson, 1991).

$$NO_2 + O_2 + h\nu \leftrightarrows NO^- + O_3$$
$$CO + 2O_2 + h\nu \rightarrow CO_2 + O_3$$
$$CH_4 + 4O_2 + 2h\nu \rightarrow HCHO + H_2O + 2O_3$$
$$RH + 4O_2 + 2h\nu \rightarrow R'CHO + H_2O + 2O_3$$

Figure 2.2: Chemical reactions of O_3 formation in the troposphere (Rao *et al*., 2000).

It is known that H_2O_2 and NO function as signalling molecules in plants, and that a wide range of biotic and abiotic stresses from various sources result in their generation (Neill *et al*., 2002). H_2O_2, a form of reactive oxygen species (ROS), is generated as a result of oxidative stress. H_2O_2 is a major ROS contributing to the oxidative burst (Wojtaszek, 1997b), and apparently plays a role in the induction of the defence responses (Alvarez *et al.,* 1998). Oxidative stress arises from an imbalance in the generation and metabolism of ROS, with more ROS (such as H_2O_2) being produced than what is metabolised (Neill *et al*., 2002). H_2O_2 generation, *via* electron transport, is increased in response to environmental stresses such as excess excitation (light) energy, drought and cold (Dat *et al*., 2000). Given that H_2O_2 is produced, in response to such a variety of stimuli, it is likely that H_2O_2 mediates cross-talk between signalling pathways. It is, therefore, a signalling molecule contributing to the phenomenon of “cross-tolerance”, in which exposure of plants to one particular stress offers protection against another (Bowler & Fluhr, 2000). During this study H_2O_2 production was measured to investigate the role of ROS in defence against ozone stress.

Ozone has strong oxidising properties and causes injury and premature mortality of plant tissues. When susceptible tobacco was exposed to ozone, white flecks soon appeared on the upper leaf surfaces, followed by chlorosis and wilting (Wohlgemuth *et al*., 2002). Similar symptoms were described as early as 1938 by Homan, however it was only in the 1950's that ozone was recognised as the cause of this and many other serious plant defects (Darley & Middleton, 1996). Although the specific symptoms of ozone injury vary among plant species and varieties, certain general expressions form a common thread, or similarity. While, initially, limited to the upper leaf surfaces, lesions may extend through the leaves when O_3 concentrations become higher. The symptoms may, however, vary between different plants (Hill *et al*., 1970).

Depending on the concentration of O_3 and the plant species concerned, O_3 causes two different types of plant response, commonly referred to as acute and chronic. Acute exposure, which involves higher concentrations of O_3, (150-300ppb), for relatively short periods (4-6h), rapidly causes visible injury (necrotic) symptoms on the leaf surfaces.

The necrotic lesions and plant responses induced by acute O_3 exposures are reminiscent of the hypersensitive response (HR) that occurs as a result of incompatible plant-pathogen interactions (Rao *et al*., 2000). Inhibition of photosynthesis, respiration and nutrient uptake may subsequently lead to reduced yields of agricultural crops (Aunan *et al.*, 2000). Chronic exposures involve low concentrations of O_3 (≤100ppb) with exposure over longer periods (days to months). Chronic injury is subtler, and depending on the plant species, may include symptoms such as chlorosis and premature senescence (Pell *et al*., 1997).

The phytotoxicity of O_3 was already known in the mid 1950s (Richards *et al*., 1958). Its discovery prompted widespread studies of the effects of O_3 on plant physiological processes, under both laboratory and field conditions. National and international limits on the regulation of ambient O_3 concentrations (Rao *et al.,* 2000), have already been suggested, but the implementation of such regulations seems impossible.

2.2 THE PRACTICAL SIGNIFICANCE OF INTERACTIONS BETWEEN AIR POLLUTION AND OTHER ENVIRONMENTAL STRESSES

From the preceding section it is clear that air pollution is an environmental stress or causing responses in plants that have characteristics, which are common to other stress responses. The ability of plants to adapt to environmental stress conditions must include biochemical, molecular and physiological aspects. The crucial factors of plant stress responses are mostly studied from the stage of signal perception and transduction, to the appearance of accumulative and protective mechanisms leading to adaptation or death. The ideal is to implement a control system that will result in the control of the quantity of pollution that is released into the atmosphere. This could include the implementation of legislation stipulating measures for the emission of cleaner industrial and automobile pollution. That will mean that plants will be protected and adverse effects in crops and natural plant communities will not occur (Weinstein & McCune, 1979).

Because each plant species acts differently to ozone pollution, it is difficult to specify air quality standards applicable to all possible conditions. Therefore, it remains difficult to predict the effects of air pollution under different environmental conditions. The interaction of air pollution with other environmental stresses that might occurs, is therefore a major problem and makes research on the effects of air pollution on plants very difficult (Weinstein & McCune, 1979).

2.3 EFFECTS OF OZONE ON PLANT ACTIVITIES

2.3.1 ABSORBANCE OF OZONE

2.3.1.1 Ozone effect on the stomata

Ozone phytotoxicity results from biochemical changes within a cell or on its surface. The ease, with which ozone moves from the ambient air to the target sites, is therefore, a key factor in controlling plant response. O_3 enters the plant through the stoma and it is assumed that, once O_3 has entered the leaf, radicals produced from O_3, alters the integrity of the cells. As O_3 reacts, presumably instantaneously, with the

cellular components such as the cell wall and plasma membrane, reduced oxygen species such as super oxide radicals (O_2^-), hydroxyl radicals ($OH^\bullet$) and hydrogen peroxide (H_2O_2) are formed (Grimes *et al*., 1983).

At high concentrations, ozone causes cells to collapse, resulting in visible foliar injury. Effects on the plasma membrane cause changes in membrane function, which in turn reduces the photosynthetic processes in the chloroplast. Increased dark respiration often occurs, probably due to the increased respiration associated with maintenance and repair (Amthor & Cumming, 1989). The reduced CO_2 assimilation and increased respiratory CO_2 losses leads to the overall reduction of assimilate production in leaves of crop species such as maize.

Soil stress, water stress and enhanced atmospheric vapour pressure deficit can cause a reduction in stomatal conductance and hence in O_3 uptake, which may lead to a reduction in the impact of ozone on yields (Fangmeier *et al*., 1993). The stomatal control of O_3 uptake is controlled by intrinsic and environmental factors that can partially or completely exclude ozone stress form the plant. Some intrinsic factors include stomatal opening and closing. When the stomata are closed, no or little O_3 is able to enter the plant and no injury occurs. Problems arise when the stomata size varies under changing experimental conditions during ozone exposure. Based on the measurements of O_3 flux in leaves, Liask *et al*., (1989) suggested that O_3 does not penetrate deeply into intracellular spaces but rather decomposes at the cell wall and plasma membrane. Evans and Ting (1974) studied the water potential, leaf resistance, stomata spacing and other leaf characteristics of primary bean leaves in relation to ozone sensitivity and injury. They found that leaf water potential decreased during O_3 exposure. After O_3 treatment abaxial leaf stomata resistance initially increased, but then decreased. After 1 hour, abaxial resistance returned to its pre-fumigation level. At high O_3 concentrations, abaxial leaf resistance decreased steadily.

It has been suggested that even if the stomata should remain open, in some cases the plant may not necessarily be injured. For example, Ting & Dugger (1971), found no closure of stomata and a slight stimulation of photosynthesis when the Pinto

beans (*Phaseolus*) were exposed to O_3, and there was no evidence of visible injury. However, the contrary could also be true, as many other studies have shown. Hill & Littlefield, (1969), found only partial closure of the stomata in oats after exposure to ozone. Although little visible injury was observed, they did observed severely reduced rates in photosynthesis and transpiration.

2.3.1.2 Ozone in the extracellular spaces (apoplast)

Once the leaf has absorbed ozone, it comes into direct contact with the leaf interior and has to move from the extracellular spaces to the target sites.

The extracellular matrix (apoplast) of cells is the first active aqueous defence line against gaseous air pollutants such as ozone (Padu *et al*., 1999). In order to affect the plant the O_3 must dissolve in the aqueous layer lining the cells, diffuse across the cellular membrane, and so influence cellular components and metabolic processes (Grobbelaar & Mohn, 2002). Exposure to ozone, however, alters the permeability of the plants' plasma membrane (Heath, 1988), making normal cellular activities difficult.

The plant cell wall contains many phenolic groups, olefinic compounds and amide proteins. In addition, the adjacent plasma membrane contains many unsaturated lipids. It is likely that the first set of bio-molecules which can react with O_3 will be encountered within the cell wall regions just outside the plasma membrane and form highly toxic ROS (Heath, 1987). These O_3 derived ROS are believed to alter the physicochemical properties of the plasma membrane by initiating lipid peroxidation (Pauls & Thompson, 1980), and altering Ca^{2+} and ion fluxes (Castillo & Heath, 1990) which together disrupt the cellular machinery causing a reduction in net photosynthesis (Reich & Amundson, 1985). It is possible that either the O_3-derived ROS or the intermediates generated due to the reaction of O_3 with cellular components are propagated throughout the cell causing a variety of biochemical changes.

2.3.1.2.1 Reactive oxygen species ‘Oxidative burst’

The oxidative burst is an integral component of plant resistance to stress, be it biotic or abiotic. These include extreme temperatures, UV radiation, EEE (excess excitation energy), ozone exposure, wounding and eliciting pathogens (Prasad *et al*., 1994). It is generally defined as a rapid production of high levels of reactive oxygen species (ROS) in response to external stimuli (Wojtaszek, 1997a).

It has been observed that plant-pathogen interactions cause an active controlled oxidative burst and formation of self-propagating apoplastic ROS production in plants.

ROS serve as signalling intermediates in programmed cell death (PCD), which is a organised disassemblement of cells, which eventully leads to localised cell death (Lamb & Dixon, 1997; Bolwell, 1999). Previous studies have shown that the major ROS contributing towards the oxidative burst is H_2O_2, with possible participation of O_2^- (Levine *et al*., 1996). The mitochondrion is a major source of ROS formation and it is possible that this organelle could participate in the oxidative burst in plants (Tiwari *et al*., 2002). Plants, as aerobic organisms, require oxygen for the efficient production of energy. During the reduction of O_2 to H_2O, ROS such as O_2^-, H_2O_2 and $OH^\bullet$ are generated. Initially the reaction requires an input of energy, whereas subsequent steps are exothermic and can occur spontaneously, either catalysed or not (Vranová *et al*., 2002).

In the mesophyll cells the O_3 is converted to superoxide anion (O_2^-), hydroxyl radicals ($OH^\bullet$) and H_2O_2. O_3 is broken down in the water, and during its reactions with the constituents of the apoplast ROS are generated (Grimes *et al.,* 1983, Neill *et al*., 2002). These ROS is very harmful to the plant and H_2O_2 is especially toxic because of its ability, even at low concentrations, to inhibit the Calvin-cycle. Given that H_2O_2 is produced in response to such a variety of stimuli, it is likely that H_2O_2 mediates cross-talk between signalling pathways and is an attractive signalling molecule to the phenomenon of ‘cross-tolerance’, in which exposure of plants to one stress provide protection towards another (Bowler & Fluhr, 2000).

Another way, in which ozone may act, is by inhibiting phosphorylation of leaf mitochondria (Lee, 1967). Therefore, plants have to be able to metabolise these active oxygen species and this is achieved through the antioxidative defence system. These deleterious compounds are inactivated by antioxidants. Several natural products have the potential to exhibit antioxidant properties. Among them are specialised pigments where they can capture radiant energy, using sensory pigments i.e. carotenoids (Götz *et al*., 1999). Carotenoids are effective quenchers of triplet-state and protect against singlet oxygen and peroxide radicals (Krinsky, 1989).

They are pigments that appear red/orange, are present in all photosynthesising cells and they absorb light form the blue/green range of the visible spectrum. Their colour in the leaves is normally masked by chlorophyll, but in the autumn when the chlorophyll disintegrates the carotenoid pigments become visible.

Carotenoids contain a conjugated double bond system of the polyene type (Hall & Rao, 1972). They are usually either hydrocarbons (carotenes) or oxygenated hydrocarbons (carotenols or xanthophylls). The carotenoids are situated in the chloroplast lamellae in close proximity to the chlorophyll. The energy absorbed by the carotenoids may be transferred to chlorophyll *a* for photosynthesis (Hall & Rao, 1972).

In addition, the carotenoids may protect the chlorophyll molecules from too much photo-oxidation in excessive light, thus their primary role is to neutralise harmful compounds created during photosynthesis (Hall & Rao, 1972). These compounds, often H_2O_2 and singlet oxygen, both attack and destroy cell membranes, and ultimately damaging the cell.

Some response of plants to O_3, are given in Table 1, where (+) indicate positive and (-) negative responses. Overall O_3 cause a positive response in terms of activating antioxidant or defence responses, as well as activating the various signalling molecules. The most prominent negative response plants display is the inhibition of photosynthesis, which eventually causes plant death.

Table 1: A summary of the similarities in plant responses, at the morphological, physiological and molecular level to O_3 and pathogen exposure (Rao *et al*., 2000).

Morphological Responses	Physiological Responses	Antioxidant/defense Responses	Signalling molecules
Chlorotic lesions (+)	ion fluxes (+)	Ascorbate,glutathione (+/-)	
Necrotic lesions (+)	Photosynthetic Pigments(-)	APX, DHR, AR (+/-)	Jasmonic acid (+)
	Photorespiration (+)	GR, GST, GPX, POX (+/-)	Ethylene (+)
	Photosynthesis(-)	Phenolics, ASA, NOS (+)	$\bullet O_2$-, H_2O_2 (+)
	Photoinhibition (+)	LOX, AS, Lignin (+)	NO (+)
	Lipid peroxidation (+)	PAL, CAD, Phytoalexins (+)	Ca^{2+}fluxes (+)
	ATP depletion (+)	STS, Lignin, Callose (+)	Calmodulin (+)
	Programmed cell death (+)	LOX, NOS (+)	ABA, MeJA (+)
		Polyamines (+)	C-6 volatiles (+)

APX (ascorbate peroxidase), AR (acquired resistance), GST (glutathione-*S*-transferase), GR (glutathione reductase), GPX (glutathione peroxidase), POX (peroxidase), ASA (ascorbate), NOS (nitric oxide synthase), LOX (lipoxygenase). PAL (phenylalanine ammonia lyase), O_2^- (superoxide anion), H_2O_2 (hydrogen peroxide), NO (nitric oxide), ABA (abscisic acid), MeJA (methyl jasmonate),

Thus the photoprotective function of carotenoids is essential for photosynthetic organisms. Non-photosynthetic organisms suffer form photooxidative stress caused

by light and near-UV radiation, which requires the presence of antioxidative protective systems (Moradas-Fereira *et al*., 1996).

2.4 EFFECT OF OZONE ON PHOTOSYNTHESIS

Ozone can inhibit the photosynthetic activity of plants due to decreased stomatal conductance and/or by reducing the capacity of mesophyll cells to fix CO_2 (Grobbelaar & Mohn, 2002). Photosynthesis however, is far from the only metabolic process influenced by ozone, but it is intimately linked to productivity. During photosynthesis under high light flux, especially in the saturation range of the photosynthetic light curve, the photosynthetic apparatus absorbes more light. Ozone affects and can destroy chlorophyll, leading to reduced photosynthesis. Mostly at higher concentrations, it causes visible injury (Runeckles & Resh, 1975; Knudson *et al*., 1977). While net photosynthesis can be impaired, without the development of visible symptoms, earlier research first suggested that photosynthesis tends to return to normal when the exposure ends (Pell & Brennan, 1973). Even low ambient O_3 concentrations may reduce net photosynthesis in O_3-sensitve tree and crop species (Reich & Amudson, 1985). Rubisco is the major leaf protein in plants. In potato, O_3 resulted in a decline in photosynthetic carbon fixation through loss of Rubisco activity, associated with a reduced concentration of Rubico protein and diminished photosynthetic capacity (Dann & Pell, 1989). Thus, most probably O_3-induced loss of Rubisco contributes significantly to the accelerated senescence process (Pell *et al*., 1994).

Ozone affects so many related processes, that it is difficult to distinguish which is first affected. One vital process inhibited is electron transport, in the water splitting light reaction, whereby O_2 is released and energy is made available to drive the ‘non-cyclic’ reactions, in which carbon dioxide (CO_2) is reduced (hydrogen is added) and carbohydrates are formed. This is accomplished by the coenzyme, nicotinamide adenine dinucleotide phosphate ($NADP^+$). The most important sources of ROS during photosynthetic electron transport are the reduced electron acceptors of PS I, which transfer individual electrons to O_2 (Asada, 1999).

$NADP^+$ can capture an electron from chlorophyll, and accept a hydrogen ion from the splitting of water molecules becoming NADPH or reduced NADP. NADPH then takes part in the sugar-building reactions of the carbon cycle. ATP and total adenylate are increased immediately following O_3 exposure (Pell & Brennan, 1973). The increased energy is derived form lipids and proteins inside the cell membranes, once the normal carbohydrate reserves are exhausted (Skärby *et al*., 1987). ATPase which in turn is associated with ion pumps in the membrane, can be rendered inactive by ozone and all these changes may lead to disruption of normal cell activities (Dominy & Heath, 1985).

These are the first, detectable, effects of O_3 on photosynthesis, which is then followed by the inhibition of electron transport between the different photosystems (Schreiber *et al*., 1978). Membrane permeability, particularly the chloroplast membrane is also altered by O_3 (Nobel & Wang, 1973). Ozone has been found to reduce the activity of the carboxylase enzyme, which is vital to CO_2 fixation and thereby, limits the production of essential sugars. Generally, the quantum requirement for CO_2 reduction is great, especially when the end products of photosynthesis are organic molecules other than simple sugars. Metabolic demands can often require that light-dependent ATP production be increased, relative to $NADP^+$ reduction (Nakamura & Saka, 1978).

There is evidence that short moderate to high doses (acute) ozone can be more harmful to a plant, than long term (chronic) low doses. Both these chronic and acute exposures to O_3 reduce net photosynthesis and might also enhance premature senescence and thereby causing crop losses (Kangasjärvi *et al*., 1994). This includes any impairment of the intended use of the plant i.e. loss in weight, number or size of plant parts that might be harvested; changes in the chemical composition or quality; or loss in aesthetic quality, a value difficult to quantify or judge. Studies by Heagle (1972) showed that low concentrations of ozone, when exposures were extended through the growing season could cause pronounced losses in production. Ozone concentration as low as 50-100ppb, for 6 hours per day, throughout the growing season caused significant reduction in the fresh weight of corn ears, number of kernels and dry weight of the kernels.

When light strikes an organic molecule in the ground state, it absorbs radiation of certain spesific wavelengths to jump to an excited state. This excited chlorophyll molecule can revert back to the ground state in a number of ways. A part of the excitation (absorbed) energy is lost on vibration relaxation, i.e., radiationless transition to the lowest vibrational level takes place in the excited state. And eventually the molecule returns to the ground state while emitting a kind of optical energy, which is called “fluorescence”.

Chlorophyll fluorescence parameters are most commonly used for the remote sensing of plant photosynthesis (Schmuck *et al*., 1992). Chlorophyll fluorescence analysis permits the evaluation of the quantum yield of PS II (Genty *et al*., 1989a), which in turn gives estimates of the rate of linear electron transport (ETR), provided the light absorbed by the leaf is known.

There are two different photosynthetic pathways operational in plants. Plant can either make use of the C_4 or C_3 photosynthetic pathways. In plants such as maize and sugar cane the C_4 photosynthetic pathway is predominantly operative. Although the functional essence of this type of CO_2 assimilation is identical to that of the C_3 pathway, the primary mode of CO_2 capture is substantially more efficient. This is in contrast to the C_3 systems, where the carboxylating reaction occurs only in the mesophyll. C_4 photosynthesis employs two tissue types, namely the mesophyll and bundle sheath cells, to achieve the same result. CO_2 enters through the stomata and diffuses into the mesophyll tissue where it is fixed by PEP-carboxylase to form oxaloacetate, which is then converted into malate (a 4-carbon molecule), and transported into the bundle sheath cells. Here, this C_4-acid is decarboxylated and the released CO_2 refixed by Rubisco and assimilated through the enzymes of the photosynthetic carbon reduction cycle to from sucrose and starch. Because the C_4 mechanism is highly efficient at PEP (phosphoenolpyruvate) carboxylation and C_4-acid delivery, the Rubisco in the bundle sheath is super saturated with CO_2 such that photorespiration is virtually eliminated. Note, however, that the C_4 pathway incurs an extra cost in ATP. In the C_4 plants (e.g. maize), a good correlation has been found between ETR and net CO_2 assimilation (Edwards & Baker, 1993).

Fluorescence is a light emitting process by which pigments in the excited singlet stage, return to ground state if their excess energy is not funnelled into photochemistry, within the excited lifetime of the molecule (Prézelin & Nelson, 1990). Fluorescence occurs only from the lowest excited singlet state so the wavelength of the fluorescence maximum often is a few nanometers longer than the absorption maximum of the pigment. The majority (>90%) of *in vivo* fluorescence, at room temperature arises from back reactions of primary photochemical events occurring in the reaction centres and the light harvesting (LH) chlorophyll of the PS II (Prézelin & Nelson, 1990). Light absorbed in the antenna complexes, which is in excess has to dissipate, to avoid excess excitation energy within the PS II. Thus, the excited singlet state of chlorophyll is subjected to a number of competing, de-excitation reactions including photochemical trapping energy transfer, radiation-less excitation and fluorescence emissions (Flexas *et al*., 2002). Any changes to these reaction results in corresponding changes in fluorescence yield (Schreiber *et al*., 1998).

The most prominent pigments absorbing lighy energy are chlorophyll *a* and *b*. When light energy is absorbed by a chlorophyll molecule, the electron configuration of the molecule is temporarily altered. Plants are continually in danger of absorbing more light energy than they can productively use for photosynthesis. Therefore, acclimation to environmental conditions induces the development of mechanisms for dissipating the accumulation of such excess energy. Acclimation can be due to many signal transduction pathways, which would be initiated by the reception of excess excitation energy, both inside and outside the chloroplast (Mullineaux & Karpinski, 2002). The light energy received by plants, in excess of what they need for photosynthetic productivity, is termed excess excitation energy (EEE). EEE is ever present in land plants. Failure to dissipate or avoid accumulation of EEE leads to photo-oxidative damage of the photosynthetic apparatus. This is often manifested as bleaching, chlorosis or bronzing of leaves. Immediate responses to the conditions promoting EEE initiate signalling pathways leading to plant acclimation. Dissipation of EEE in plants is achieved by a combination of, so-called, non-photochemical and photochemical quenching (Prézelin & Nelson, 1990).

Fluorescence yield is measured amongst others with a modulation fluorometer. Depending on the light conditions different states can be distinguished and are characterised by fluorescence yield notations (e.g. F_O, F_M) and quenching coefficients (q_P and q_N) that are derived from F_0 and F_M, as described in Table 2. In order to obtain useful information about the photosynthetic performance of a plant, from measurements of chlorophyll fluorescence yields, it is necessary to distinguish between the photochemical and non-photochemical contributions to quenching (Bilger & Schreiber, 1986). Quenching can be explained in two ways. Firstly, there is an increase in the rate at which electrons are transported away from PS II. This is due mainly to the light induced activation of enzymes involved in carbon metabolism and the opening of the stomata. Such quenching is called 'photochemical quenching' (Bilger & Schreiber, 1986). Photochemical processes are those processes that utilise absorbed energy for photochemistry, during which electron donation from pigment to an acceptor molecule occurs. Such processes direct the energy needed for the chemical work involved in photosynthesis.

Secondly, there is an increase in the efficiency at which energy is converted to heat, and this process is termed 'non-photochemical quenching' (Johnson *et al*., 1993). Non-photochemical processes are those processes where energy is dissipated from the photosynthesis apparatus in a manner, which does not drive photosynthesis. The notation q_N is termed 'non-photochemical quenching to indicate that it quantifies a decrease in fluorescence of an origin different from that of the photochemical quenching $q_P = (F_M'-F)/(F_M'-F_O')$, as seen in Table 2. However the naming and symbolisation led to confusion, as they give the impression that the two terms are complementary and moreover, that they refer to the same state. In addition the characterisation 'non-photochemical' as such is quite misleading since q_N contains as much photochemical information as non-photochemical information (Strasser *et al*., 1995), and therefore, is not at all a specific index for non-photochemical events. The competition between these processes ensures that reduction in the rate of one process would be associated with a corresponding increase in the rates of competing processes. That would imply that a reduction in the dissipation by non-photochemical processes such as heat production will be reflected in an increase in energy

dissipation by non-photochemical processes, such as heat production and chlorophyll fluorescence (Mullineaux & Karpinski, 2002).

Photochemical quenching (q_P) is directly defined by the relative variable fluorescence as: $1 – q_P = V = (F- F_O)/(F_M- F_O)$. q_P refers only to one physiological state and depends on the redox state Q_A^-/Q_A of the sample in a given physiological state. While q_N refers to two physiological states i.e. index of the change from the dark-adapted to a light adapted state. q_N does not refer to any intermediate redox state Q_A^-/Q_A (Strasser, 1997). Q_r (reduced reaction centres) was calculated as: $Q_r = (F_s-F_0')/(F_M'-F_0')$. The quantum yield for primary photochemistry (φ_P) is defined as the ratio of the total energy flux trapped by the PS II reaction centres (RCs) and used for primary photochemistry. φ_{po} is the maximum quantum yield of primary photochemistry, when all the RCs are open and the relative variable fluorescence is zero (Strasser 1978):

$$\varphi_{po} = 1- (F_O / F_M)$$

Paillotin (1976) derived the equation $\varphi_P = \varphi_{Po}\ [(F_M-F)/(F_M-F_O)]$. The expression in brackets in this equation, is identical to 1-V, where V is the relative variable fluorescence, and moreover, it is also identical to the so-called photochemical quenching q_P, as defined for the steady state of the Kautsky transient, i.e. $F= F_S$.

Hence Genty's equation: $\varphi_e = \Delta F/F_M$ (Genty *et al*., 1989b) for the quantum yield of electron transport, is equal to the quantum yield of primary photochemistry (since it refers to the steady state). Steady state fluorescence yield (F_S) is a function of the competition between photochemical and non-photochemical de-excitation of the energy absorbed by the light-harvesting complexes (Schreiber *et al*., 1998). Steady state fluorescence measures the proportion of the light, absorbed by chlorophyll associated with PS II that is used in photochemistry. It can give a measure of the rate of linear electron transport and also an indication of overall photosynthesis (Fryer *et al*., 1998).

It was felt that the many different descriptions of especially chlorophyll fluorescence parameters measures with so-called 'saturation pulse method' (Quick & Horton, 1984; Dietz *et al*., 1985; Schreiber *et al*., 1986) has caused unnecessary confusion.

The different components that are usually measured with this technique are depicted in Figure 2.3. The nomenclature is defined in Table 2 (van Kooten & Snel, 1990).

Tabel 2: Definition of chlorophyll fluorescence nomenclature (van Kooten & Snel, 1990).

a: *Fluorescence intensity indicators*	
F_t fluorescence intensity	Actual fluorescence intensity at any time (t)
F_O minimal fluorescence (dark)	Fluorescence intensity with all PSII reaction centers open while the photosynthetic membrane is in the non-energized state, i.e., dark adapted q_P=1 and q_N=0. It can also be used for the O level in the (O-I-D-P-T nomenclature).
F_i fluorescence at I level	Fluorescence intensity at I level (O-I-D-P-T nomenclature).
F_p fluorescence at P level	Fluorescence intensity at P level (O-I-D-P-T nomenclature).
Fs or F fluorescence in steady state	Fluorescence intensity at steady state, i.e., T level in O-I-D-P-T nomenclature. Steady state is defined as a period within which the fluorescence intensity does not change while external circumstances remain constant.
F_M maximal fluorescence (dark)	Fluorescence intensity with all PSII reaction centers closed (i.e., q_P=0) all non-photochemical quenching processes are at a minimum (i.e., q_N=0). This is the classical maximum fluorescence level in the dark or low light adapted state.
F_M' maximal fluorescence (light)	Fluorescence intensity with all PSII reaction centers closed in any light adapted state, i.e., q_P=0 and $q_N \geq 0$.
F_O' minimal fluorescence (light)	Fluorescence intensity with all PSII reaction centers open in any light adapted state i.e., q_P=1 and $q_N \geq 0$.
F_V variable fluorescence (dark)	Maximum variable fluorescence in the state when all non-photochemical processes are at a minimum, i.e. (F_M-F_O).
F_V' variable fluorescence (light)	Maximum variable fluorescence in any light adapted state, i.e. (F_M'-F_O').
b: *Fluorescence quenching parameters*	
q_P photochemical quenching	(F_M'-F_s) / (F_M'-F_O')
q_N non-photochemical quenching	1-(F_M'-F_O') / (F_M-F_O)

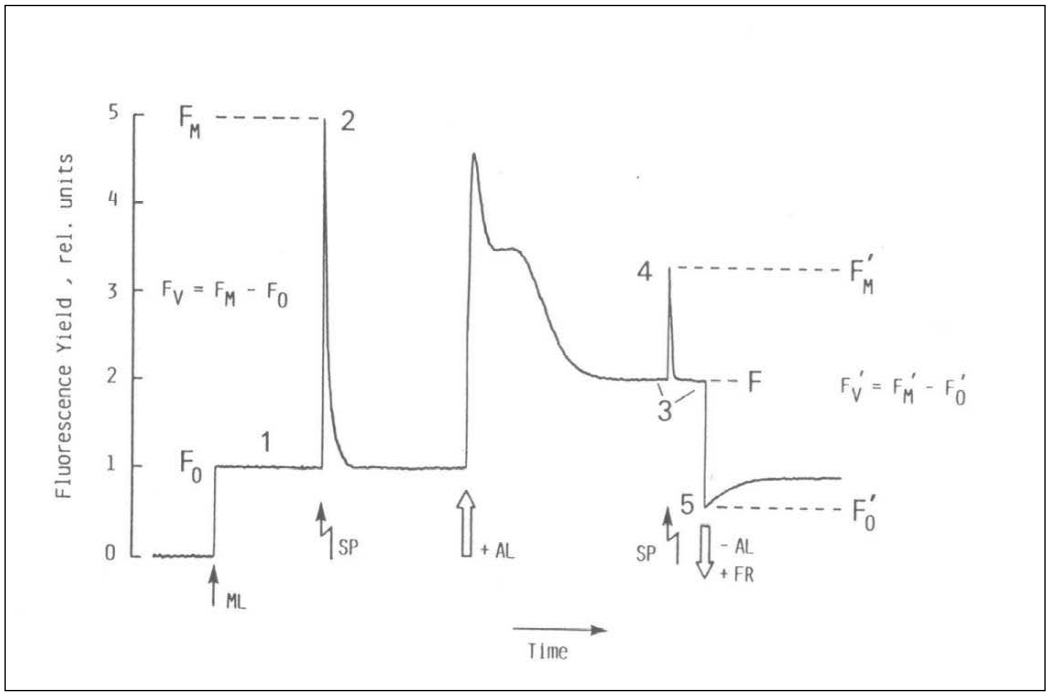

Figure 2.3: Principles of quenching analysis by the saturation pulse method. Fluorescence yield is measured with a modulation fluorometer. Depending on the light conditions 5 different states are distinguished and the corresponding points in the induction curve characterized by fluorescence yield notations (e.g. F_O, F_M) and quenching coefficients (q_P and q_N). Fluorescence quenching at a given time following the onset of actinic illumination (at point 3) is evaluated by comparison with a dark-adapted reference state (1), which is characterized by q_P=1 and q_N=0. In both cases a pulse of saturating light is applied to close all PSII reaction centers, thus eliminating photochemical quenching (q_P=0) (points 2 and 4). It is assumed that non-photochemical quenching is not affected during a saturation pulse. q_P and q_N are quenching *coefficients*, designated to *relative* decrease in *variable* fluorescence yield. The fluorescence yield F_O', i.e., in the energized state with all centers open, is determined briefly after switching-off actinic light in the presence of weak far-red illumination (point 5). ML, weak modulated measuring light (approx. 6 nmol m^{-2} s^{-1} at 660 nm); SP, saturating light pulse (approx. 10 000 μmol m^{-2} s^{-1}, 400 nm < λ < 700 nm, applied for 0.5-2 s); AL, continuous actinic light; FR, far-red light (approx. 6 μmol m^{-2} s^{-1}, λ > 700 nm) (van Kooten & Snel, 1990).

Change in chlorophyll fluorescence, was observed as early as 1931 by Kautsky (Kautsky & Hirsch, 1931). They found that upon transferring photosynthetic material, from the dark into the light, an increase in the yield of chlorophyll fluorescence occurred over a period of about 1 second.

The light energy absorbed, by the chloroplast, first excites pigment molecules of the light harvesting chlorophyll (LHC) proteins. These LHC proteins then transfer their

energy to either PS I or PS II. Fluorescence changes occurring in green leaves correlate with photosynthetic electron transport through PS II and PS I, leading to oxidation of water, oxygen production, the reduction of $NADP^+$ to NADPH, membrane protein transport and eventually ATP synthesis (Castanga *et al.,* 2001).

The flow of electrons from the electron donor site of PS II to the electron acceptor site of PS I are evident of the highly organised interaction between the photosynthetic components. The two photosystems are linked, in series, by a transport chain of electron and hydrogen carriers. This creates a pathway for the flow of electrons and this flow of electrons from water to $NADP^+$ is termed 'non-cyclic' electron transport.

Light energy initially absorbed by the LHC and transferred to the reaction centres is lost *via* a number of different mechanisms. The loss of light energy from the reaction centres (RC), as fluorescence (Farage *et al*., 1990). Approximately 1-2% of the light energy absorbed by the chlorophyll pigment is re-emitted from the excited state as fluorescence. Fluorescence yield can therefore, be quantified by exposing a leaf to light at a defined wavelength and then measuring the quantity of light re-emitted, at longer wavelengths (Darrall, 1989), because the emission peak is of a longer wavelength than the excitation energy. The absorption of quanta and subsequent transduction of excitons to PS I or PS II must be completed within a nanosecond if photochemistry is to take place. One modification to the basic measuring devices which has been instrumental in revolutionising the application of chlorophyll fluorescence has been the use of a modulated measuring system (Quick & Horton, 1984).

In such systems, the light source used to measure fluorescence is modulated (switched on and off at high frequency) and the detector is tuned to detect only fluorescence excited by the measuring light. The fate of light energy absorbed by chlorophyll molecules in a leaf can be one of three:

(a) it can be used to drive photosynthesis (photochemistry),
(b) excess energy can be dissipated as heat or,
(c) it can be re-emitted as light chlorophyll fluorescence.

These three processes occur in competition, in such a manner that any increase in the efficiency of one will result in a decrease in the yield of the other two. Thus by measuring the yield of chlorophyll fluorescence, information about changes in the photochemistry and heat dissipation can be gained (Maxwell & Johnson, 2000). These measurements can only be relative, as light is inevitably lost. Although fluorescence measurement may sometimes provide a useful measure of the photosynthetic performance of plants, its real strength lies in its ability to supply information which is not readily available by the use of other techniques and methods. Fluorescence, in particular can give insight into the ability of a plant to tolerate environmental stresses and into the extent to which those stresses have damaged the photosynthetic apparatus (Maxwell & Johnson, 2000).

With the PAM-fluorometer (Pulse Amplified Modulation-fluorometer) the efficiency (yield) of photosynthesis is measured. The PAM measures by means of difference in fluorescence, the efficiency in electron transport of the photosystems. Under dark adaptive conditions, three fluorescence analysis parameters can be measured using the PAM; namely, F_0 (minimal fluorescence), measured after dark adaptation, F_M (maximal fluorescence), measured in dark after giving a strong saturated light pulse and Fs. The strong advantage of the pulse-modulated techniques is that it enable fluorescence under ambient light to be measured.

Using the F_0 and F_M parameters, one can therefore calculate the yield obtained in the dark-adaptive conditions. Once the F_O' and F_M' values are obtained, the yield produced in light-adaptive conditions can therefore be calculated (www.walz.com/mini.htm).

Another tool that can be used to calculate several structural and functional parameters of the intact plant is the JIP-test (Strasser *et al*., 1995). The polyphasic chlorophyll *a* fluorescence rise gives a fair indication of photosynthetic rates. Several parameters of PS II can be examined simultaneously. The measurements are rapid and inexpensive. The JIP-test is being used extensively in stress physiology in a range of plant species. This data, in conjunction with the available data banks of

physiological traits and crops can then be used to interpret the effect of stress on crops.

One major advantage of the JIP test is that repeated measurements, even on a single leaf of the test plants at defined times points, can be made during prolonged stress periods, followed by recovery. Due to these advantages, the JIP test can be used for the stress mapping of many cultivars, which can reveal their behaviour with respect to stress factors (Strasser *et al*., 1995). Here only PAM measurements were made

2.5 BIOCHEMICAL EFFECTS OF OZONE

2.5.1 Defence strategies

The stress caused by air pollution is largely chemical and is the result of either direct poisoning by toxic materials, or the effect of secondary substances created in the air or in the plants. Several of these reactions form part of the plant defence systems toward oxidative stress e.g. ascorbic acid, peroxidases, phenolic compounds and polyamines (Langebartels *et al*., 1990).

Resistance, in plant-pathogen interactions, is accompanied by the rapid employment of a multi-component defence responses. The individual components of this defence response include the HR, chemical weapons and structural defensive barriers (Dixon *et al*., 1994). Signals for the activation of these various defences are initiated in response to recognition of elicitors by plant receptors. The sequence of events in a defence response can be thought to include host cell death and necrosis, accumulation of toxic phenols, modification of cell walls by phenolic substitutes of physical barriers such as appositions or papillae, and finally the synthesis of specific antibiotics (Nicholson & Hammerschmidt, 1992). The defence response may be induced specifically or non-specifically by a range of biotic and abiotic elicitors (Dixon *et al*., 1994).

In the case of pathogenesis the defence mechanism has two parts. Firstly it is assumed to involve the rapid accumulation of phenols at the infection site, which slows down or even halts the growth of the pathogen and secondly allows for the activation of 'secondary' strategies that would inhibit the pathogen. Secondary responses would involve the activation of specific defences such as the *de novo* synthesis of phytoalexins, phenols or other stress related substances (Matern & Grimmig, 1994). Ozone has been found to resemble fungal elicitors, and it can induce signal molecules such as ethylene and salicylic acid, as well as certain genes and biosynthetic pathways associated with pathogen and oxidative defence. The action of ambient ozone on the plant defence system may predispose the plant to enhance and induce resistance. These results mean that ozone is also an elicitor of stress responses (Sandermann *et al.,* 1998).

At low levels O_3 is also known to affect growth and development of plants when the period of exposure lasts for weeks or months. Exposure of plants to sub-acute levels of O_3 is known to induce many biochemical and physiological changes (Pleijel *et al*., 1999). Ozone exposure often causes a surge in the production of the plant hormone ethylene, as well as changes in polyamine metabolism, and increases in the activities of several phenylpropanoid and flavonoid pathway enzymes. Pathogenesis-related (PR) proteins, β-1,3-glucanses, chitinase and protein 1b are induced by ozone (Ernst, 1996).

The increases in expression, of the genes for β-1,3-glucanase and chitinase in response to O_3, is supported by an increase in activities of these enzymes. The role of these proteins in O_3 induced cells is not clear, but they have been associated with loosening of the cell wall during development, that may allow for the escape of degradation products, which otherwise may be trapped within the dead cell (Pell *et al.,* 1997). Tingey *et al*., (1973) noted that the level of soluble protein only rose 24 hours following exposure (at high ozone concentration), but there were no changes associated with lower O_3 concentrations. Craker & Starbuck (1972) claimed that the protein content declined in beans following exposure to ozone. It does appear that the change in total protein, if changes occur at all, is small and occurs only after

many hours. Larger changes might be observed for specific classes of proteins, especially several hours after ozone fumigation.

The ability of ozone to mimic other stresses has previously been observed and has been termed ‘cross-induction’ (Eckey-Kaltenbach *et al*., 1994). Molecular studies have revealed that there is an overlap in the signalling pathways as well as in the defence-related genes that are induced by ozone, and other stresses such as; pathogen infection (Sharma *et al*., 1996), UV (Rao *et al.,* 1996), cold, drought and heavy metal toxicity (Sharma & Davis, 1997). If the concentration of O_3 is very high and unregulated, cell death will occur. So a central question is whether O_3 induced necrotic lesions are a result of ramped oxidation and subsequent unregulated cell death? This question was answered by Pell *et al*., (1997), when they found that the cell wall and membrane become oxidised during the initial O_3 exposure. When the doses are high and the stomata are open, loss of semi-permeability can rapidly occur followed by plasmolysis, which ultimately leads to cell death. Smaller levels of ROS may provide the signals to the nucleus leading to induction of a suite of responses, which will lead to an increase in the oxidising stress in the chloroplast. As a leaf ages this stress increases, due to an inherent decline in antioxidants. Rubisco normally degrades after oxidative modifications and in the O_3 treated foliage the processes will occur more rapidly.

Since Rubisco is central to leaf longevity, O_3 induced acceleration in the loss of this protein, may contribute significantly to the increased role of ageing and leaf loss observed in plants subjected to chronic exposure to the pollutant (Pell *et al.,* 1997).

2.5.2 Pathogenesis related (PR) proteins

Among the most frequently observed biochemical events, which follow plant infections by pathogens, are the production and accumulation of a family of proteins known as PR- proteins. PR-proteins display very characteristic physiochemical properties, which aid in their detection and isolation (Stintzi *et al.,* 1993):

- they are very stable at low pH and remain soluble (for instance in an extraction buffer of pH 2.8), whereas most other proteins are denatured,
- they are relatively resistant to the action of proleolytic enzymes and are endogenous, but may also be exogenous in origin,
- they are monomers,
- they are localised in compartments such as the vacuole, the cell wall and/or apoplast.

Higher plants accumulate several types of PR proteins in response to pathogenic infections, from viruses or fungi. The hypersensitive reaction (HR) in response to pathogen attack is one of the most efficient defence mechanisms in nature and leads to the induction of numerous plant genes coding these proteins. PR proteins were first described in tobacco plants. The involvement of these PR proteins, in plant defence against pathogens, has been extensively demonstrated (Van der Westhuizen *et al.,* 1994). Plants develop a complex variety of events that involve synthesis and accumulation of new proteins that can have a direct, or an indirect action during pathogenesis. The co-ordinated induction of several PR proteins which may act synergistically, are part of the defence strategy that plants activate against the invading host and may limit the colonisation of the plant inhibiting fungal growth (Caruso *et al*., 1999).

Several members of the five classes of PR proteins have been shown to mediate host plant pathogen resistance, by over expression of their genes in transgenic plants, but these hydrolysing proteins have received less attention, in cereals, over the years. PR proteins are expressed constitutively at low levels and their regulated expression in healthy plants suggests that PR proteins also play a role in plant development (Caruso *et al*., 1999; Kitajima & Sato, 1999).

Following the probable initial effects of ozone on membranes and photosynthesis, a number of secondary responses might be expected. Both increases and decreases in e.g. sugars have been reported, and it was largely dependent on the ozone concentrations the plant received. These variable ozone concentrations may be responsible for changes related to the activity of enzymes in the glycolytic pathway as well as for the stimulation of the pentose phosphate pathway (Tingey *et al*., 1976).

Ozone also affects polyunsaturated fatty acids by oxidative mechanisms. These oxidation's, in turn, can change the properties of membranes (Heath, 1975).

2.6 ELICITING EVENTS DURING DEFENCE REACTIONS

The term 'elicitor' refers to compounds causing phytoalexin production in plants, and known elicitors also stimulate plants to activate other defence reactions. This includes synthesis of cell wall-association phenylpropanoid compounds, the deposition of callose (1,3-β–glucan), the accumulation of hydroxyproline-rich glycoproteins, and the synthesis of certain hydrolytic enzymes (i.e. β–glucanases and chitinases) (Ebel, 1986).

Pathogen recognition takes place through elicitors. These elicitors can be released from invading fungal, or bacterial pathogens prior to, or during, ingression (Dixon *et al*., 1994). It is unclear whether the wounding or the surface-contaminant micro-organisms carried into the wound, elicit the low levels of phytoalexin accumulation during wounding (Scheel, 1998). Plant defence mechanisms include processes resulting form transcriptional activation of defence-related genes, such as the production of lytic enzymes, phytoalexin biosynthesis and systematic acquired resistance (Hammond-Kosack & Jones, 1996). Other plant responses, associated with pathogen defence, result from allosteric enzyme activation initiating cell wall lignins and the production of ROS (Lamb & Dixon, 1997).

The activation of plant defences in incompatible plant-microbe interaction results from recognition by the plant, of either cell surface constituents of the pathogens or factors that are produced and secreted by the pathogen, upon contact with the host plant. Plant-derived elicitors released from the plant by fungal hydrolytic enzymes are thought to act in a way similar to pathogen-derived elicitors. Receptors, for pathogen-derived signals function either on the plant cell surface or intracellular, mediating the conversion of an extra cellular signal (Nürnberger, 1999).

2.7 PHENOLIC ACIDS

All phenolic compounds have an aromatic ring containing various attached groups, such as hydroxyl, carboxyl and methoxy (–O–CH_3) groups, and often non-aromatic ring structures. Phenols play a variety of roles in the plant. Many of them have some role in defence against herbivores, pathogens, biotic and abiotic stresses. Others function in mechanical support, in attracting pollinators to fruit and flowers by releasing distinctive fragrances, or in reducing the growth of nearby competing plants (Taiz & Zeiger, 1991). The shikimic acid- and malonic acid pathways are the two basic pathways in which plant phenolics are biosynthesised.

2.7.1 Physical and chemical properties

Phenols are colourless in the pure form and they tend to be sensitive to oxidation and may turn brownish or dark when exposed to air. These phenols, unless completely esterified or glycosylated, are normally soluble in polar organic solvents.

Water solubility increases with the number of hydroxyl groups present. Phenolic substances are aromatic and therefore have intense absorption in the UV region of the spectrum (Van Sumere, 1989). Phenolics make up a vast class of compounds comprising of anthocyanins, leucoanthocyanins, anthoxanthins, hydroxybenzoic acids, glycosides, sugar esters of quinic and shikimic acids, esters of hydroxycinnamic acids and coumarin derivatives (Goodman *et al*., 1967). Figure 2.4 (a-e) show the ring structures of the phenolic acids examined during this study.

Phenolic substances are known to participate in a number of physiological processes, which are essential for growth and development, such as oxidation-reduction reactions, lignification and stimulation of various biochemical reactions, as well as inhibition of auxin activity. Phenols and their oxidation products (quinones) are also potent uncouplers of oxidative phosphorylation, inhibitors of enzymes, and chelators of metal co-factors (Misaghi, 1982).

2.7.2 Synthesis and induction of phenols

The response of plants to pathogens, based on host and non-host interactions are characterised by the early accumulation of phenolic compounds at the infection site, which as a result of hypersensitive cell death, limits pathogen development (Nicholson & Hammerschmidt, 1992). Rapid accumulation of phenols may result in effective isolation of the pathogen (or non-pathogen) at the original site of ingress. These responses include the formation of lignin, the accumulation of cell-wall appositions such as papillae and the early accumulation of phenols within the host cell walls (Sherwood & Vance, 1976). Low molecular weight phenols, such as the benzoic acids and the phenylpropanoids, are formed during the initial response to infection.

Evidence strongly suggests that the esterification of phenols to cell wall materials is a common theme in the expression of resistance. The accumulation of polymerized phenols occurs as a rapid response to attack. A common host response is the esterification of ferulic acid to the host cell wall and it has been suggested that cross linking of such phenylpropanoid esters leads to the formation of lignin like polymers (Nicholson & Hammerschmidt, 1992). The shikimic pathway (Figure 2.5) participates in the biosynthesis of most plant phenolics (Taiz & Zeiger, 1991).

Unlike animals plants cannot defend themselves against a stress condition (e.g. ozone) or microbial attack by producing circulating antibody proteins or specialised cells. Instead they offer resistance through physical and chemical defence. That may either be performed (cuticle and cell wall) or induced after they have been subjected to a relevant stress. As we have seen through out this chapter, induced defences may include production of ROS, cell wall strengthening phytoalexin biosynthesis, the induction of various phenols and the accumulation of defence related proteins such as PR-proteins (Rivera *et al.,* 2002).

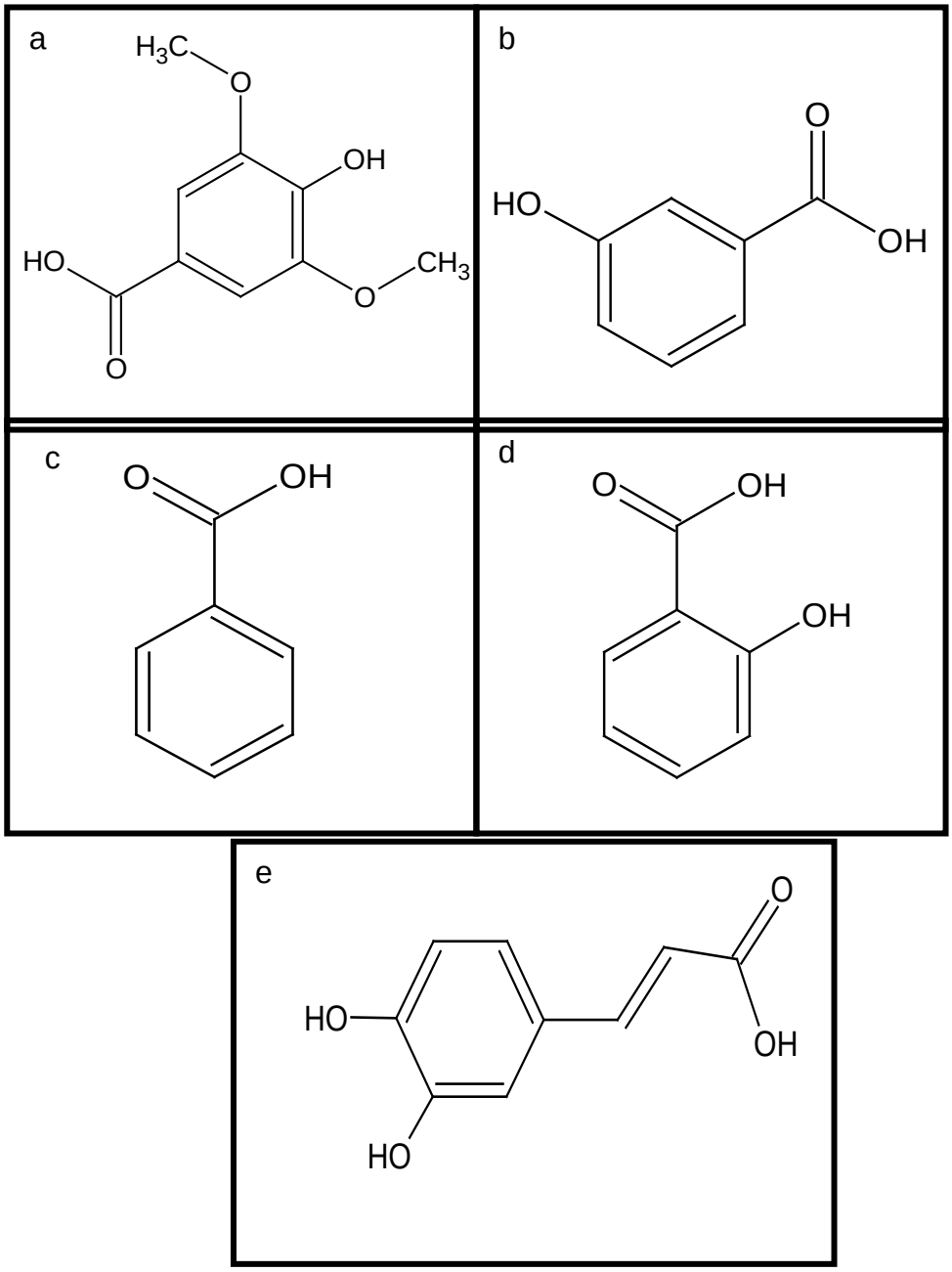

Figure 2.4: The ring structures of the phenolic acids examined during this study, (a) 4-hydroxy-3,5-dimethoxybenzoic acid (syringuc acid), (b) 3-hydrobenzoic acid, (c) Benzoic acid, (d) Salicylic acid, (e) 3,4-dihydroxycinnamic acid (caffeic acid).

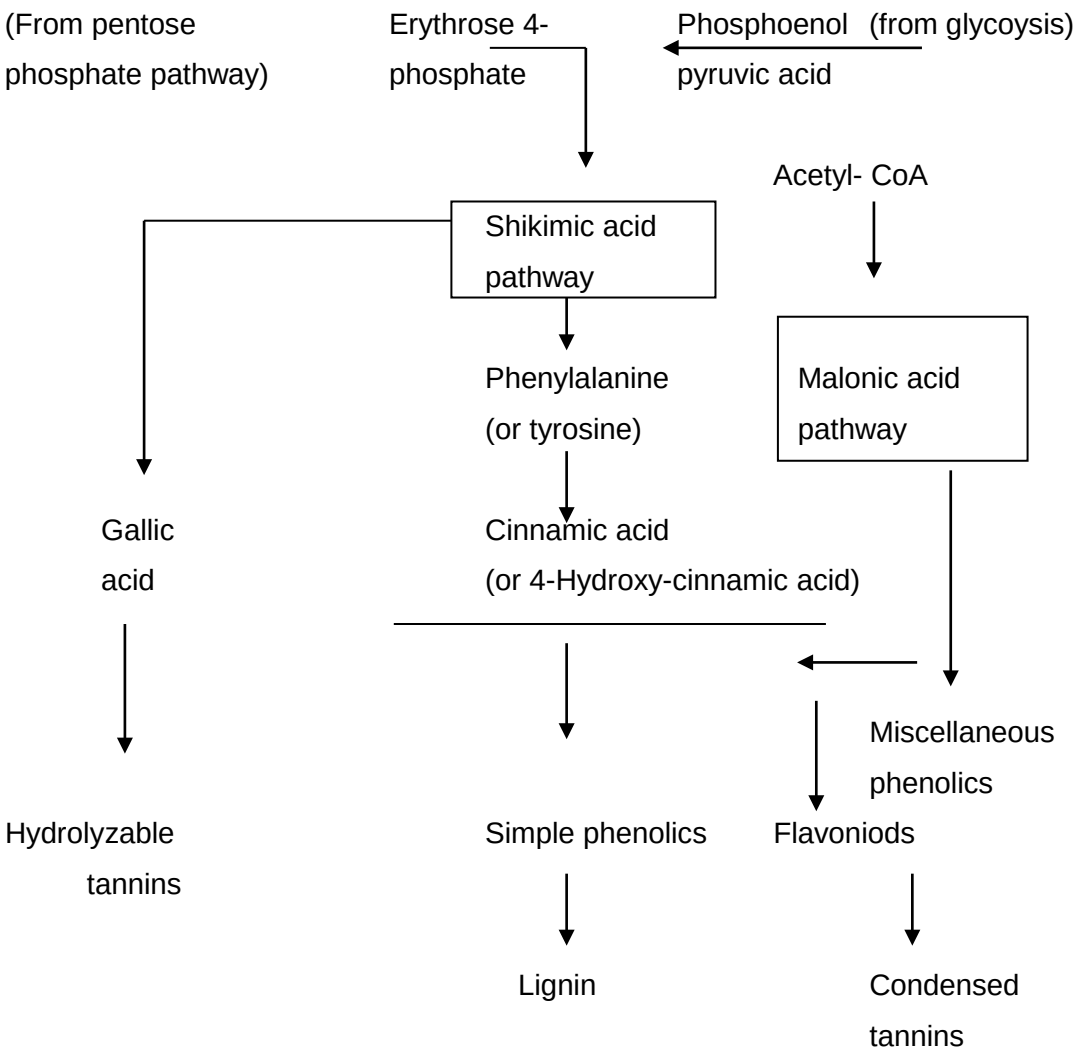

Figure. 2.5: Plant phenolics biosynthesis pathway. In higher plants, most secondary phenolics are derived at least in part form phenylalanine, a product of the shikimic acid pathway (Taiz & Zeiger, 1991).

CHAPTER 3

MATERIAL AND METHODS

3.1 Chemicals

All chemicals used were of analytical grade.

3.2 Plant material

The *Zea mays* (*33A13*) seeds used in this study were kindly supplied by Pioneer Ltd. (Pioneer Hi-bred R.S.A., Jan Kempdorp). This cultivar (*33A13*) was used in all the experiments. Choice of this cultivar was based on the fact that commercial farmers regularly plant it in South Africa.

3.2.1 Growth conditions

The plants were grown, under controlled conditions, in a glasshouse with a day/night temperature of ±28 °C/±22 °C. The plants were grown in pots (13 cm) containing 50 % potting soil and 50 % good soil mixture, watered three times a week with tap water, without any chemical or nutrient additions. The plants were closely monitored and were kept in the glasshouse up to the age of 4 weeks when they reached the two leaf stage. At 4 weeks the best plants were selected and transferred to a Controlled Environment Conviron, Model CC24 growth camber, where they were allowed to acclimatise to the conditions before ozone fumigation.

3.2.2 Ozone treatment

A Controlled Environment Conviron Model CC24 growth chamber fitted with an O_3 generator was used during this study for fumigating the plants. The light intensity inside the chamber was 360 µmol photons $m^{-2}s^{-1}$ with a day/night cycle of 16/8 h.

Prior to fumigation, the surface of each pot was individually covered with aluminium foil, to limit the reaction of O_3 with the organic compounds in the soil. No more that six pots were placed, at any given time, in the chamber. The plants were exposed to 150 ppb O_3 for 6 hours. Directly after O_3 fumigation, the selected leaves were dark-adapted (using special clips), for 15 minutes. Control plants were exposed to the same growth conditions in a second similar growth chamber, with the exception that no fumigation with O_3 took place.

3.3 Methods

3.3.1 Determination of hydrogen peroxide (H_2O_2) concentration

H_2O_2 was determined using the $TiCl_4$ method. (Ferguson *et al.*, 1983). One gram of fresh leaf material was homogenised in 5ml cold acetone, where after the extract was centrifuged (1 250 g) at –4 °C for 20 min. The supernatant was collected and 0.5 ml titanium chloride ($TiCl_4$) reagent (diluted in HCL to a 20 % concentrated) was added to it. After drop-wise addition of 3.5 ml NH_4OH (25 %), the solution was mixed. The sample was centrifuged at 1250 g and –4 °C for 15 min. The supernatant was discarded and the precipitate was washed repeatedly with 5 ml volumes of acetone until the supernatant was colourless. The washed precipitant was dissolved in 20 ml 2N H_2SO_4, and filtered prior to measurement of absorbency at 415 nm against a blank sample, using a Hitachi, U-2000 spectrophotometer. The H_2O_2 concentration was calculated as (mmol.g^{-1} fresh mass).

3.3.2 Determination of protein concentration

Protein extractions where made from 1 g pieces of the ozone fumigated maize leaves. The maize leaves where grinned to a fine powder in liquid nitrogen. This powder was then transferred to a cold centrifuge tube. 1.5 ml of extraction buffer [Tris-HCl (50 mM) pH 7.5, EDTA (2 mM), Mercapto-ethanol (10 mM), Polyvinylpyrrolidone (2 mM) (in EtOH)] were then added to the powder.

This mixture was then centrifuged for 15 min at 10 000g. The supernatant was then removed and used for further analysis.

Protein content of the enzyme extract was determined according to the method of Bradford (1976) as modified by Rybutt and Parish (1982). The assay mixture consisted of 160 μl distilled water, 40 μl Bio-Rad (Bio-Rad laboratories GmbH), and 10 μl enzyme extract or standard. The absorbency was measured at 595 nm using the Bio-Rad microplate reader model 3550. Bovine γ-globulin (0.5 $\mu g.\mu L^{-1}$) was used as a standard.

This enzyme extract was also used for the determination of β-1,3-glucanse and chitinase activities.

3.3.2.1 Determination of β-1,3-glucanase activities

The colorimetric assay of β-1, 3-glucanase was done according to Fink *et al*., (1988). The reaction mixture consists of 240 µl Na-acetate buffer, 250 µl laminarin ($2\ mg.ml^{-1}$) and 10 µl enzyme extract. It was then incubated, in a test tube, at 37 °C for 10 minutes. Then 500 µl Somogyi reagent (Somogyi, 1952) was added and heated at 100 °C for 10 minutes. Somogyi reagent comprised of $CuSO_4$ (0.4 g), $NaSO_4$ (18 g), Na_2CO_3 (2.4 g), $NaHCO_3$ (1.6 g), Na-tartrate (1.2 g) and dH_2O (100 ml). The mixture was then cooled, under tap water, and 500 µl Nelson's reagent (Nelson, 1944) added. It was then mixed until a dark blue colour appeared. The blank and the glucose standards were subjected to the same procedure after which the absorbency was read at 50 nm.

The β-1, 3-glucanase activity was expresses as mg glc/mg protein.

3.3.2.2 Determination of chitinase activity

Chitinase activity was measured according to the method of Wirth & Wolf (1990). The method is based in the perceptibility of the indigested substrate CM-Chitin-RBV (Carboxymethyl-chitin-remazol brilliant violet 5R: Loewe Biochemica GmbH) in buffered solutions with HCl.

The reaction mixture consisted of 490 μl 50 mM Na-acetate buffers (pH 6.5), 1.5 mg chitin and 10 μl enzyme extract. The reaction mixture was incubated at 37 °C for 30 min. The mixture was then centrifuged at 1 000 g at 5 °C for 1 min. 300 μl of the supernatant was withdrawn and transferred to a clean eppendorf. 20 μl of 1.5 % (w/v) cytohelicase and 30 μl of 1M K-phospate buffer (pH 7.1) were added to it. This mixture was again incubated at 37 °C for 30 minutes. After 30 min 250 μl was once again withdrawn and 50 μl 0.8 M K-tetraborate buffer (pH 9.1) was added before heating in a vigorously boiling water bath for exactly 3 min. It was cooled under running tap water before adding 150 μl diluted 4- dimethylaminobenzaldehyde (1 DMBA: 9 HCl), and incubation at 37 °C for 20 min followed. The sample was cooled under running tap water. Before reading the absorbency at 585 nm, the sample was diluted 3x with distilled water (Wirth & Wolf, 1990).

3.3.3 Separation and quantification of phenolic compounds

Leaf material was frozen in liquid N_2 and grounded to a fine powder. This material was hydrolysed in 2 N HCl for 30 minutes at 85 ˚C after which the solution was cooled and centrifuged for 20 minutes at 12 000 g. The pellet was extracted with diethylether (3x), whereafter the extract was dried in a Bucchi rotavapor under reduced pressure. The residue was dissolved in 95 % (v/v) ethanol.

The phenolic compounds were separated by means of HPLC using a 5 μ C-18 reverse phase column (250 x 4.60 mm), and detected with a UV detector at 270 nm.

The column was developed at 1 ml/min^{-1} with a mixture of acetonitrile (A): methanol (B): 0.57 % acetic acid(C). The following gradient was applied: starting conditions; 6 % A, 88 % B, 6 % C for 60 minutes after which it was changed to 6 % A: 48 % B and 46 % C (Baiocchi *et al*., 1993).

The following phenolic standards were used: 3,4-dihydroxycinnamic acid (caffeic acid), salicylic acid, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), and *p*-hydroxybenzoic acid.

3.3.4 Chlorophyll fluorescence

Chlorophyll fluorescence was measured using an OS-500 Modulated fluorometer from Opti-Sciences, USA and an FMS-2 fluorometer from Hansatech Instruments, UK. Both these are versatile pulse-modulated fluorescent instruments, designed to measure chlorophyll fluorescence emission from dark-adapted samples or under ambient light conditions in the field or laboratory. Chlhrophyll fluorescence has widely been used as a fast measuring method to assess stress and the photosynthetic properties of green plants.

Research is often done with chlorophyll flourometers, measuring leaf areas of approximately 1 cm^2 (Lootens & Van der Casteele, 2000). Chlorophyll fluorescence transients were measured on dark-adapted samples (Schreiber & Bilger, 1987), where dark adaptation meant using the specially designed clips provided and always allowing 15 minutes for dark adaptation after ozone fumigation. As described by Grobbelaar & Mohn (2002), we were aware that it would take much longer for all the RC's to be fully re-oxidised, but it was assumed that most RC's would be 'open' and care was taken to keep the time constant for all measurement. Measurements were made on intact leaves and the results were from about 1 cm^2 surface areas of the topside of the leaves. Five to seven plants were used each time. Each measurement lasted about 2 minutes.

Fluorescence measurements were performed on intact maize leaves in the growth chamber, field or the green house, depending on the particular experiment. Following dark adaptation the leaves were excited, with a weak-modulated beam, to obtain the minimum dark chlorophyll fluorescence yield (F_0). A saturating white light pulse (0.7s) was then applied and the maximum fluorescence (F_M) when all PS II reaction centres were closed was measured. An actinic light source was then switched on for measurements under steady state. While the actinic light was on a series of saturating light pulses was applied and when the fluorescent signal reached steady state. F_M' (maximum light adapted fluorescence) was measured. Values of minimum light adapted fluorescence yield (F_0') were determined after the actinic illumination

was switched of and far-red illumination applied, which preferentially excites PS I and re-oxidise PSII (Carrasco-Rodriguez & del Valle-Tascon, 2001).

Accurate determination of fluorescence requires a detection system sensitive to fluorescence wavelengths, yet blind to the actinic (or excitation) light that drives photochemistry. In continuous excitation with instruments such as the *Plant Efficiency Analyser* (Hansatech), the signal discrimination is achieved by using a custom designed 650nm actinic light source, to drive photochemistry. Optical filtering of the detector prevents detection of non-fluorescence wavelengths of light. The FMS 2 uses an electronic method, commonly referred to as modulated fluorometry, to separate actinic light from the fluorescence signal (Ögren & Baker, 1985). During measurement, the tissue sample is exposed to a pulsed amber (or optimally blue) LED source (the modulating beam), which induces a pulsed fluorescence signal from the sample, under conditions where ambient light is excluded. When ambient light is applied, the optical filtering of the FMS 2 allows for three types of light signals to reach the detector:

- ambient light of fluorescence wavelengths,
- non-pulsed fluorescence signal induced by the ambient light,
- and pulsed fluorescence signal induced by the modulating beam.

3.3.5 Determination of chlorophyll and Carotein concentration

The chlorophyll concentrations chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and total carotenoids (Tot_{car}) of the tested plants were measured using the spectrophotometric method as described by Lichtenthaler (1984).

Pieces of the top leaves, about 10 cm^2 pieces, of the plants were cut off, weighed and used for the analyses. 1 g of the fresh leaf material was ground to a fine powder using acid washed sea sand in 5 ml of 80 % acetone. Grounding continued, with further additions of 5 ml of 80 % acetone at regular intervals until 20 ml of 80 % acetone was added. Temprature was kept at 5 °C.

The extract was removed using a Pasteur pipette and was transferred to centrifuged tubes. Prior to centrifugation care was taken to keep the extracts in the dark. The combined extract was then centrifuged at 12 000 g for 10 min at 5 °C. The absorbency of the supernatant was measured at 663 nm, 646 nm and 470 nm using a Hitachi U-2000 spectrophotometer. The absorbancies were then used to calculate the pigment concentrations using the formulas as given below.

Equations used in pigment concentration determination according to Lichtenthaler (1984).

$$\text{chl } a \text{ (mg/g)} = \frac{[12.12\,(A663) - 2.18\,(A646).\,V]}{1000 \times W}$$

$$\text{chl } b \text{ (mg/g)} = \frac{[20.13\,(A646) - 5.03\,(A663).V]}{1000 \times W}$$

$$\text{Tot}_{car} \text{ (mg/g)} = \frac{[20.13\,(A470) - 2.27\,C_a - 104\,C_b]}{229}$$

A = absorbency of the chlorophyll extract at the indicated wavelength.
V = final volume of the extract in ml.
W = mass of the fresh leaf material used, in grams.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chlorophyll Fluorescence analyses

4.1.1 Multiple light pulse analyses

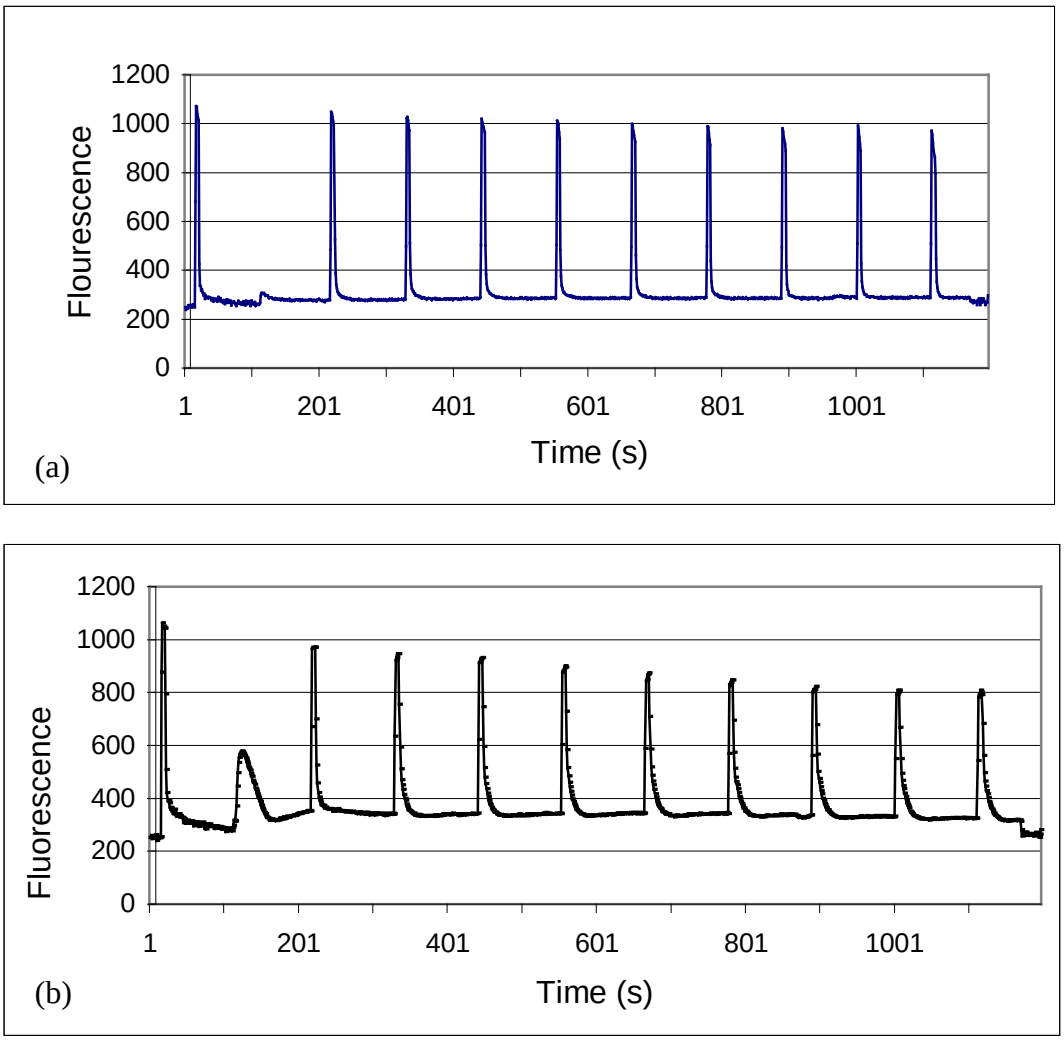

Figure 4.1a & b: Two typical fluorescence traces. Figure 4.1a represent a healthy maize (*33A13*) plant grown in the laboratory. Figure 4.1b show the same plant directly, after ozone exposure. In both graphs the average of 5 to 7 repetitions were used. Every peak is the result of a saturating light flash. The lower the fluorescent peaks, the more stressed the plants are (lower photosynthetic potential) and this is clearly seen in the bottom graph.

Prior to testing the response of maize (*33A13*) to ozone fumigation, their fluorescence characteristics were determined, against which the treatments were compared. The results are shown in Figure 4.1a & b. The ratio F_V/F_M, or the maximal photochemical quantum efficiency of PS II was significantly reduced by the O_3 treatment (Figure 4.1b and Table 4.1), when compared to the control. When the F_V/F_M of the field plants are compared to those grown in controlled environments, a reduction could be observed, allthough the reduction was not as severe as after O_3 exposure. From the results it is also clear that the outdoor plants were more stressed than the control plants (lower F_V/F_M). O_3 fumigation resulted in an increase in F_O, compared to the control and outdoor plants. Noticeable is also the lower F_M value of the outdoor plants. Mulitpule saturating pulses wre applied during the steady state phase of the measurements, in order to determine whether O_3 also influenced the relaxing kinetics of fluorescence. As can be seen O_3 had an overall affect and because of this the last pulse was used for the quenching analyses. Fluorescence emission was monitored from the upper surface of the leaves. The measuring modulated light intensity was kept between 149 to 159 µmol photons m^{-2} s^{-1}. This helped no to produce any significant variable fluorescence.

The photochemical quenching of the fumigated plants were significantly lower than the control and field plants, where the opposite is seen for non-photochemical quenching (Table 4.1). These results clearly indicate that the re-oxidation of Q_A was impaired by O_3 as indicated be the decrease in q_P, but there is also the possibility of q_P being low due to electorn flow blockage before Q_A.

Table 4.1: Multiple light pulse analysis of Chlorophyll fluorescence parameters as measured on the top leaves of four week old maize plants, during laboratory and field experiments.

	Control	**Variance**	**n**	**O_3 treated**	**Variance**	**n**	**Field**	**Variance**	**n**
Minimal fluorescence (F_O)	280	0.632	5	329	1.002	5	250	0.722	5
Max. fluorescence (F_M)	1010	2.445	7	898	1.630	7	887	1.111	7
Light adapted max fluo (F_M')	962	1.671	7	804	2.770	7	763	1.053	7
Steady state (F_s)	287	1.981	5	324	2.143	5	255	1.201	5
F_o'	262	0.188	5	252	1.051	5	239	1.465	5
Yield (Y)	0.702	0.952	5	0.597	0.763	5	0.666	0.532	5
Quantum efficiency of PSII (ΦPSII)	0.702	0.001	5	0.600	0.035	5	0.692	0.243	5
F_V/F_M	0.722	0.381	5	0.634	0.575	5	0.718	0.223	5
PAR (µmol photons m^{-2} s^{-1})	159	-	5	149	-	5	153	-	5
Photochemical quenching (q_P)	0.964	0.382	5	0.870	0.224	5	0.990	0.570	5
Non-photochemical quenching (q_N)	0.066	0.014	5	0.165	0.231	5	0.194	0.112	5
ETR	111.618	3.613	5	89.4	1.556	5	101.89	3.022	5

4.1.2 Single saturated light pulse analyses

Figure 4.2a & b are results obtain when a single saturated light pulse was applied during exposure to actinic light of dark-adapted leaves of maize. Indicated on the

graphs are the various chl *a* fluorescence parameters measured. F_O F_M and F_M' values measured in the ozone treated plants were lower, in comparison with the non-fumigated control plants.

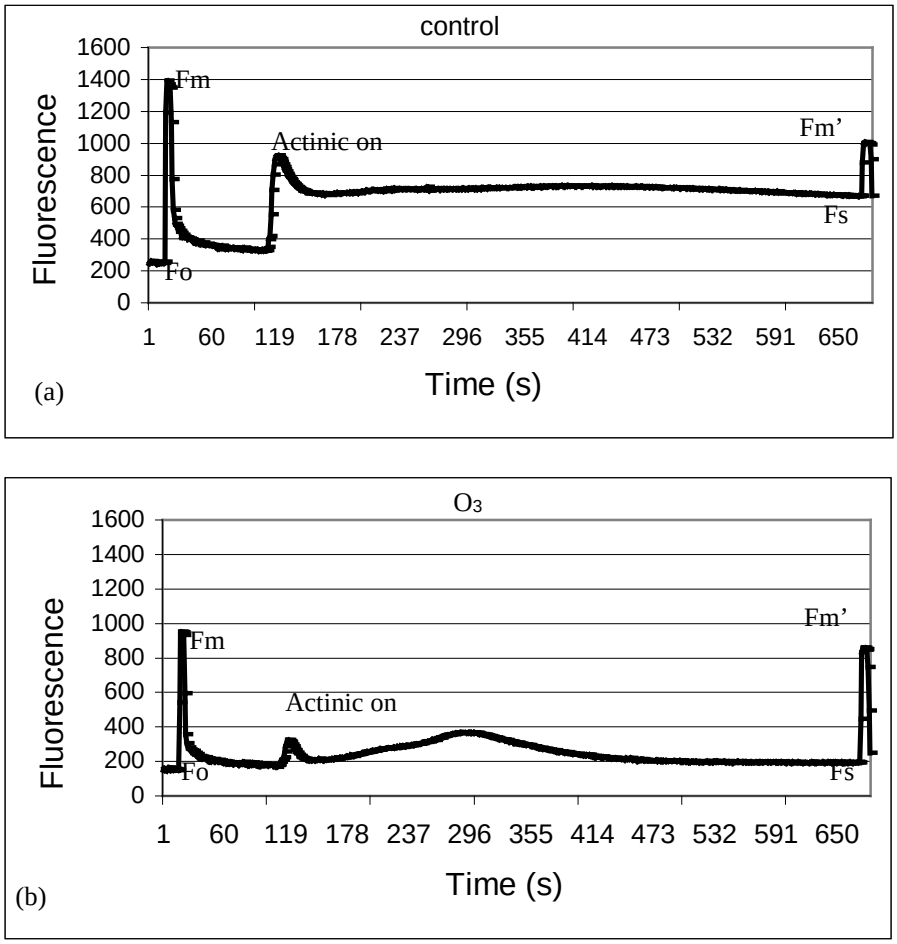

Figure 4.2a & b: Effect of O_3 fumigation on maize leaves, as was measured with single saturated light pulse analyses. Figure 4.2a represent the non-treated control plant, while Figure 4.2b represents a O_3 fumigated plant. [Minimum fluorescence (F_O), maximum fluorescence (F_M), minimum steady state fluorescence (F_S) and maximum steady state fluorescence (F_M')]

The effect of ozone treatment on the various chlorophyll fluorescence parameters measured following exposure is shown in Table 4.2. As with the previous experiments where multiple saturating flashes were given during the actinic light phase, it was seen that the chlorophyll fluorescence parameters were greatly influenced by O_3. Ozone exposure resulted in a decrease in the F_V/F_M ratio, while photochemical quenching (q_P) also underwent significant decreases. However, the non-photochemical quenching (q_N) was higher in the treated plants, compared to the

non-fumigated control. It was noted that F_0 decreased following O_3 exposure, which is contrary to the results presented in Table 4.1. A possible explanation is the overall reduction in all the fluorescence parameters measured following O_3 exposure.

Table 4.2: Representative single light pulse chlorophyll fluorescence values, as was measured on intact four-week-old maize leaves, before and after exposure.

	Control	**Variance**	**n**	**O_3 treatment**	**Variance**	**n**
Minimal fluorescence (F_O)	251	1.155	5	192	1.000	5
Max. fluorescence (F_M)	1381	1.000	5	949	1.527	5
Light adapted max fluo (F_M')	1004	1.000	5	857	1.000	5
Steady state (F_s)	670	2.000	5	152	1.523	
Max. quantum efficiency (F_V/F_M)	0.818	0.010	5	0.798	0.015	5
PAR ($\mu mol\ m^{-2}\ s^{-1}$)	139	-	5	109	-	5
Photochemical quenching (q_P)	0.943	0.025	5	0.444	0.038	5
Non-photochemical quenching (q_N)	0.107	0.0015	5	0.375	0.0015	5
Quantum efficiency of PS II (ΦPS2)	0.776	0.0016	5	0.333	0.0027	5
ETR	107.864	3.567	5	36.297	1.577	5

4.2 Chl *a* content and maximal quantum efficiency

Chlorophyll *a* and chlorophyll *b* were closely monitored following ozone exposure. As indicated in Figure 4.3 Chl *a* underwent a significant reduction, following and during the exposure. The losses of Chl *a* content, relative to the control, ranged from 35 to 50%. Chl *b* was unaffected by the O_3 fumigation (results not shown).

Five replicas were used in this experiment. Following the O_3 exposure the plants were allowed a 2 days recovery time. Twenty-four hours following the initial O_3 exposure, the Chl *a* content showed tendency to return to levels observed prior to fumigation. At 48h after the initial exposure the Chl *a* content in the second leaf of the

treated plants was even higher, and had almost recovered to pre-fumigation concentrations (Figure 4.3). The increase in chlorophyll *a* content, relative to the ozone fumigated plants, was more than 60%.

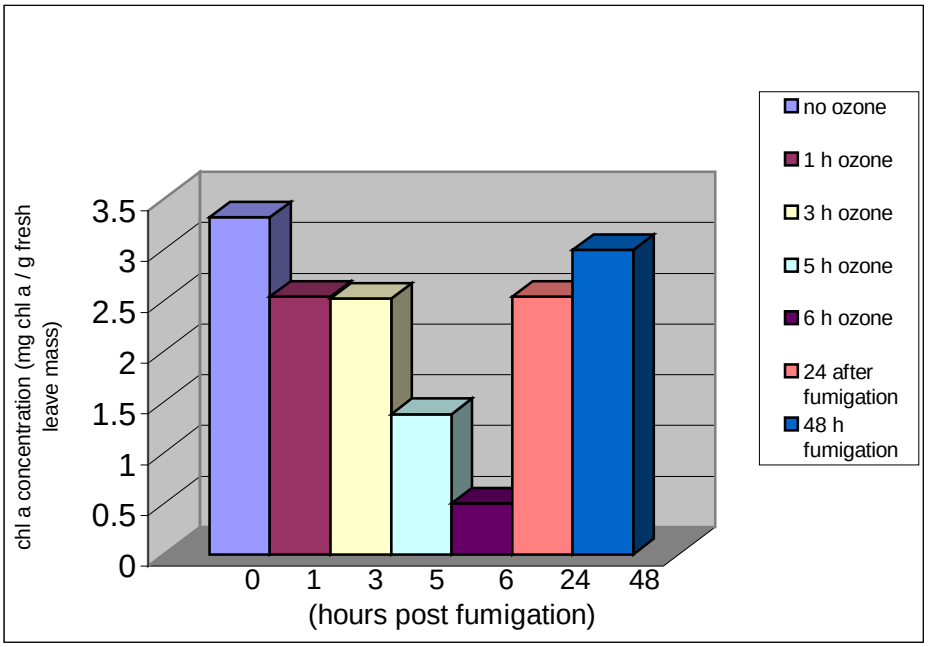

Figure 4.3: Chlorophyll *a* concentration of the biomass following exposure to O_3 for 6 hours, at 150ppb. Following fumigation the plants were allowed to recover and measurements were made after 24 and 48 hours.

4.2.1 Variable maximal fluorescence ratio (F_V/F_M)

F_V/F_M, ratio to or the maximum yield of primary photochemistry, was significantly affected by ozone fumigation. The average F_V/F_M ratio of the control plants was 0.722 and only changed slightly over the experimental period. From time zero up to 8h after exposure a sharp decrease in the F_V/F_M was observed. After 8h of exposure, levels as low as (0.560) were measured, in the ozone treated plants. F_V/F_M levels then remained contant at 0.575. From 10 to 30h after exposure the F_V/F_M remain to increase rapidly and forty-eight hours after the initial exposure the increase that was observed meant that the F_V/F_M levels were almost back to levels seen prior to fumigation, indicating a return in the efficiency the PS II photochemistry.

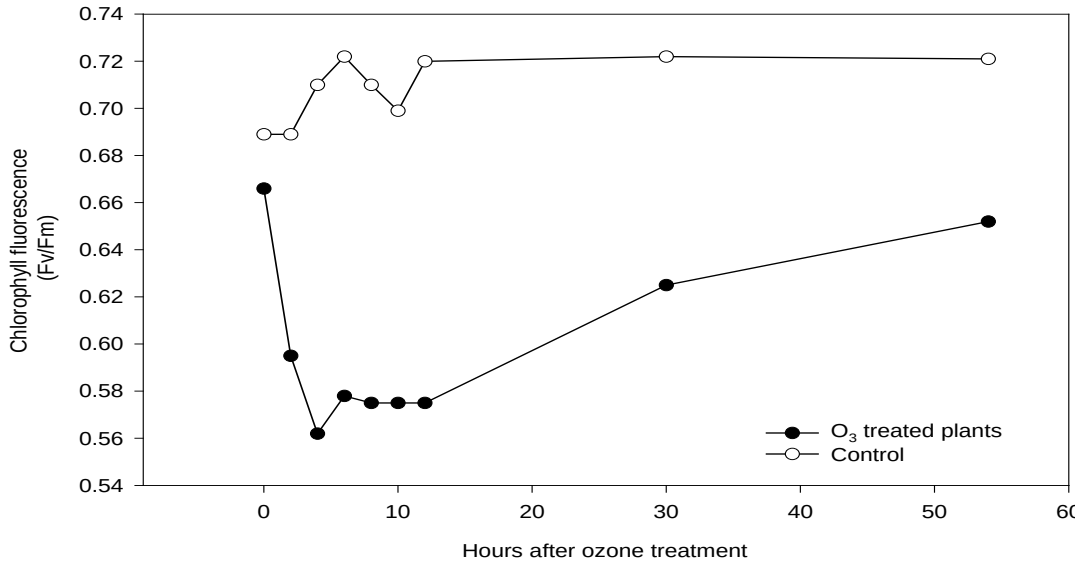

Figure 4.4: Illustration of the maximum yield of primary phototchemistry over a time period of 54 hours. O_3 was applied in the first 6 hours, and then the plants were allowed a recovery of 24h following exposure before measurements were again made (n=5)

4.2.2 Visible injury index

Exposure to O_3 (85 and 150ppb for 6h a day and over 8 consecutive days) resulted in clear visible discoloration of the leaves (Table 4.5). Significant differences were seen depending on the time of exposure to O_3 and the concentration, either 85ppb or 150ppb ozone. Visible injury increased during the exposure period and was greater in the plants exposed to a higher ozone concentration.

A subjective visible injury index was compiled, which depended on the extent of damage of each leaf. The leaves that were exposed to 85ppb were then compared to those that received 150ppb, and the results are illustrated in Figure 4.5. The damage was typical small chlorotic lesions, which were confined to the top part of the leaves. The lesions only started appearing after the third day of treatment.

However, the plants exposed to 150ppb ozone showed signs of injury form day two onwards. These plants showed symptoms as yellow necrotic lesions, and they were

spread over the entire leaf surface. These leaves failed to recover and eventually died. The control plants did not show any visible injury.

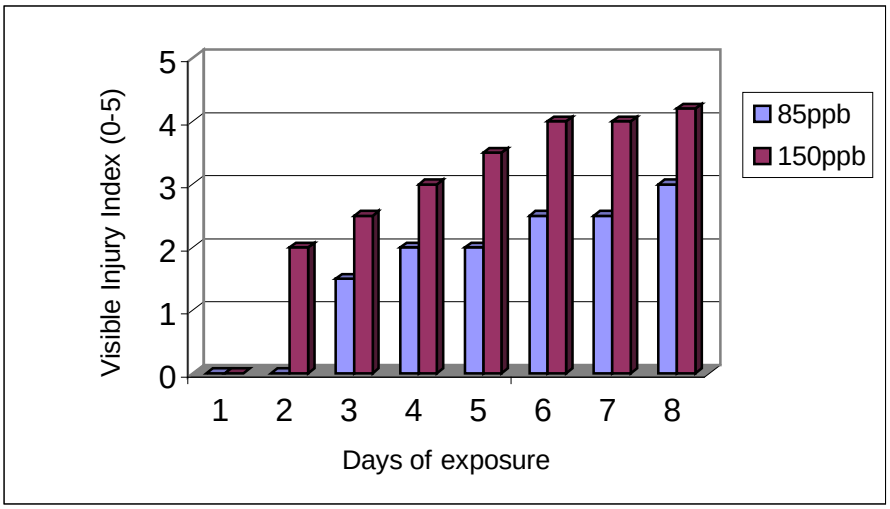

Figure 4.5: Visible injury index of the top leaves of maize (*33A13*) plants after exposed to 85 or 150ppb O_3 respectively. Both sets of plants were exposed for 6h a day for 8 consecutive days.

Significant differences were seen depending on time of exposure to O_3 and the concentration. As necrotic lesions increased with ongoing treatment, considerable decreases in the chl *a* content was also measured.

When comparing leaves of the non-treated control and treated plants with each other, major differences were evident. Results obtained (photos v-vii), show the major damage done to the leaves following exposure to O_3 (150ppb). These leaves eventually turned completely yellow and died.

Early stages of leaf injury following O_3 exposure (85ppb) are shown in (Photos i - iii). Photo (vi), is the leaf of the control plant. Photos (i – iii) were taken after the plant was exposed to 150ppb ozone. The mature leaf (second leaf) of the plant were the most affected. The most affected leaf area appeared to be the leaf tip. The necrotic spots then gradually spread to the base of the leaf. On the leaf injury index scale these leafs were classified as 3 to 3.5, which indicate medium to more severe damage. The leaf in photo (iv) represents a healthy (control) plant, and was not exposed to any ozone.

Photo (i)

Photo (ii)

Photo (iii)

Photo (iv)

Photo (v)

Photo (vi)

Photo (vii)

The leaves on the left in photos (v) and (vi) were not exposed to any ozone, while those on the right were. These leaves were severely damaged by the ozone, and on the leaf injury index they were classified as 5. They did not recover after the plant was removed from the ozone chamber. After a few days they turned yellow and died.

The leaf (photo vii) did not recover, in fact, the whole plant was so badly stressed that it eventually died.

4.3 Effect of ozone treatment on PR-protein enzyme activities

4.3.1 β-1,3-glucanase activity

Ozone induced a 5.2-fold increase in the β-1,3-glucanse activity 5h after exposure to 150ppb ozone. Following this increase, a sharp decrease was seen. Despite this decrease, levels of β-1,3-glucanse activity remained higher in the fumigated plants, compared to the non-fumigated control plants. β-1,3-glucanse activity in the control plants maintain low and relative unchanged for the entire period (Figure 4.6)

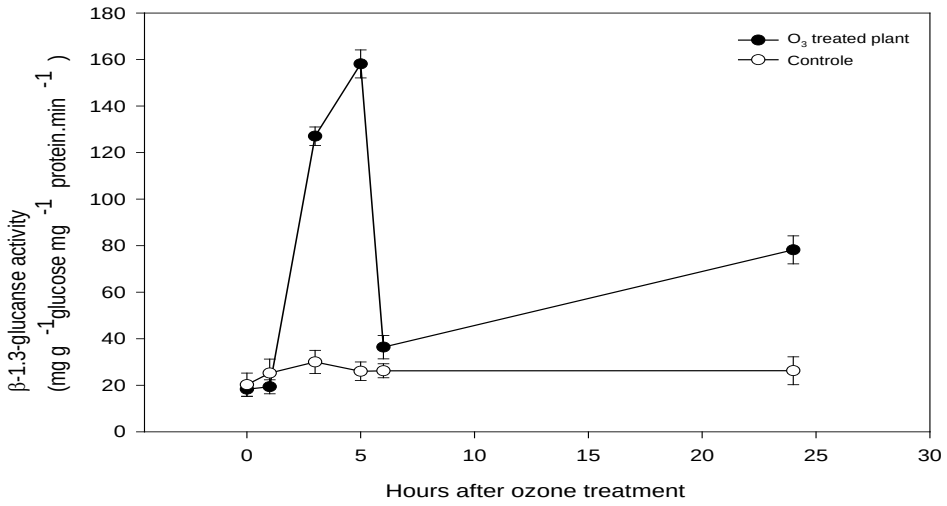

Figure 4.6: Effect of O_3 treatment on ß-1,3-glucanase activity (expressed as mg g^{-1}glucose mg^{-1} protein.min^{-1}) in maize (*33A13*). Error bars indicate standard deviation of, (n=3).

4.3.2 Chitinase activity

Similar to the β-1,3-glucanse activity, chitinase activity was also induced following 6h-ozone expose. A 2.5-fold increase in chitinase activity was observed 6h after the initial exposure period.

Form 6 to 25h after exposure, a slight decrease in chitinase activity was measured. Despite this decrease, the chitinase activity still remained significantly higher compared to the activity observed in the non-treated control plants (Figure 4.7).

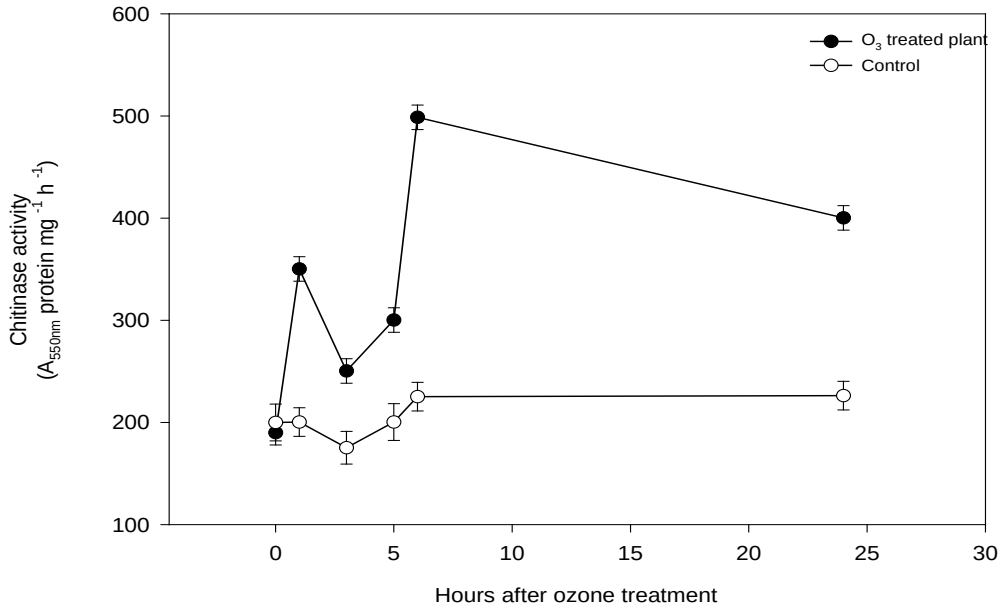

Figure 4.7: Effect of ozone treatment on chitinase activity (expressed as A_{550nm} mg^{-1} protein h^{-1}) in maize (*33A13*) plants. Error bars indicate standard deviation, (n=3).

4.4 Phenolic compounds

4.4.1 Effect of ozone treatment on *p*-hydroxybenzoic acid concentration

The level of *p*-hydroxybenzoic acid concentration was substantially increased following 6h of treatment. It reached a peak 7h after exposure and represent a 4-fold increase. After the initial peak, *p*-hydroxybenzoic acid concentration decreased but still remained two times higher than in the control plants. The *p*-hydroxybenzoic acid concentrations of the control plants remained constant throughout the entire period of testing (Figure 4.8).

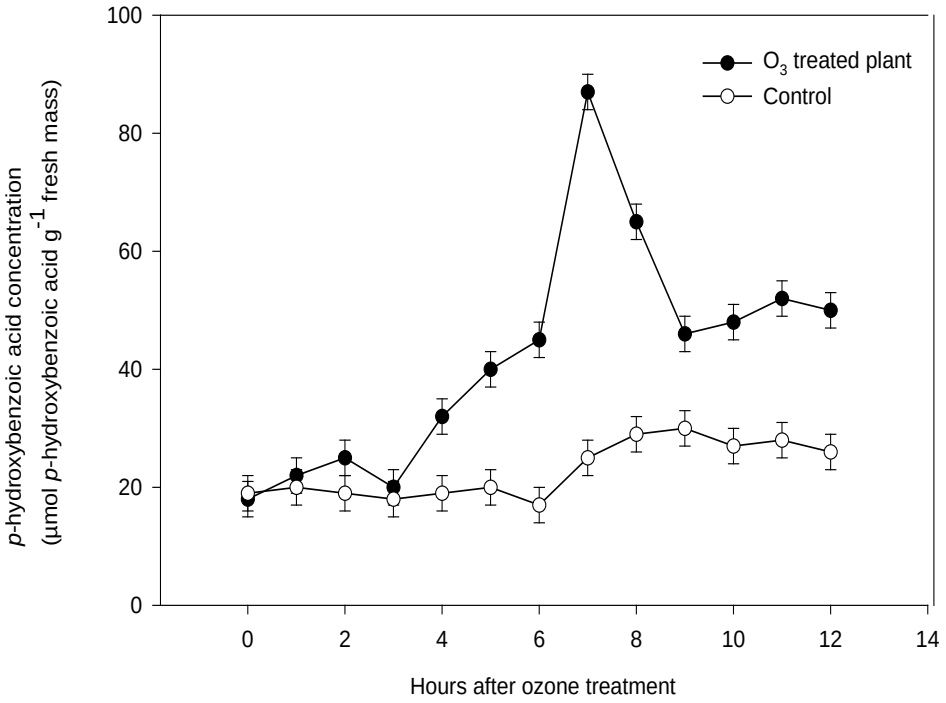

Figure 4.8: Effect of (6h at 150ppb) O_3 treatment on the concentration of *p*-hydroxybenzoic acid in maize (*33A13*). Error bars indicate a standard deviation of, (n=3).

4.4.2 Effect of ozone treatment on salicylic acid concentration

Fumigation with ozone resulted in an increase in salicylic acid concentration in the younger leaves of the maize plants. Salicylic acid concentration levels were induced to levels 4.5 times higher compared to the control. The significant induction observed in the fumigated plants were in sharp contrast with results obtain form the non-treated control plant, where salicylic acid concentrations remained remarkable constant throughout the tested period. Even though the concentration of salicylic acid in the treated plants decreased substantially from the 7th hour onward, concentrations were still higher than in the control plants (Figure 4.9).

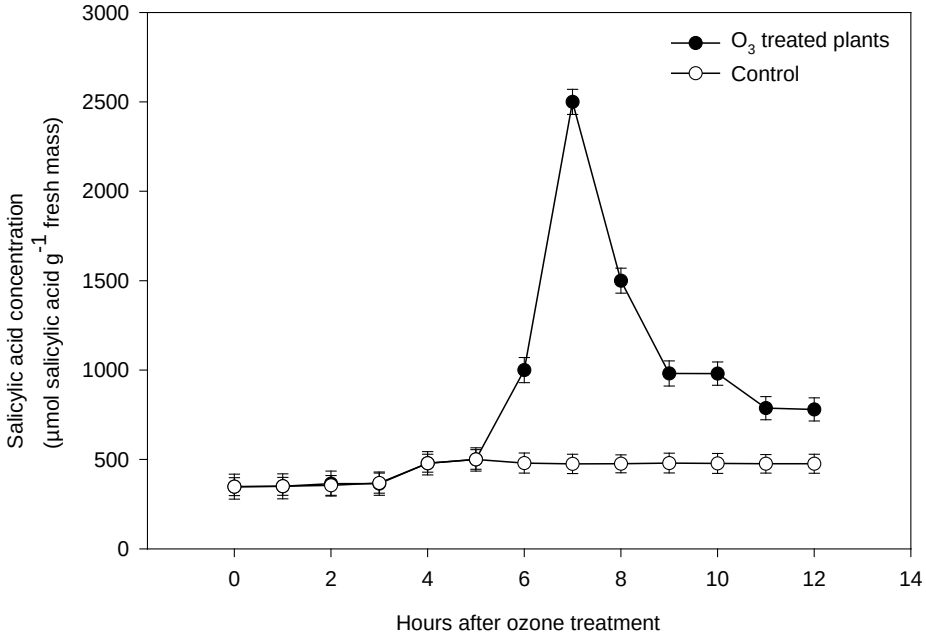

Figure 4.9: Effect of O_3 fumigation on salicylic acid concentration in maize (*33A13*) plants. Treatment lasted for 6h and was conducted at 150ppb. Error bars indicate standard deviation of, (n=3).

4.4.3 Effect of ozone treatment on caffeic- and syringic acid concentrations

Ozone fumigation at 150ppb for 6h resulted in an induction of caffeic- (Figure 4.10) and syringic acid concentrations (Figure 4.11) in the top leaves of 4-week-old maize plants. These high concentrations were maintained for the entire experimental period. These concentrations were low in the non-treated control plants and remained relative constant over the period of investigation.

Caffeic acid (Figure 4.10) reached a peak value 12h following ozone treatment. A 6-fold increase in caffeic acid concentration was observed, compared to the control.

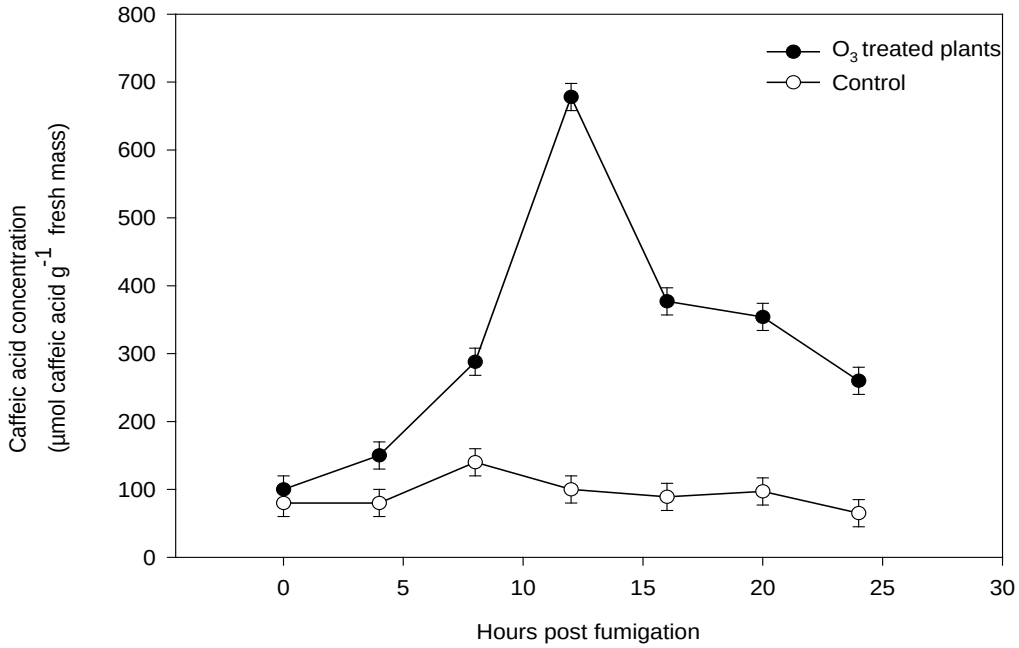

Figure 4.10: Effect of ozone treatment on the activity of caffeic acid concentration in the top leaves maize (*33A13*) plants. Error bars indicate standard deviation, (n=3).

Similar to caffeic acid, induction of syringic acid concentration (Figure 4.11) was observed, following the prolonged exposure to ozone. A 6.5-fold induction of syringic acid concentration was obtained 8h after fumigation, when compared to the non-induced control plants. Substantial decreases in both caffeic- and syringic acid concentrations were observed following the initial induction, but levels still remained higher than in respective control plants.

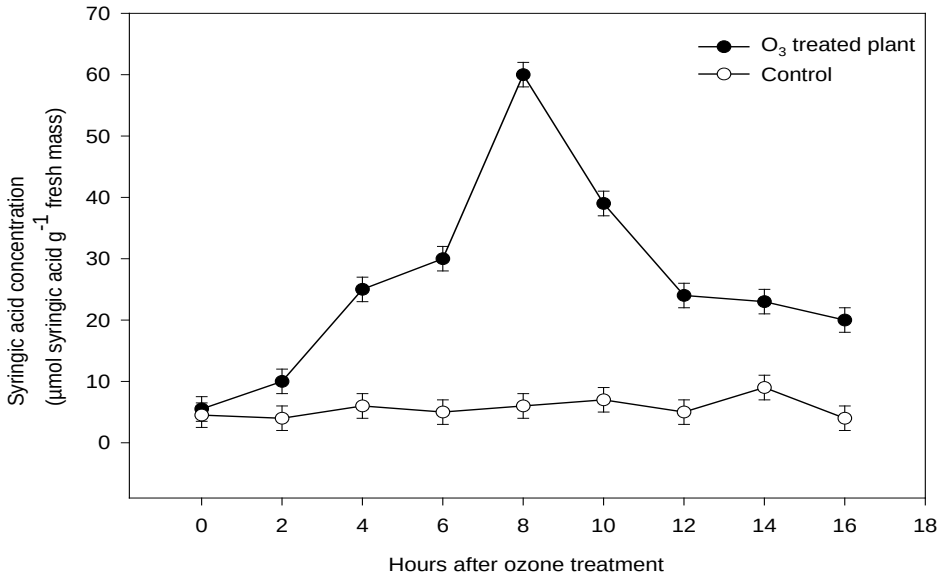

Figure 4.11: Effect of O_3 treatment on syringic acid concentration in the top leaves of 4 week old maize (*33A13*) plants Error bars indicate standard deviation, (n=3).

4.5. Effect of ozone exposure on Hydrogen Peroxide (H_2O_2) concentration

Ozone exposure are induced a substantial increase in the H_2O_2 concentration in the mature leaves of 4 week old maize plants. H_2O_2 concentration reached a peak 8h after exposure. Compared with the non-exposed control plants, H_2O_2 concentration remained high in the treated plants, despite the fact that a decrease occurred after seven hours (Figure 4.12).

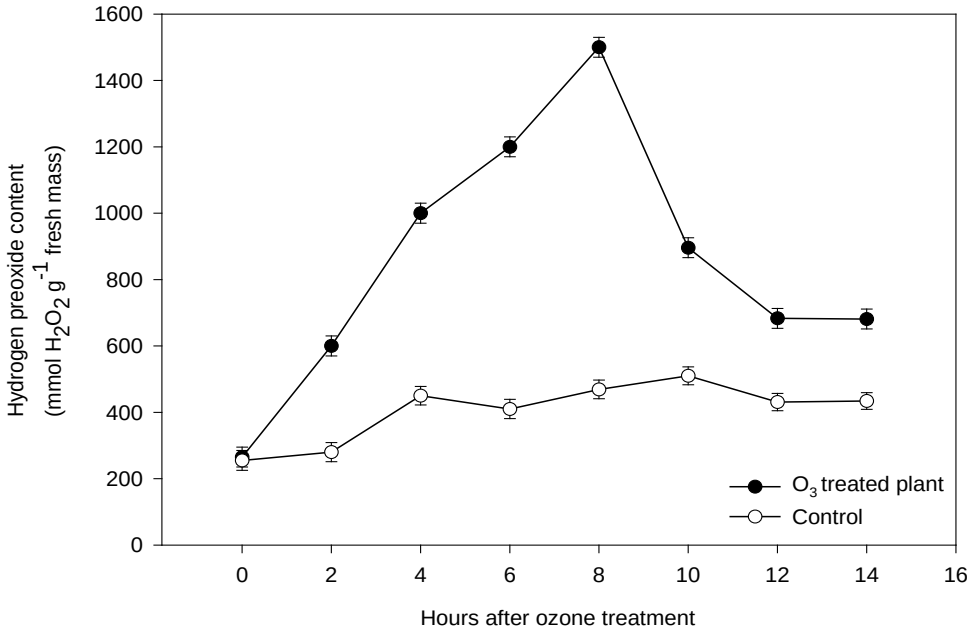

Figure 4.12: Effect of O_3 treatment on the H_2O_2 concentration in the top leaves of 4-week-old maize (*33A13*) plants. Leaves were harvested with 2h intervals following exposure. H_2O_2 concentration was read at 415nm, and H_2O_2 concentration was expressed as ($mmol^{-1}$ g fresh mass). Error bars indicate standard deviation, (n=3).

CHAPTER 5

GENERAL DISCUSSION

Plants integrate many environmental parameters and represent useful tools to show environmental problems revealing the dysfunction of ecosystems (Godefroid, 2001). There has been a definite increase in ozone concentrations in the troposphere over the past decade. Plant growth and production is not only stressed from exposure to high-elevated ozone concentrations, but by emitting volatile organic carbon compounds, they also contribute to ozone build-up in the troposphere. To fulfil the overall purpose of this study, which was to investigate the impact of aboitic stress on crop plants in South Africa, ozone was chosen as the stressor. The effects of ozone treatment on the photosynthesis mechanism of 4-week-old maize plants were examined to assess the overall response across a suite of measured variables.

The adverse effects of ozone, as with other stresses, depend on the dose (i.e. concentration) and exposure time, and were found to influence the plant response independently of leaf age. This suggests that young and mature leaves behaved in a similar way, as Castanga *et al*., (2001) found while exploring the effects of ozone on photosynthesis in pumpkin (*Cucurbita pepo*) plants.

The influence of ozone, on the functioning of PS II was assessed non-destructively, by chlorophyll fluorescence measurements and analysis (Nussbaum *et al*., 2001). Our results indicated that plants responded fairly quickly to ozone exposure (Figure 4.1a & b). The multiple saturated light pulse fluorescence traces (Figure 4.1a & b) clearly indicated the major differences in the non-exposed control and exposed maize plants.

The efficiency of primary photochemistry of PS II can be expressed by the ratio of variable to maximum fluorescence (F_V/F_M) of dark-adapted leaves exposed to saturating light pulses. This is also known as the maximum quantum yield of dark-adapted photosynthesis. F_V/F_M was significantly affected by the ozone treatment, indicating an alteration of the PS II photochemistry as a consequence of exposure to

O_3. A decrease in this parameter, which is commonly considered a sign of photo-inhibition (Krause, 1988), has been frequently observed in O_3 treated plants (Reichenauer *et al*., 1998; Soldatini *et al*., 1998; Shavini *et al*., 1999). Similar to results obtained from multiple light pulse analysis (Figure 4.1a & b), results from single light pulse analysis (Figure 4.2a & b) support the notion of F_V/F_M to decline following ozone exposure.

F_V/F_M decreased from (0.722) in the control plants to (0.575) in the treated plants. These results support similar findings made by Grandjean Grimm & Fuhrer (1992), who found that F_V/F_M was reduced following ozone exposure. The possibility that the effect of ozone on F_V/F_M may depend on the time of dark adaptation (Farage, 1996) could not be excluded. During this study 15 minutes of dark adaptation were allowed. But as Grobbelaar and Mohn (2002) stipulated, we were aware that it would take much longer for all the RC's to be fully re-oxidised, but it was assumed that most RC's would be 'open' and care was taken to keep the duration for dark adaptation constant for all measurements. The reduction of F_V/F_M ratio observed in ozone treated plants confirmed that the primary target site of ozone fumigation to be PS II photochemistry. Chlorophyll a fluorescence analysis showed that the F_V/F_M ratio, which indicates the efficiency of exitaion capture of PS II of dark adapted leaves, was significantly effected by the fumigation treatment. This indicated an alteration of the PS II photochemistry as a consequence of exposure to O_3. A decrease in this parameter, which is commenly consedered a sign of photoinhibition has been frequently observed in O_3 treated plants (Sharvin *et al*., 1999; Soldatini *et al*., 1998). An increase in F_O is an indication of inactivation of the reaction centres and it appears as if this is the primary site of O_3 stress.

Photochemical quenching (q_P) is often calculated from steady state fluorescence measurements (Maxwell & Johnson, 2000), where q_P gives an indication of the proportion of PS II reaction centres that are open.

Shown in both single and multiple light pulse analysis (Figure 4.1b & 4.2b) exposure to ozone resulted in a decrease in q_P, indicating that the rate of re-oxidation of Q_A was less effective, thus leading to an increased fraction of closed PS II centres during

actinic illumination. Castanga *et al.,* (2001) confirmed this after analysing the effect of ozone on the photosynthetic apparatus of pumpkin (*Cucurbita pepo*) plants. These observations therefore allow us to conclude that the RC's closes almost immediately following exposure and that the closed centres, who are unable to undergo charge separation and take part in linear electron transport, will lead to a decline in the actual quantum yield of PS II.

Such a reduction in q_P has also to be attributed to the increase in q_N following exposure to ozone. In fact, the increase in q_N, which is associated with dissipation of excess excitation energy, reduces the quantum efficiency of linear electron transport. A major reduction of more than 50 % in linear electron transport (Table 4.2) was observed following 6h of ozone fumigation. According to Ranieri *et al*., (1997) the resulting reduction in electron transport efficiency could lead to excessive excitation of the reaction centres of PS I and PS II. Therefore, the leaves may have to tolerate an excess of excitation energy in the pigment antennae of the photosystems, which can result in the damage of the particular sensitive PS II reaction centres, giving rise to P680 triplet and singlet oxygen. According to our results the plants used in the field trails were more stressed than those used as control. This could be due to more fluctuating wheather conditions outdoors, appose to the controlled and optimum conditions of the glass house. Thus the stress field plants experienced could be attributed to lots of factors and not just to stress due to O_3 exposure.

The light adapted maximal fluorescence (F_M') of both single and multiple light pulse analysis (Table 4.1 & Table 4.2) revealed major photosynthetic differences following ozone treatment. A decrease of between 12–15%, compared to the non-fumigated control plants was evident. In order to compare the results to outdoor grown plants in the field, measurements were made on such plants (Table 4.1). The results obtained during these field trails, compared quite favorably with the results obtain for the control plants, showing minimal stress under the outdoor conditions, at that particular point in time.

Chlorophyll *a* content of the maize (*33A13*) leaves, decreased significantly following fumigation, while the Chl *b* content was unchanged. According to Castanga *et al*.,

(2001), the decreases in Chl *a* (Figure 4.3), may be due to damage of acclimatization. In the first case, ozone derived reactive oxygen species could simply initiate chlorophyll breakdown. In the second case, the decrease in chlorophyll levels could represent an attempt to avoid excessive light interception in the antennae. Mulholland *et al*., (1997) however, found that relative low ozone concentrations had little effect on chlorophyll content in spring wheat (*Triticum aestivum* L.). Based on that, it could, therefore, be argued that the decline in the Chl *a* content seen in this study, was specifically due to fumigation with high concentrations of ozone, since at lower O_3 concentrations the Chl *a* content was less affected.

After the prolonged ozone treatment, the plants were allowed a two-day (48h) recovery period (Figure 4.3). Twenty-four hours following the initial exposure, the Chl *a* content was again determined. A 5-fold improvement in contents was seen after 6h following exposure. These observations allow us to speculate that even though the reaction centers of PS II became damaged during exposure (Dann & Pell, 1989; Grobbelaar & Mohn 2002), repair and recovery did take place even if only a miniscule amount of the leaf chlorophyll occures in the RCs.

Two days (48h) after the fumigation period the Chl *a* content and F_V/F_M ratio (Figure 4.4) were determined again and were found to be even higher, compared to results obtained after 24h following treatment. Both the Chl *a* and F_V/F_M ratio, exhibited values close to those measured before fumigation. It therefore, furthermore supports the repair and reversibility of the damage caused by ozone.

From the data obtained during this study, it is clear that ozone did reduce photosynthetic performance in leaf tissue and this seems to be consistent with ozone inducing the premature onset of leaf senescence. Ozone exposure clearly does have a detrimental effect on the ability of the leaf to maintain chlorophyll and protein contents, photosynthetic capacity and efficiency.

Changes observed in the photosynthetic characteristics of ozone-treated leaves, during this study, were found to be consistent with ozone having a negligible effect on the development of photosynthetic competence but inducing the onset of

premature loss of activity in leaves, as found by Nie *et al*., (1993). Cooley & Manning (1987) established that in wheat crops alteration of allocation resulting form ozone exposure is likely to reduce energy reserves, which may have important implications for winter survival and re-growth when energy reserves are needed. Skärby *et al*., (1995) found that necrotic lesion appeard on tobacco leaves that had been exposed to relatively low levels of ozone (90 or 100ppb/8h per day) for 20 consecutive days. Visual foliar injuries have been reported for air pollutants on several species. Gaseous pollutants enter through the stomata and causes alterations first in the extracellular aqueous phase and later in cell membranes, followed by the intracellular structures (Pasqualini *et al*., 2003).

Taking this into consideration, we subjected two sets of maize plants to prolonged ozone treatments. One set of plants was exposed to 85ppb ozone and the other to 150ppb ozone. In both cases, exposure lasted for consecutive days (fumigating for 6h/day). Not surprisingly, it was found that the leaves of plants exposed to 85ppb eventually showed necrotic and chlorotic spots. It was significantly less severe than damage seen on leaves exposed to 150ppb ozone (Figure 4.5). As seen on the leaves (Photos i-iii), exposure to 85ppb ozone led to injuries mostly confined to the leaf surfaces. When these plants were allowed some recovery time following the exposure, they recovered well and resumed their normal photosynthetic activities. However, the leaves of the plants exposed to 150ppb ozone (Photos v-vii) did not recover, even after extended recovery times were allowed. Necrotic and chlorotic spots at 150ppb were also no longer confined to the leaf surfaces, but appeared all over the leaves, and indicating damage to deeper lying tissues.

These observations, therefore, allow us to propose that there is a strong correlation between the decline in Chl *a* content and the visible injury appearing on the leaf. Our findings were inline with findings of Pelloux *et al*., (2001) where they clearly showed that ozone induced a decrease in the chlorophyll content and an increase in necrosis, as well as carbon metabolism alterations. Based on our findings we can therefore, conclude that exposure to ozone has a significant damaging effect on maize.

In recent years the use of plants exhibiting differential ozone tolerance has led to rapid advances in our understanding of plant responses to oxidative stress. It was found that several ozone-induced responses resemble those of plant-pathogen interactions (Sandermann *et al*., 1998). Pathogen-related (PR) proteins are, therefore, not only induced by biotic factors, but they also respond to a variety of abotic stimuli e.g. organic chemicals, heavy metals and air pollutants (Bol *et al*., 1990). The air pollutant ozone has been characterised as an important abiotic elicitor of PR-proteins (Brederode *et al*., 1991; Schraudner *et al*., 1992). Accumulation of PR-proteins is one of the most common markers for active plant defence. Among them, the hydrolytic enzymes β-1,3-glucanase and chitinase have been identified to be involved in plant resistance, especially against fungal pathogens.

Based on the results from this study, β-1,3-glucanse (Figure 4.6) and chitinase (Figure 4.7) enzyme activities increased following 6h of ozone exposure. β-1,3-glucanse activity in the young leaves of the 4-week-old maize (*33A13*) plants, showed an 5.2-fold increase following the ozone treatment and remained 2 to 3-times higher than in the control plants after the period of exposure (Figure 4.6). A peak value was obtained 5h after exposure. Similar to β-1,3-glucanase, a clear induction in chitinase activity was also evident.

Chitinase enzyme activity reached a peak 6h following exposure. Even though the chitinase activity decreased over time, it still remained 2-times higher in the ozone treated plants when compared to the control plants. It is doubtful whether β-1,3-glucanase has any direct role against ozone. However, the fact that the β-1,3-glucanase activity is induced to much higher levels in ozone treated plants that in control plants is indicative of its involvement in the general resistance responses of the plants. As mentioned, ozone penetrates the leaf easily and reaches the target sites quickly. One key feature of ozone is its ability to dissociate in the aqua's apoplast, leading to the formation of amongst others, O_2^- and H_2O_2. The fact that many defence mechanisms are evoked in the apoplast, and given that many defence-related proteins are subsequently found there (v/d Westhuizen *et al*., 1994), supports the results regarding the PR-proteins (Figure 4.6 & 4.7). The results also emphasises the important role of PR-proteins, ROS and phenols in plant defence.

The role of these PR-proteins in O_3-induced cell death is not entirely clear, but they have been associated with loosening of the cell wall during development (Slakeski & Fincher, 1992). Loosened cell walls may allow for escape of degradation products that might otherwise be trapped within the dead cell. It has been shown that O_3 itself is not deleterious to the plasma membrane (Grimes *et al*., 1983), but rather the fact that it degraded into ROS (e.g. O_2^-, H_2O_2, $OH^\bullet$) (Kangasjärvi *et al*., 1994) and forms singlet oxygen's when reacting with biological molecules (Kanofsky & Sima, 1991).

During this study the plants that were not subjected to any ozone produced little if any H_2O_2. However, after ozone treatment a considerable induction in H_2O_2 was evident (Figure 4.12). H_2O_2 remained high in the leaves of the ozone-fumigated plants throughout the test period, contrary to the non-fumigated control plants where every low H_2O_2 concentrations were observed. As shown in Figure 4.12 H_2O_2 concentration reached a peak 8h following exposure.

Ozone exposure stimulates an oxidative burst in leaves of sensitive plants, resulting in the generation and accumulation of reactive oxygen species (Wohlgemuth *et al*., 2002). In addition to triggering defence responses (the induction of β-1,3-glucanse (Figure 4.6) and chitinase (Figure 4.7)), ROS are involved, particularly in high concentrations, in the most drastic plant response namely the suicide of pathogen-invaded cells (Lamb & Dixon, 1997; Finkel, 1998). A further level of complexity regarding H_2O_2 signalling is the fact that H_2O_2 does not function alone, but rather in combination with other signalling molecules. Such molecules may be constitutively present, or increase in concentration/activity during stress (Neill *et al*., 2002) and may include e.g. salicylic acid (Figure 4.9). Systematic responses to excess excitation energy stress were found to be mediated by H_2O_2, indicating that it can also function as a signal during abotic stress (Mullineaux *et al*., 2000). Therefore, we can conclude that the H_2O_2 generated in the leaves of the ozone treated plants could be eventually responsible for stomatal closure and photo-oxidative damage, as seen with the decrease in electron transport rates, photosynthetic activities and increased fluorescence.

A direct dose-effect relationship between air pollutants (e.g. ozone) and phenol contents may be assumed (Pasqualini *et al*., 2003). The positive correlation between total phenols and ozone concentration indicates that high ozone concentrations induce the phenolic content in plants. According to Howell (1970), high levels of ozone influenced enzymatic activity intervening in phenol metabolism. However, according to Kainulainen *et al*., (1994) there was no relation between total phenol concentrations and high ozone concentrations. The impact of ozone on phenolic acids in plants has given rise to many contradictory results and, therefore, we came to the conclusion that the reaction to ozone depends on the particular plant species. In this context it is also important to state that the enzymes which take part in these biosynthetic pathways, may differ in sensitivity to pollutants (Loponen *et al*., 2001).

Phenolic acids are carbon-based compounds present in plants. They are perhaps the compounds most noted for their ability to bind to proteins *in vitro*, forming soluble and insoluble complexes (Singh *et al*., 2002). These phenolic-protein interactions are thought to be, in part, responsible for the putative function of phenolics as plant defence compounds (Coley, 1983). Phenolics are toxic to pathogens (Hammerschmid & Kuc, 1982), and their polymerisation makes cell walls more difficult to penetrate and degrade (Ride, 1980).

Since the initial reactions with ozone occur in the apoplastic compartment of the cell, the reaction of the ozone with the cell wall phenolics (e.g. salicylic acid, *p*-hydroxybenzoic acid, caffeic acid, syringic acid) leads to the formation of ROS (Runeckles & Vaartnou, 1997; Pasqualini *et al*., 2002). According to Yalpani *et al*., (1994) an increase in salicylic acid (Figure 4.9) is a likely trigger for the synthesis of PR-proteins (Figure 4.6 & 4.7) and resistance responses throughout the plant. We found a direct correlation between ozone and phenolic acid concentrations.

During the course of this project we found that fumigation with ozone resulted in increased salicylic acid accumulation (Figure 4.9) in the second leaf of the tested plants, with the greatest induction of salicylic acid found 7h following exposure to ozone. A 4.5-times increase in salicylic acid concentration was measured, compared to the control. It is known that salicylic acid has been implicated for its a role in

defence responses by inhibiting catalase activity with the resultant accumulation of hydrogen peroxide (Chen *et al*., 1993). Thus, since much higher levels of salicylic acid had accumulated in the ozone treated leaves; inhibition of catalase at these sites may yield significant quantities of H_2O_2, possibly promoting hypersensitive response-associated cell death.

Our results clearly indicate that when maize is exposed to ozone several defence-related pathways are stimulated. This is indicated in the biosynthesis of salicylic acid and accumulation of PR-proteins. The effect of this abiotic stressor on salicylic acid levels and disease resistance appeared to mimic that of narcotising pathogens, suggesting that biotic and abiotic inducers of salicylic acid accumulation and disease resistance may share a common signal-transduction pathway (Yalpani *et al*., 1994). For example, it is known that UV light, ozone and narcotising pathogens elicit a burst of activated oxygen species in plant tissue (Imbrie & Murphy 1984; Heath 1988; Apostal *et al*., 1989; Dixon & Lamb, 1997). However, it remained to be determined if oxygen radicals are linked to the induction of salicylic acid biosynthesis and acquired disease resistance.

A large difference in *p*-hydroxybenzoic acid concentration was observed between the control and ozone treated plants (Figure 4.8). Levels of *p*-hydroxybenzoic acid remained consistently low in the control plants, appose to the ozone treated plants where concentrations were induced too much higher levels. *p*-Hydroxybenzoic acid reached peak values 7h following exposure, where it was 3.5-times higher than in the control.

Several papers cited in this study indicated close similarities between ozone-induced and general stress reactions in plants. This is no different for caffeic (Figure 4.10) and syringic acid (Figure 4.11) concentrations. They were found to respond positively (induced) after ozone exposure. Ozone-induced increases of both these phenols were clearly seen in the leaves of O_3 exposed plants. Bradley *et al.,* (1992) observe a similar phenomenon in the leaves of wheat plants after infestation with the Russian wheat aphid. They also suggested that these phenols could be responsible for the process leading to lignification during incompatible interactions. Phenolic acids and

lignin are major components of plant tissues and can influence plant-pathogen relationships. Some plants treated with O_3 showed increased enzyme activity that finally led to the formation of these phenolic compounds, suggesting that increased biosynthesis of lignin and related products might also occur (Quesada *et al*., 2002). Moerschbacher *et al.,* (1990) found that in the hypersensitive response wheat plants showed towards fungi, the mechanism of response resistance could be attributed to cellular lignification. Vance *et al*., (1980) confirmed that lignification might also function in the reduction of fungal growth, by increasing resistance of the cell wall against fungal penetration.

During this study it was found that caffeic acid (Figure 4.10), was induced 7-times higher than the controls after 11h following exposure. Whilst syringic acid concentrations, were 5.5-times higher, after 8h following exposure, when compared to the control plants. The concentration of both phenols in the leaves of the ozone treated plants decreased after reaching their initial peak value. These concentrations, however, were still higher than the concentrations observed in the control plants. We, therefore, confirm the importance of phenolic acid as biological indicator of air quality in maize, since all the phenolic compounds analysed for this study responded positively to ozone fumigation.

It is an undeniable fact that O_3 is a major air pollutant effecting plant growth and productivity. By exploiting the ability of O_3 to generate ROS, significant progress has been made towards the understanding of the wide spectrum of plant defence responses. The understanding of the unique ability of O_3 to mimic several pathogen-induced responses was helpful during this study and promoted the understanding of various interacting signalling pathways. There is no doubt, that further studies using O_3 as an abiotic elicitor will provide more detailed information on the interaction of different signalling pathways during oxidative stress.

In this study, we have examined the problem of air pollution and the biological consequences of the associated increases in tropospheric concentration of O_3. In comparison with levels documented in some European and Asian countries, levels of tropospheric ozone in South African still seem to be relatively low. However, our fast growing coal and motor industries and the ever increasing population are putting

severe strains on the environment and this could eventually lead to major environmental changes, which include the increase of trophosperic ozone.

We have shown that plants have evolved various mechanisms to avoid or tolerate ozone stress. Avoidance factors relate to the uptake of ozone *via* the stomata and to detoxification processes, whereas tolerance factors include energy dependent processes of metabolic readjustments once ozone reaches the target sites. Responses of plants to ozone may be regarded as the culmination of a sequence of biochemical and physiological events, which may eventually result in injury. It is also important to note that the relative importance of avoidance and tolerance factors in determining resistance to ozone varies vastly among plants. Differences in ozone sensitivity, are generally the result of various avoidance and tolerance mechanisms acting at the same time. Furthermore, other stress factors can influence the role of each of these mechanisms and thus also determine the type and degree of plant response to ozone.

At the beginning of the 21st century, we are faced with a rapid changing environment. There can be no doubt that under future conditions, agro-ecosystems and their management will substantially differ from today, and potential ozone impacts in the agricultural sector will depend largely on changes in other factors. Hence, for the evaluation of future trends in ozone research we cannot rely solely on trends at ground-level ozone concentrations predicted by atmospheric models.

In many countries where the demand for food is stabilised or only slowly increasing, the importance of domestic agricultural production and constrains might be declining. Whether or not agricultural production will be able to keep pace with the growing food demand in rapidly expanding populations will depend on many factors, including the future levels of trophosperic ozone. It is now clear that ozone fumigation have serious implications for crop production and this may create the need for the development of new crop varieties that are better adapted to with-stand high ozone concentrations. Thus, to be effective in a policy or in a technological context, the results from present and future research should be funnelled into an appropriate knowledge transfer scheme to gain added value.

However, in this quest of transforming knowledge concerning this problem into practical implementation plans, there have to be continuous research, both fundamental and applied. The aim remain to continuously improve our tools and data to assess areas of risk and to develop pollution abatement strategies that are optimised in terms of their feasibility and effectiveness in using integrated assessment models. That man will ever devise an artificial, or any kind of system, which will approach, let alone surpass, the effectiveness of the plant as a photosynthetic system seems extremely doubtful. We must therefore also seriously consider what effect Man’s activities may have on photosynthesis in the future and what use we may make of our increasing knowledge of nature’s mechanisms, and ultimately to enables us to control and direct it with greater effect.

SUMMARY

The impact of elevated ozone concentrations on plants has important global implications for the environment and especially for crop production. The fact that ozone can cause serious damage to crop production served as motivation for the research done during this study. One of South Africa's most common agricultural crops, *Zea mays*, was chosen as test organism. The aim was to get a better understanding of the effect of ozone on maize.

During this study we investigated the photosynthetic and metabolic responses of *Zea mays* after it had been exposed to moderate concentrations of ozone. The plants were grown in a green house until they reached the two-leave stage. Thereafter they were placed in a controlled environment growth chamber, where they were fumigated with ozone at 150ppb. After fumigation, leaves of the exposed plants were dark adapted for 15 minutes, before chlorophyll fluorescence measurement were made.

Ozone fumigation resulted in an increase in the F_O, compared to the control. Noticeable was also the lower F_M values of fumigated plants. The photochemical quenching of the fumigated plants was significantly lower than the controls, while the opposite was seen for non-photochemical quenching. Several fluorescence indicators and quenching parameters were used to determine the photosynthesis potential following ozone exposure. We could also evaluate the quantum yield of PS II, which gives an estimate of the rate of linear electron transport.

The effect of ozone fumigation on PR proteins (β-1, 3-glucanase and chitinase), phenolic acid composition and the hydrogen peroxide (H_2O_2) concentrations were also measured. H_2O_2 production was induced following the ozone exposure. The increase in H_2O_2 corresponded with the increases in β-1, 3-glucanase and chitinase activity and we concluded that if corresponded to an activation of the defence genes. Down-stream defence responses continued after exposure, with the synthesis of phenolic compounds, as indicated by increased phenolic acid concentrations. This was seen as a manifestation of the hyper sensitive response of *Zea mays*, which

forms part of this crop’s defence response against ozone. We also conclude that ozone is a major stressor that could influence crop yields significantly.

OPSOMMING

Die impak van verhoogde osoonkonsentrasies op plante, het verrykende gevolge vir die omgewing en spesifiek vir die produksie van landbougewasse. Die feit dat osoon, gewas produksie ernstig kan benadeel, het gedien as motivering vir hierdie studie. Een van Suid-Afrika se mees algemene verboude gewasse, *Zea mays*, is gekies as proefplant. Die doel was om ‘n beter insig te bekom vir die interaksie en die invloed van osoon op *Zea mays*.

Gedurende die studie het ons ondersoek ingestel na die fotosintese en metaboliese reaksies van *Zea mays*, nadat dit aan osoon blootgestel is. Eksperimentele plante is in ‘n glashuis gekweek tot die tweeblaar-stadium bereik is. Daarna is hulle uit die glashuis verwyder en in groeikabinette, onder beheerde toestande, geplaas. In die groeikabinette is osoon teen 150 dele per biljoen toegedien. Na blootstelling aan osoon, is die blare vir 15 minute lank donkeraangepas, voordat chlorofilfluoressensie bepalings gedoen is.

Blootstelling aan osoon het gelei tot ‘n toename in die F_O-waarde, in vergelyking met die kontrole. Daar was ook ‘n aansienlike afname in die F_M-waarde van die blootgestelde plante. Die foto-chemiese blusing van die osoonblootgestelde plante was aansienlik laer as die van die kontrole plante, terwyl die teenoorgestelde vir die nie-photochemiese blussing waardes gesien is. Verskeie fluoresenssie-intensiteit indikatore en blussingparameters is gedurende die studie gebruik om die invloed van osoon op fotosintese na te gaan. Dit het ingesluit die bepaling van die kwantumopbrengste van PS II.

Die effek van osoon op die patogeenverwante proteine, (β-1,3-glukanase en kitinase), fenolsuur samestelling en waterstof peroksied (H_2O_2) konsentrasie, is ook bepaal. H_2O_2 produksie is geinduseer na osoonblootstelling. Die toename in H_2O_2 stem ooreen met die toename in aktiwiteit van β-1,3-glukanase en kitinase, en ons kon aflei dat dit was a.g.v aktivering van verdedigingsgene. Stroom-af verdedigingsreaksies is bepaal met die sintese van die fenoliese verbindigs soos aangedui in die verhoging van konsentrasies van die onderskeie ure. Dit kan gesien

word as ‘n manifestasie van die hipersensitiewe reaksie van *Zea mays*, en maak deel uit van die verdedigingsreaksie wat die gewas teen osoon toon. Ons kon dus die afleiding maak dat osoon ‘n belangrike stressor is, en dat dit gewas produksie baie kan beinvloed.

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