EFFECTS OF FLUCTUATING LEVELS OF OZONE OR NITROGEN DIOXIDE ALONE AND IN SEQUENCE ON PLANT GROWTH AND PHOTOSYNTHESIS

by

UPENYU MAZARURA

B.Sc. Agric. (Hons), The University of Zimbabwe, 1987
M.Phil, The University of Zimbabwe, 1989

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Plant Science)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 1997

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of PLANT SCIENCE

The University of British Columbia
Vancouver, Canada

Date 29 AUG. 1997

DE-6 (2/88)

ABSTRACT

Radish, *Raphanus sativus* L., plants were exposed daily to ozone, O_3, or nitrogen dioxide, NO_2, or sequences of the two pollutants. The exposure profiles for both gases approximated sine waves with peak concentrations of 120 ppb (parts per billion by volume; nl l^{-1}). In the case of O_3, this is close to the reported threshold for adverse effects while with NO_2 it is below the reported threshold. The sequences involved different combinations of exposures to NO_2 from 06:00 to 10:00h and/or 18:00 to 22:00h and O_3 from 10:00 to 18:00h. O_3 alone caused no significant effects on growth after 21 days but NO_2 alone early in the day caused a small growth increase. Increases in the quantum yield of chlorophyll fluorescence and in photochemical quenching observed in both treatments after 6 days of exposure indicated increased photosynthetic CO_2-assimilation that, in the case of O_3, was presumably offset by increased maintenance/repair respiration since no increase in dry matter occurred. Exposures to NO_2 in sequence with O_3 had increasingly negative effects on growth. Since O_3 tended to prevent stomatal closure early and late in the day, the estimated NO_2 fluxes in the sequential treatments were greater than those with NO_2 alone. However, since growth reductions were observed with the sequences, the mechanisms of stimulation of assimilation were counteractive.

No visible necrotic injury was observed in any treatment and O_3 alone resulted in a significant effect on ethylene release, a decrease. This suggests that O_3-induced increases in ethylene release reported in the literature may have reflected the occurrence of incipient or actual foliar injury.

The lack of growth effects due to O_3 alone but their occurrence with NO_2-O_3 sequences indicates that exposures to other pollutants in ambient air must be considered in establishing objectives or standards of air quality to protect vegetation.

Studies with a C-3 grass, *Poa pratensis* L., Kentucky bluegrass, and a C-4 grass, *Setaria viridis* Beauv., green foxtail, treated with similar sine-wave exposures to O_3 within the modified cavity of an electron paramagnetic resonance (EPR) spectrometer, permitted observation of the effects of exposure on both the free radical signals in photosystems I and II and on chlorophyll fluorescence attributable to photosystem II. Both Signal I (from $P700^+$ in PSI) and Signal II (from Tyr-160 in the D2 protein of PSII) were stimulated by O_3. However, the fact that Signal I observed in white light in bluegrass during exposure to O_3 rose to the level of Signal I in far-red light indicates reduced electron flow through PSI. These effects and concomitant effects on chlorophyll fluorescence confirm a major effect of O_3 on the water-splitting side of PSII.

In Kentucky bluegrass, the level of Signal I in white light was approximately one half of that in far-red light, while in foxtail the situation was reversed presumably because of a contribution by PSII light-harvesting to PSI in the bundle-sheath chloroplasts. Measurements of the relative levels of Signal I in white or far-red light may therefore provide a means of assessing the extent of such contributions in other C-4 species.

TABLE OF CONTENTS

ABSTRACT II

LIST OF TABLES VII

LIST OF FIGURES VIII

ABBREVIATIONS XI

ACKNOWLEDGEMENT XII

1. INTRODUCTION AND STATEMENT OF OBJECTIVES 1

2. LITERATURE REVIEW 5

2.1 Origin of atmospheric O_3 and NO_X 5

2.1.1 The formation of atmospheric O_3 5

2.1.2 The origins of atmospheric NO_2 8

***2.2* Uptake of gaseous pollutants by plants** 9

2.3 General considerations of plant exposure to gaseous pollutants 12

2.4 Plant response to O_3 and NO_2 15

2.4.1 Plant responses to O_3 15

2.4.1.1 Injury, growth and yield 15

2.4.1.2 Photosynthetic gas exchange 16

2.4.1.3 Stomatal response 17

2.4.1.4 Other physiological processes 19

2.4.2 Plant responses to NO_2 21

2.4.2.1 Injury, growth and yield 21

2.4.2.2 Photosynthetic gas exchange 23

2.4.2.3 Stomatal response 23

2.4.2.4 Other physiological processes 24

2.4.3 Plant response to NO_2-O_3 mixtures and combinations 24

2.5 Cellular mechanisms of response to O_3 and NO_2 25

2.5.1 Potential for free radical production from O_3 25

2.5.2 Potential reactions of O_3, oxyradicals and NO_2 with cell components 27

2.5.3 Photosynthetic electron transport 28

2.5.4 Photosynthetic carbon assimilation 32

2.5.5 Nitrogen metabolism 34

2.5.6 Other metabolic processes 35

3. RADISH STUDIES 37

3.1 Introduction 37

3.2 Methods and materials 39

3.2.1 Growth chamber exposures 39

3.2.2 Plant materials 45

3.2.3 Growth measurements 45

3.2.4 Stomatal resistance measurements and estimation of pollutant fluxes 45

3.2.5 Statistical analyses 46

3.2.6 Fluorescence measurements 46

3.2.7 Endogenous ethylene production measurements 48

3.3 Results 51

3.3.1 Effects of NO_2/O_3 sequences on growth 51

3.3.2 Stomatal Conductance and Flux 56

3.3.3 Fluorescence 59

3.3.4 Effects on ethylene production 70

3.4 Discussion 72

4. GRASS STUDIES. 81

4.1 Introduction 81

4.2 Materials and methods 83

4.2.1 Plant material and growing condition 83

4.2.2 Exposure to O_3 84

4.2.3 Electron paramagnetic resonance (EPR) 84

4.2.4 Infiltration of spin traps into leaves 86

4.2.5 Experimental design, spectra data handling, normalization and statistical analyses 88

4.2.6 Fluorescence measurements 91

4.3 Results 93

4.3.1 Bluegrass 93

4.3.1.1 Effects of O_3 on photosynthetic radicals 93

4.3.1.2 Effects of O_3 on photosynthetic radicals in the presence of spin traps 96

4.3.1.3 Effects of O_3 on chlorophyll fluorescence 101

4.3.1.4 Effects of O_3 on chlorophyll fluorescence in the presence of spin traps *103*

4.3.2 Foxtail ***106***

4.3.1.1 Effects of O_3 on photosynthetic radicals *106*

4.3.1.2 Effects of O_3 on photosynthetic radicals in the presence of spin traps *106*

4.3.1.3 Effects of O_3 on chlorophyll fluorescence *115*

4.3.1.4 Effects of O_3 on chlorophyll fluorescence in the presence of spin traps *115*

4.4 Discussion **119**

5. GENERAL DISCUSSION **130**

6. SUMMARY AND CONCLUSIONS **135**

7. BIBLIOGRAPHY **138**

APPENDIX A **156**

APPENDIX B **162**

APPENDIX C **164**

LIST OF TABLES

Table 3.1 Federal Air Quality 1-hour Objectives for Canada for NO_2 and O_3, and National Ambient Air Quality Standards for the United States 42

Table 3.2 Maximum 1-hour average concentrations (ppb) of NO_2 and O_3 measured at non-urban locations in the Fraser Valley of British Columbia. 42

Table 3.3 Definition of chlorophyll fluorescence nomenclature used in this study. 47

Table 4.1 Effects of 120 ppb O_3 on kinetics of signal I in bluegrass subjected to different sequences of illumination. 96

Table 4.2 Effects of 120 ppb O3 on signal I kinetics in bluegrass infiltrated with 4-POBN, subjected to different sequences of illumination. 101

Table 4.3 Effects of 120 ppb O_3 on kinetics of Signal I in foxtail subjected to different sequences of illumination in foxtail. 110

Table 4.4 Effects of 120ppb O_3 on kinetics of Signal I in foxtail infiltrated with DMPO, subjected to different sequences of illumination. 110

Table 4.5 Effects of 120 ppb O_3 on kinetics of Signal I in foxtail infiltrated with 4-POBN, subjected to different sequences of illumination. 113

Table 4.6. Summary of the changes in EPR signals and chlorophyll fluorescence parameters in bluegrass and foxtail after 6h exposure to O_3 with 120ppb maximum at 4h. All changes are relative to controls in medical air. 124

LIST OF FIGURES

Fig. 2.1 Ozone formation in the troposphere 6

Fig. 2.2 Diagrammatic summary of formation of O_3 in a, normal, and b, air polluted with oxides of nitrogen (NO, NO2) and hydrocarbons 7

Fig.2.3 The Z scheme 29

Fig. 3.1 The exposure regime for the NO_2/O_3sequences 43

Fig. 3.2 Typical time-courses of the levels of O_3, NO_2 and NO in the control chamber and the ambient air illustrating the effectiveness of activated charcoal and Air Repair in reducing the gases in the air supplied to the chambers 44

Fig 3.3. The relationship between the measured fluorescence parameters 48

Fig 3.4 A typical trace of Y, qP, and qN, from which the last four data points were selected for data analysis 50

Fig. 3.5. Effects of treatment on total dry mass 52

Fig. 3.6. Effects of treatment on shoot dry mass 53

Fig. 3.7. Effects of treatment on hypocotyl dry mass 54

Fig. 3.8. Effects of treatment on shoot/root ratio 55

Fig. 3.9 Time course of relative diffusive resistance by treatment 57

Fig. 3.10 Time course of estimated fluxes of NO_2 (broken line) and O_3(solid line) by treatment 58

Fig. 3.11 Fm and Fo vs. treatments for radish 60

Fig. 3.12 Fluorescence quantum yield (Y) vs. treatments for radish 60

Fig. 3.13 Fluorescence quantum yield (Y) vs. treatments for radish 61

Fig. 3.14 Time course of fluorescence (Ft) by treatment 62

Fig. 3.15 Time course of maximal fluorescence (light adapted; Fm')by treatment 63

Fig. 3.16. Effects of treatment on mean quantum yield (Y), qP, qN and NPQ 65

Fig. 3.17 Time course of fluorescence quantum yield (Y) by treatment 66

Fig. 3.18 Time course of photochemical fluorescence quenching (qP) by treatment. 67

Fig. 3.19 Time course of non-photochemical fluorescence quenching (qN) by treatment. 68

Fig. 3.20 Time course of non-photochemical fluorescence quenching (NPQ) by treatment. 69

Fig. 3.21. Effects of treatment on ethylene release. 71

Fig. 4.1 The microwave cavity showing the adaptation of the threaded collar to accommodate the fibre-optic cable from the fluorometer, and provide an additional illumination port. 85

Fig. 4.2. Longitudinal (a) section and cross-sectional (b) views of the cavity, showing the arrangement of the leaf and leaf holder, the ozone sampling tube, the fluorometer fibre-optic, and the illumination areas of the leaf. 87

Fig. 4.3. Typical first derivative signals in a bluegrass leaf in a, darkness; b, white light; c, 710nm light, illustrating signal height measurements and *g*-value. 89

Fig. 4.4 Trace of Signal I low-field maximum in a bluegrass leaf in changing light conditions. 90

Fig. 4.5 The coefficient of non-photochemical quenching (qN) for foxtail for the O_3 treatment and the control; a. before and b. after normalization to 11:00h. The shaded triangle indicates the O_3 exposure period. 92

Fig. 4.6 Effects of O_3on relative signal height in the dark or in white and light for bluegrass vacuum-infiltrated with distilled water. 94

Fig. 4.7 Typical EPR kinetic Signal I traces for bluegrass leaves (after infiltration with water) in medical air or during exposure to 120 ppb O_3, with different sequences of illumination. 95

Fig. 4.8 Effects of O_3 on relative signal height in the dark or in white and light for bluegrass vacuum-infiltrated with distilled water or 20mM DMPO. 97

Fig. 4.9 Effects of O_3 on relative signal height in white and 710nm light for bluegrass vacuum-infiltrated with distilled water or 20mM 4-POBN. 99

Fig. 4.10 Typical EPR kinetic Signal I traces for bluegrass leaves (after infiltration with 20 mM 4-POBN) in ambient air or during exposure to 120 ppb O_3, with different sequences of illumination. 100

Fig. 4.11 Effects of O_3 on a. quantum yield (Y); b. photochemical quenching (qP); and non-photochemical quenching c, qN and d, NPQ in bluegrass. 102

Fig. 4.12 Effects of DMPO on the time course of a. quantum yield (Y); b. photochemical quenching; (qP) and non-photochemical quenching c, and d, in bluegrass. 104

Fig. 4.13 Effects of 4-POBN on the time course of a. quantum yield (Y); b. photochemical quenching; (qP) and non-photochemical quenching c, and d, in bluegrass. 105

Fig. 4.14 Effects of O_3 on relative signal height in white and 710nm light for foxtail vacuum-infiltrated with distilled water. 107

Fig. 4.15 Typical EPR kinetic signal I traces for foxtail leaves (infiltrated with distilled water) in medical air or during exposure to O_3, with different sequences of illumination. 108

Fig. 4.16 Effects of O_3 on relative signal height in the dark or in white and light for foxtail vacuum-infiltrated with distilled water or DMPO. 109

Fig. 4.17 Typical EPR kinetic Signal I traces for foxtail leaves (after infiltration with DMPO) in medical air or during exposure to 120 ppb O_3, with different sequences of illumination. 111

Fig. 4.18 Effects of O_3 on relative signal height in white and 710nm' light for foxtail vacuum-infiltrated with distilled water or 4-POBN. 112

Fig. 4.19 Typical EPR kinetic Signal I traces for foxtail leaves (after infiltration with 4-POBN) in medical air or during exposure to 120 ppb O_3 with different sequences of illumination. 114

Fig. 4.20 Effects of O_3 on a. quantum yield (Y); b. photochemical quenching (qP); and non-photochemical quenching c, qN and d, NPQ in foxtail. 116

Fig. 4.21 Effects of DMPO on the time course of a. quantum yield (Y); b. photochemical quenching; (qP) and non-photochemical quenching c, and d, in foxtail. 117

Fig. 4.22 Effects of 4-POBN on the time course of a. quantum yield (Y); b. photochemical quenching (qP); and non-photochemical quenching c, qN and d, NPQ in bluegrass. 118

Fig. 4.23 Signal I decay kinetics; a. in 710nm light in the control; b. in white light in the control; c. in 710nm light during exposure to 120 ppb O_3; and d. in white light during exposure to120ppbO_3 123

ABBREVIATIONS

4-POBN	α-(4-pyridyl-1-oxide) -N-t-butylnitrone
ATP	Adenosine triphosphate
DMPO	5,5-dimethyl-1-pyrroline-N-oxide.
EPR	Electron Paramagnetic Resonance
GDH	Glutamate dehydrogenase
GOGAT	Glutamine oxoglutarate aminotransferase
GS	Glutamate synthetase
LHCI	Light-harvesting complex I
LHCII	Light-harvesting complex II
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NiR	Nitrite reductase
NR	Nitrate reductase
OEC	Oxygen-evolving complex
PAR	Photosynthetic active radiation
PEPC	Phosphoenolpyruvate carboxylase
PG	True photosynthetic CO_2 assimilation
PN	Net photosynthetic CO_2 assimilation
PPFD	Photosynthetic Photon flux density
PSI	Photosystem I
PSII	Photosystem II
Rubisco	Ribulose 1,5-*bis*phosphate carboxylase/oxygensae
RuBP	Ribulose 1,5-bisphospahte
SOD	Superoxide dismutase

ACKNOWLEDGEMENT

This thesis is dedicated to:
My wife, Elsie and my daughters,
Rangarirai Jocelyn and Ropafadzai Grace Mazarura

I would like to thank my wife and daughters for both support and especially tolerance over the long period of time, and my wife for help in typing some of the many drafts of my thesis.

I would also like to extend my special thanks to my research supervisor, Dr. V.C. Runeckles for advice, encouragement throughout the course of the research, and editorial help with the manuscript.

In addition, I would like to thank the Commonwealth Fellowship Plan for financial assistance, my employer for giving me the opportunity to pursue my studies, and in particular the director, Dr. L.T.V Cousins.

Lastly, I would like to thank the Almighty for seeing my family through the numerous difficult moments during the entire course of the research.

1. INTRODUCTION AND STATEMENT OF OBJECTIVES

Ozone (O_3), an allotrope of oxygen (O_2), is a very reactive gas composed of three oxygen atoms. In unpolluted environments reactions that involve nitrogen oxides (NO_X), atmospheric O_2, and biogenic volatile organic compounds (VOCs) in the presence of sunlight form O_3 in the ambient air of the troposphere. Under polluted conditions the same processes occur but with anthropogenic sources of NO_X and VOCs added to the natural sources, leading to increased O_3 levels. The additional NO_X and VOCs are emitted from combustion and utilization of coal and petroleum products.

Since nitrogen dioxide (NO_2) is a significant component of NO_X, NO_2 and O_3 are intimately related in ambient air. In regions subjected to inputs of the anthropogenic precursors it has been observed that the diurnal cycles for NO_2 and O_3 usually result in higher levels of NO_2 occurring in the morning and late evening, with O_3 reaching a maximum at midday to late afternoon. Regardless of the other pollutant gases that may be present, this means that under ambient conditions a plant may be exposed to a complex combination of these two pollutants and thus observations of plant response to either pollutant alone may not be relevant to the 'real world situation'. Furthermore, much of the information on the effects of NO_2 and O_3, particularly on physiological processes, has come from "sudden exposures" to steady concentrations of pollutant rather than to fluctuating levels simulating the 'real world'.

NO_2-induced foliar injury symptoms are rare even at high concentrations but NO_2 has been reported to decrease net photosynthesis, cause stomatal closure, increase dark respiration (particularly at high concentrations) and to decrease ethylene release. Biomass stimulation is known to occur, which has been interpreted as resulting from the use of NO_2 as an N source.

On its own, O_3 is also known to be responsible for a wide variety of adverse effects on plants, ranging from foliar injury involving necrosis of the mesophyll, to premature senescence, reduced shoot and root growth, and decline in overall growth and crop yields. At the physiological level, O_3 has been reported to inhibit photosynthesis and many other processes. Ozone has been reported to affect stomatal behaviour (usually causing closure), to cause increased maintenance respiration, and to induce ethylene production.

Although exposure of plants to mixtures of NO_2 and O_3 has revealed various interactive effects, little is known of the interactions when the exposures are sequential. Furthermore, the nature of the actual mechanisms involved in plant response to either pollutant are still largely speculative.

Negative effects of NO_2 are thought to be the result of perturbation of normal cell function because of increased cellular acidification and nitrite levels and possibly because of effects on reaction centers, enzymes and other cellular processes and competition with carbon fixation for reducing power. Effects attributable to O_3 are thought to occur through its reaction products, especially the oxyradicals, hydroxyl and superoxide anion, although its high oxidative reactivity may also cause direct chemical changes, particularly in the apoplastic space of the leaf.

Although there is recent direct evidence for the O_3-induced formation of the superoxide anion radical based on electron paramagnetic resonance (EPR) spectroscopy, a role for other free radicals such as hydroxyl is largely based on theoretical grounds. In situations where direct EPR evidence of free oxyradicals cannot be obtained, usually because of the low equilibrium levels present resulting from their reactivity, the use of spin traps (chemicals which form relatively stable adducts with free radicals) has provided the means for investigating the appearance of transient radicals in many biological systems *in vitro*.

EPR spectroscopy permits the detection of radicals in photosystem I and II. The performance of photosystem II has also been investigated through measurements of chlorophyll fluorescence. Hence the combination of EPR and chlorophyll fluorescence provides the means for detecting changes in the photosynthetic process resulting from exposure to air pollutants, and the possibility of determining the mechanisms involved. Since it is impossible to follow changes in EPR signals in excised leaf tissue over several hours because of the appearance of a large signal attributable to tissue wounding that appears after about one hour, the use of EPR in studies of the effects of pollutant exposures over several hours limits such studies to species with narrow, intact leaves such as the grasses.

Although numerous studies have reported effects of O_3 on the growth of species with C-3 and C-4 types of photosynthesis, little is known of the ways in which the different photosynthetic mechanisms are involved in such responses.

The objectives of this exploratory study were therefore;

a) to determine the overall effects of various sequential exposures to NO_2 and O_3 on plant growth, chlorophyll fluorescence and endogenous ethylene production, using exposures to rising and falling pollutant levels simulating the situation in ambient air;

This led to the following hypotheses:

1. O_3 (120ppb max.) would: reduce growth, reduce photosynthesis (based on fluorescence) and increase ethylene production.
2. NO_2 (120ppb max.) would: increase growth, have no effect on photosynthesis, and increase ethylene production.
3. O_3-NO_2 sequences would: reduce growth (synergism), possibly reduce photosynthesis, and increase ethylene production.

b) to elucidate the effect of the sequences of NO_2 and O_3 on photosystem functioning by means of EPR measurements of free radical intermediates in photosystems I and II, and chlorophyll fluorescence transient kinetics;

This led to the following hypotheses:

1. O_3 (120ppb max.) would: increase photosynthetic free radical signals, and reduce photosynthesis (based on fluorescence).
2. C3 and C4 species would show similar responses.

c) and, to use selective infiltrated spin traps to attempt to detect the presence of pollutant-induced free radicals in intact leaves.

This led to the following hypothesis:

1. O_3 would give rise to $OH^{\cdot}$ and $O^{\cdot}_2$ which would be revealed as spin adducts.

Because of time limitations largely related to ongoing problems with the reliable operation of the EPR spectrometer, the scope of the studies reported had to be limited to work with radish in meeting objective a, and to work on the responses of a C-3 and a C-4 grass to O_3 alone in meeting objectives b and c.

2. LITERATURE REVIEW

2.1 Origin of atmospheric O_3 and NO_X

2.1.1 The formation of atmospheric O_3

Schoenbein, De la Rive, Houzeaum and Soret discovered ozone in the 19th century (Chameides and Lodge, 1992). It exists in both the troposphere and the stratosphere. Stratospheric O_3 protects the earth from harmful ultraviolet (UV) radiation from the sun. Chapman (1930) first identified the series of reactions that maintain stratospheric O_3. In the Chapman mechanism, O_3 is produced as a result of UV photolysis of oxygen,

$$O_2 + h\nu \rightarrow O + O \qquad (R1)$$

followed by a reaction between atomic and molecular oxygen

$$O + O_2 (+M) \rightarrow O_3 (+M) \qquad (R2)$$

(M is a molecule like N_2 required to stabilize the reaction but does not actively participate in the chemical reaction) (Chameides and Lodge, 1992). In the Chapman mechanism, O_3 loss is achieved by the following reactions:

$$O_3 + h\nu \rightarrow O + O_2 \qquad (R3)$$

$$O + O_3 \rightarrow 2O_2 \qquad (R4)$$

The rate constants of the reactions R1 to R4 lead to the establishment of an O_3 concentration equilibrium. Additional gas-phase reactions occur in which hydrogen oxides, nitrogen oxides and especially halogenated hydrocarbons may catalyze O_3 loss (Molina and Rowland, 1974).

Both natural processes and human activities govern the concentration of tropospheric (ambient air) O_3 in polluted and unpolluted environments. Some O_3 is transported into the troposphere from the stratosphere and other complex photochemical reactions contribute to the low O_3 level in the free troposphere, typically about 50 ppb. In polluted air, additional O_3 is produced by the "photochemical smog" reactions summarized in reactions R5, R6 and R7:

$NO_2 + hv \rightarrow NO + O$ (R5)

$O + O_2 \rightarrow O_3$ (R6)

$O_3 + NO \rightarrow O_2 + NO_2$ (R7)

The mechanisms by which NO and NO_2 are involved in these reactions with oxygen, hydrocarbons and a number of oxygenated hydrocarbons are very complex and not completely understood and involve several extremely reactive free radicals (Fig. 2.1).

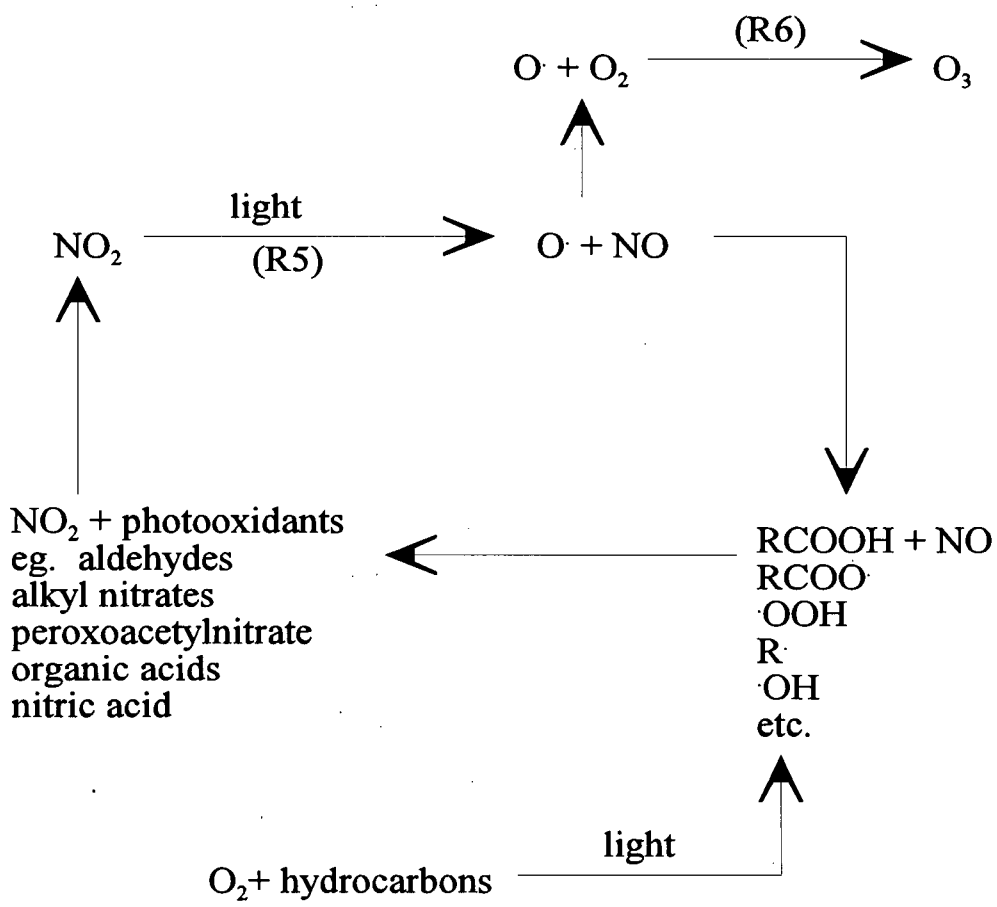


Fig. 2.1 Ozone formation in the troposphere (Weise, 1985).

A key feature of the reactions in polluted air is that the natural scavenging of O_3 by NO (reaction R7) is reduced by the photochemical reactions of NO with organics and other radicals, permitting the level of O_3 to increase. The overall processes involved are summarized diagrammatically in Fig. 2.2.

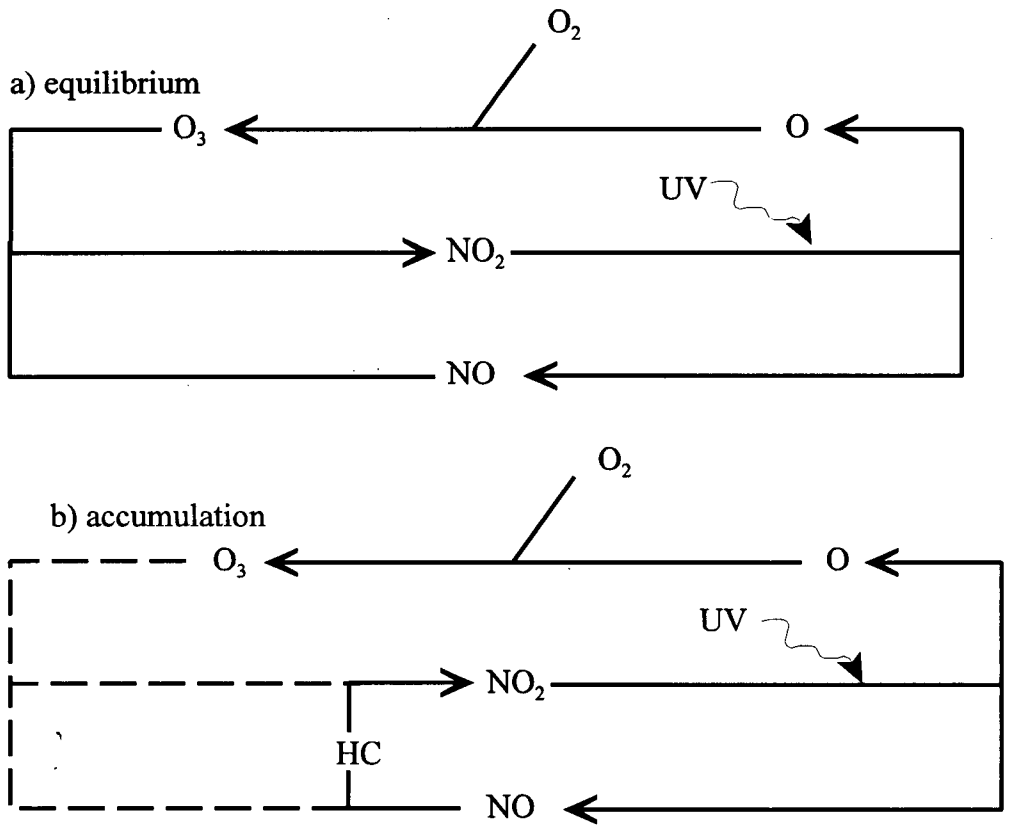


Fig. 2.2 Diagrammatic summary of formation of O_3 in A, normal, and B, air polluted with oxides of nitrogen (NO, NO_2) and hydrocarbons (HC).

As a consequence of these complex reactions, concentrations of NO_X, O_3, aldehydes, peroxyacyl nitrates, alkyl nitrates and nitrites (arising from reactions involving volatile hydrocarbons) may be elevated to levels that are detrimental to both plant and animal health. Since the process of O_3 formation is photochemically driven, it ceases at night and the O_3 level is reduced largely by scavenging by NO. As a result, in areas subjected to anthropogenic emissions, the ambient O_3 level

follows a pronounced diurnal cycle, with its maximum in the early afternoon. At night the O_3 level typically falls to levels below those found in air not subjected to anthropogenic pollutants, because of residual scavenging by NO and other reactions.

2.1.2 The origins of atmospheric NO_2

Of the numerous oxides of nitrogen only NO and NO_2 are important as pollutants and are designated NO_X. Although nitrous oxide (N_2O) has significant concentrations even in the natural unpolluted atmosphere, it is not classified as a pollutant since it is the consequence of many natural chemical and biological processes. The other oxides of nitrogen that can occur in polluted atmospheres, although at low concentrations, are nitrogen trioxide (symmetrical), asymmetrical nitrogen trioxide (O-O-N-O), dinitrogen trioxide (N_2O_3), dinitrogen tetroxide (N_2O_4; the dimer of NO_2) and dinitrogen pentoxide (N_2O_5).

The bulk of the nitrogen oxides in the troposphere is biologically produced NO (Robinson and Robbins, 1970). Bacteria produce global amounts in excess of 50×10^7 tonnes year^{-1} while human activities (internal combustion engines, boilers, incinerators, etc.) produce 5×10^7 tonnes year^{-1} (Taylor et al., 1975). The nitrogen in NO_X may come from the fuel (e.g. coal) or from ambient air. At high combustion temperature nitrogen and oxygen react and establish the equilibrium NO_X concentration:

$$N_2 + O_2 \rightarrow 2NO$$

$$2NO + O_2 \rightarrow 2NO_2$$

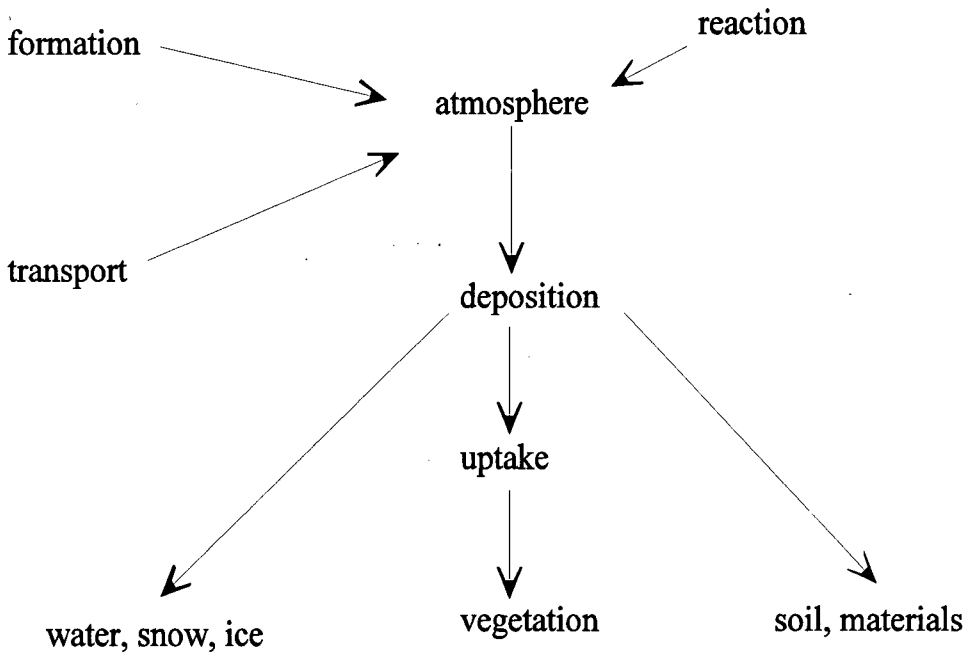
Exhaust gases contain both NO and NO_2, with NO predominant. Nitric acid manufacturing also produces NO from the reaction of ammonia and oxygen:

$$4NH_3 + 5O_2 \rightarrow 4NO + 6H_2O$$

Natural scavenging processes may reduce excessive build-up of NO and NO_2 but these do not, under normal conditions, counteract emissions from bacterial activity, motor vehicles, chemical plants and the high temperature combustion of fossil fuels.

2.2 Uptake of gaseous pollutants by plants

Runeckles (1992) presented the following general scheme for the occurrence and removal of pollutant gases from the troposphere:



In a given air mass, O_3 or NO_X enters through various routes and exits via deposition. Although O_3 and NO_X are deposited on soil surfaces (Macdowall, 1974; Turner et al., 1974) plant uptake is a major sink for pollutant deposition.

In order to affect the internal structure of the leaf (necrotic injury) or its physiological processes, phytotoxic gases must react on the leaf surface or be taken up by the leaf as part of the normal gas

exchange process. Normal gas exchange in plants involves outflow of water vapour, uptake of carbon dioxide required for carbon fixation by the dark reactions of photosynthesis, release of oxygen from the Hill reaction of the same process, uptake of oxygen for respiration and subsequent release of carbon dioxide and water as by-products of respiration. In the presence of air pollutants the plant has little choice but to take them up by the same processes of diffusion, sorption and mass flow that govern the uptake of the gases that it requires for normal functioning.

Since the leaf is the main organ of gas exchange, it is important in the uptake of O_3, NO_X and other air pollutants. As suggested by Runeckles (1992), leaf anatomy and physical conditions together give a wide range of possible outcomes for the O_3 in contact with the leaf:

(a) entry into the cuticle and possible reaction with some of the constituents of leaf cuticle,

(b) entry into the epidermis via the cuticle,

(c) possible reactions with the epidermal cells or further movement into the interior,

(d) entry into the internal leaf air spaces via the stomata,

(e) reactions with the gases in the spongy, palisade or substomatal air spaces,

(f) reaction on the mesophyll or epidermal cells after sorption and partition into the liquid phase,

(g) movement through the cell wall to the plasma membrane, and

(h) movement of O_3 and/or its products across the membrane into the cell and cell organelles.

The same range of possibilities exists for NO_2. Of the various processes listed, regulation by the stomata (d) plays a major role in the uptake of both O_3 and NO_2.

The uptake of O_3 or NO_2 can be viewed as a pollutant flux given by:

$$F = v.\Delta C$$

where F, is flux in g $m^{-2}s^{-1}$ units; v is the deposition velocity in m s^{-1} units; ΔC is pollutant gradient in g m^{-3} units (Smith, 1984). The reciprocal of deposition velocity is resistance (s m^{-1} units).

Resistance to pollutant influx can be separated into a number of components: leaf boundary layer resistance (r_a); surface and cuticular resistance (r_c); stomatal resistance (r_s) and finally, mesophyll or internal resistance (r_m). The nature of r_a, r_c, and r_s is well understood and has been covered comprehensively by Unsworth et al. (1976). Although not a physiologically definable parameter, mesophyll resistance is obviously important in determining the passage of the active species to their ultimate sites of action within the cell (Runeckles, 1992; Heath, 1994). In spite of the major role of stomata, Garsed (1982) has also provided evidence to demonstrate the importance of mesophyll resistance in determining uptake:

(a) different species with similar stomatal resistance do not take up gases in similar proportions;

(b) differences in stomatal resistance alone fail to explain differences in flux between plant species (e.g. Klein et al., 1978) and;

(c) it is not possible to predict the rate of uptake of gases by using purely physical parameters such as solubility.

It is likely that in coming in contact with the mesophyll cells pollutants dissolve in the water film on the apoplastic cellular surfaces. Ozone dissolves at 0.012 mmol/cm^3 (Bennett et al., 1973a) while NO_2 is highly soluble and readily decomposes. In spite of Garsed's caveat about solubility, the steady state uptake of O_3, NO_2 and, NO by an alfalfa canopy has been estimated as 100, 120 and 10 µl ppb^{-1} min^{-1} m^{-2} respectively (Bennett and Hill, 1973), partly reflecting the different solubility of

the gases. These estimates are in keeping with the observations of Neubert et al. (1993). They found that uptake of O_3, NO_2 and NO were linearly dependent on stomatal conductance in sunflower (*Helianthus annuus* L.) and tobacco (*Nicotiana tabacum* L.), but mesophyll resistance limited that uptake of NO. Furthermore, the proportionality of mesophyll to stomatal resistance indicated that metabolic processes rather than solubility determine NO uptake.

Dry deposition on dry surfaces is important for both O_3 and NO_X and may be enhanced by surface wetness (Fuentes and Gillespie, 1992). Wet foliage is a major sink for NO_2 (Wellburn, 1990). Penetration of O_3 deeper into the interior spaces is unlikely due to its low solubility (Taylor et al., 1988) and its high reactivity (Runeckles, 1992; Heath, 1994). Estimations of the O_3 concentration within leaf air spaces done by Laisk et al. (1989) suggested virtually no accumulation of gaseous O_3 in the leaves of sunflower and *Perilla ocymoides* L.

2.3 General considerations of plant exposure to gaseous pollutants

Pollution caused by gases such as O_3 and NO_2 is identified as such because of harmful effects on vegetation, human health and welfare, and materials. Studies aimed at determining the types and magnitude of these effects should involve exposures, i.e. the combination of pollutant concentrations and their duration, that resemble those found to occur or projected to occur in a given location. However, in exploratory studies, particularly those focusing on elucidating mechanisms, artificial exposure regimes and conditions may be appropriate, provided that extrapolation to the "real polluted world" is made with caution.

Natural exposures to O_3 and NO_2 are episodic, and involve concentrations that vary month-to-month, day-to-day, hour-to-hour and even minute-to-minute as a result of changing meteorological and other conditions. Although pollutant levels in ambient air may be described by collapsing the time intervals

and calculating mean concentrations, such averages tend to be regarded as actual concentrations rather than mathematical summaries. Such averages eliminate the stochasticity of the data from which they were obtained.

Nevertheless, averages have their uses in ambient air monitoring; collapsing continuously collected data into 30 min or 1 hour averages is widely accepted as a means of condensing the data into a more usable form. The use of such averages for each hour of the day over a season or year is, for example, justified in demonstrating the diurnal profile of rising and falling O_3 concentrations in an average day.

For reasons of experimental simplicity, many studies of the effects of pollutants such as NO_2 and O_3 have used exposures to steady concentrations of defined duration, so-called square-wave exposures, which may or may not be repeated daily. Although such exposures were the rule in most early studies, many studies undertaken since the early 1980s have utilized exposure profiles that more closely resemble those occurring in polluted ambient air. This has been particularly true of many field studies in which enrichment of the air in a field chamber with a particular pollutant in proportion to the level in the ambient air (or its selective removal by filtration) provides a range of exposure treatments all having the same periodicity as the ambient air. This approach was used with open-top field chambers in the large U.S. National Crop Assessment Network Program (Heck et al., 1989). Nevertheless, square-wave exposures continue to be used in many investigations, which together with their use in earlier work make it difficult to interrelate effects of different exposure regimes quantitatively. Some recent "laboratory" studies have used exposure profiles involving diurnal fluctuations (Bicak, 1978; Dann and Pell, 1989; Bahl and Kahl, 1995; Wellburn and Wellburn, 1996) that more closely simulate ambient conditions.

Differences in the possible response to square-wave or fluctuating exposures to O_3 were investigated by Musselman et al. (1983). They found a significant difference in the adverse effects on the growth of bean (*Phaseolus vulgaris*) between the two types of exposure if the total daily exposures were the same and the exposures were of the same duration. Since this required the peak concentration in the fluctuating exposure to be double that of the square-wave concentration (the exposures were of the same duration) the results pointed to the importance of the peak rather than the average concentration in causing the effect. However if the levels and total exposure were the same (obtained with a longer duration of the fluctuating exposure), no significant effects were observed (Musselman et al., 1986), again illustrating the dependence of response on the maximum concentration reached during the exposure period.

Concerns over the difference in response to "sudden" rather than gradually increasing exposures are justified when integrative effects of repeated exposures in processes such as growth are of interest, or when comparing effects based on different exposure regimes over time. As discussed below, pollutants such as O_3 and NO_2 have to be taken up by leaves in order to result in effects, and the main route of entry is through stomata. Regardless of any effects on stomata *per se*, the way in which the pollutant stress builds up during the exposures will be markedly different between the different regimes (Runeckles, 1992). Square-wave profiles provide "sudden" exposure that ignores the possibility of short-term acclimation. While short-term square-wave exposure may be appropriate for elucidating details of mechanism, the use of concentrations larger than say, double those that are likely to be experienced in ambient air leads to observations that may be of little relevance. Furthermore, the use of different types of exposure regimes with different peak or steady-state concentrations requires caution in making comparisons between the observations made, and in attempting to arrive at a true understanding of the responses and the mechanisms involved. This caveat therefore has to be borne in mind in any review of literature of responses to O_3 and NO_2.

2.4 Plant response to O_3 and NO_2

2.4.1 Plant responses to O_3

2.4.1.1 Injury, growth and yield

Early work on the effect of air pollutants on plants in California reported flecking and necrosis of plant leaf tissue to be caused by some constituent(s) of air (Haagen-Smit et al., 1952). Later, necrosis, chlorosis, and/or flecking of the adaxial leaf surface became widely accepted as the visible injury symptoms of O_3 injury (Heath, 1975). The palisade cells of the leaf are normally the first to show symptoms (Dugger and Ting, 1970a, b; Evans and Ting, 1974). The mesophyll is only affected in severe injury (Heath, 1975).

The injury caused by a toxic substance can be described as acute or chronic. Acute injury results from exposure to a high concentration of a pollutant lasting a short period of time (minutes, hours) the symptoms of which usually become visible in 1-3 days. Acute injury caused by O_3 usually manifests itself as visible necrosis, frequently with associated chlorosis and epinastic curvatures of leaves and leaf stalks often followed by rapid leaf, flower or fruit abscission if the level of injury is severe. The necrotic areas are typically small (<2 mm diameter) discrete bleached flecks on the adaxial leaf surface, although in some species the flecks may range in color from tan to dark brown. In contrast, chronic injury results from continuous or repeated exposures to low concentrations of a pollutant, lasting a longer period of time (weeks, months) the symptoms of which may only become visible after several weeks. Chronic injury may appear as leaf discoloration, bronzing of the adaxial surface, stippling and/or leaf tip necrosis and lead to various disturbances of normal growth and development.

Exposure to O_3 has been found to lead to decreased growth and yield (Heagle et al., 1988) regardless of whether the exposure was sufficient to induce visible injury or not. Root growth is normally reduced more than shoot growth. Increased shoot/root (S/R) dry matter ratios have been shown in numerous reports. Miller (1987), Cooley and Manning (1987) compiled lists with a total of 26 examples of such S/R increments in 21 different crops.

The potential for the impact of O_3 varies throughout the growth of a plant. Cooley and Manning, (1987) carried out numerous studies with soybean (*Glycine max*) and found that O_3 reduced overall growth, caused increased partitioning to leaves during early growth, and reduced seed set. In tomato, Oshima et al. (1975) found fruit development less adversely affected by O_3 than the growth of vegetative parts. In peppers (*Capsicum annuum*; Bennett et al., 1979) and cotton (*Gossypium* spp. Oshima et al., 1979) the reproductive growth was severely inhibited by exposure to O_3.

The appearance of foliar symptoms of injury does not necessarily lead to decreases in measurable growth or yield. It has been shown in soybean (Tingey et al., 1973), tomato (*Lycopersicon esculentum*; Oshima et al., 1975; Oshima et al., 1977), alfalfa (*Medicago sativa*; Tingey and Reinert, 1975), annual ryegrass (*Lolium multiflorum*) and clover (*Trifolium repens*; Bennett and Runeckles, 1977), spinach (*Spinacia oleracea*; Heagle et al., 1979c), and wheat (*Triticum aestivum*; Heagle et al., 1979a), that foliar-symptom production is not closely correlated with reduction in growth or yield.

2.4.1.2 Photosynthetic gas exchange

Studies based mainly on the effect of O_3 on net photosynthesis (PN) have shown that O_3 inhibits photosynthetic gas exchange. The degree of inhibition is dependent on the O_3 concentration, duration of exposure to O_3, and the species or cultivar studied (Runeckles and Chevone, 1992). The effect of O_3

is also modified by light, humidity, ambient CO_2 concentration, other gaseous pollutants and other biological factors such as leaf age, plant water status and respiration rate.

In broadbean (*Vicia faba*), CO_2 exchange was reduced with full recovery only during O_3 episodes at low levels while at levels greater than 200ppb (for 4 hours) there was no recovery (Black et al., 1982). In poplar (*Populus* spp.), exposure to 180ppb for 3 h led to a reduction in photosynthetic rate (Gupta et al., 1991). Farage et al. (1990) observed reduced light-saturated rate of CO_2 uptake at 200-400ppb in wheat. Reduced photosynthesis with increasing O_3 concentration (50, 100, 150ppb) in radish (*Raphanus sativus*) and turnip (*Brassica rapa*) was reported by Hassan et al. (1994) while Barnes and Pfirrmann (1992) reported similar decreases in radish at 73ppb. Runeckles and Chevone (1992) presented a sampling of results obtained using a variety of techniques and indicated that regardless of the type of measurement (O_2-evolution, PG, PN) O_3 reduced photosynthesis in many crop species. Further, Darrall (1989), in a review, categorized O_3 effects into those caused by short term (< 1 day) exposure and those caused by long-term (> 1 day) exposure. In general the short-term exposures were characterized by high pollutant concentrations. Out of 20 examples there was one at 100ppb, two at 200ppb and the rest were in the range 299-720ppb. In the long-term exposure group, concentrations were more like the ones experienced in real world situations, ranging from as little as 27ppb to 150ppb. In both categories she presented numerous examples of O_3 reducing photosynthesis.

The effects of O_3 on photosynthetic electron transport and carbon assimilation are presented in Sections 2.5.3 and 2.5.4.

2.4.1.3 Stomatal response

The effect of O_3 on stomatal behavior is not well defined. Darrall (1989) concluded that stomatal opening tended to occur at low concentrations and closure occurred at injurious concentrations. At

concentrations lower than 200ppb she listed no change in one species, opening in two and closure in four. At concentrations above 200ppb stomatal closure was the normal response. In addition to the examples in Darrall (1989) there are at least eight more reports of closure and one of opening. In oats (*Avena sativa*) Hill and Littlefield (1969) reported reduced transpiration and stomatal closure. Closure was also reported by Barnes et al. (1988), Barnes and Pfirrmann (1992), Salam and Soja (1995), Natori and Totsuka (1984), Grimm and Furhrer (1992), Olszyk and Tingey (1986), and Farage et al. (1990) and opening by Hassan et al. (1994).

Engle and Gabelman (1966) studied two cultivars of onion, one tolerant and the other susceptible to O_3, and found that tolerance could be explained by the rapid stomatal closure of the tolerant cultivar. Butler and Tibbetts (1979) drew similar conclusions from work with bean cultivars exposured to 134 ppb O_3 for 1h.

A clear role for stomatal closure as a response to O_3 is made difficult by the effect of other factors particularly on long-term response. Among these are CO_2 concentration, leaf water status, fluxes of ions (especially K^+) and water (Heath, 1994), and regulation by such hormones as abscisic acid and indoleacetic acid. The complex interaction of these factors makes it difficult to differentiate between direct O_3 effects on the guard or subsidiary cells, and indirect responses such as those caused by the influence of internal CO_2 on photosynthesis (Winner et al., 1988). Further, stomatal response to a single exposure has been shown to depend on the previous exposure history (Runeckles and Rosen, 1976; Hassan et al., 1994).

2.4.1.4 Other physiological processes

Ozone has been shown to affect a host of other important physiological processes such as respiration, transpiration, reproduction, solute leakage, ethylene production and secondary metabolism.

The review by Darrall (1989) concluded that respiration, in general, increased with O_3 above a threshold concentration. Pell and Brennan (1973) reported a stimulation of dark respiration by O_3 (250-300ppb) with or without visible injury in Pinto bean (*Phaseolus vulgaris* cv 'Pinto'). Such increases have been reported in tobacco callus cultures (Anderson and Taylor, 1973). Amthor (1988) partitioned specific respiration rate into a growth and a maintenance component and found that O_3 had no effect on the growth component but increased the maintenance component by up to 25% relative to the control. Amthor and Cumming (1988) also reported similar results.

Ozone -induced decreases in transpiration may affect water-use efficiency (WUE). Greitner and Winner (1988) reported an increase in WUE in soybean, but reductions in WUE have also been reported (Temple and Benoit, 1988; Reich et al., 1985).

Reproductive yield of many crops is decreased by O_3. Changes in dry matter partitioning, reduced floral initiation and development and impaired fertilization have been reported in wheat (Heagle et al., 1979a), corn (Heagle et al., 1972, 1979b) and, soybean (Heagle and Letchworth, 1982). Feder (1968), and Harrison and Feder, (1974) noted inhibition of pollen germination and pollen tube growth in tobacco (*Nicotiana tabacum*), and Feder (1970) observed a decrease in floral initiation and development in several species exposed to O_3.

For years, it has been thought that O_3 may cause plant injury by altering cell membrane permeability and solute leakage. This is because it has been shown that O_3 may modify amino acids, proteins,

unsaturated fatty acids, and sulfhydryl residues in the plasmalemma (Heath et al., 1975). Isolated membrane fractions from bean leaves (mixed plasmalemma, endoplasmic reticulum, tonoplast fractions) pretreated with O_3 had an increased ATP-dependent uptake of $^{45}Ca^{2+}$. Vesicles showed a much greater passive release (leakage) of Ca^{2+} into the medium than those from control plants, indicating a breakdown in the ability of the membranes to maintain the normal free Ca^{2+} balance (low levels in the cytoplasm vs. high levels in the cell wall) (Heath and Castillo, 1988). Thus O_3 may cause an increase in free Ca^{2+} in the cytoplasm and this imbalance may disturb the normal functioning of scavenger systems for O_3 and its products.

Barnes et al. (1988), Beckerson and Hofstra (1980), Dijak and Ormrod (1982) and McKersie et al. (1982) found O_3 -induced increases in the ability to leach a range of ionic and nonionic solutes from pretreated leaves. Dugger and Palmer (1969) showed that after exposure to O_3 the permeability of lemon (*Citrus* spp.) leaf discs to radioactive glucose rose about 2-fold during the days following exposure (max. 6). Evans and Ting (1974) used ^{86}Rb tracer fluxes and observed increased solute permeability of bean plant cell membranes. Evans and Ting (1973) found increased O_3-induced water permeability as well as leakage of solutes out of the leaf discs pretreated with 300ppm O_3 while net water loss seemed to occur immediately upon O_3 exposure.

Perchorowicz and Ting (1974) repeated Dugger and Palmer's (1969) work but with *Phaseolus vulgaris* with the same results. In addition they showed that there was no change in permeability until several hours after the end of the exposure but it then rose uniformly to a 3-fold increase after 24 hours.

Ozone injury has also been reported to induce ethylene production (Craker, 1971). Tingey et al. (1976) found a positive correlation between stress ethylene release and plant sensitivity to O_3. Haagen-Smit et al. (1952) and Adepipe and Ormrod (1972) treated plants with ethephon or ethylene and observed

enhanced O_3 injury in a number of species. Mehlhorn and Wellburn (1987) showed that inhibiting ethylene biosynthesis with aminovinylglycine (AVG) could drastically reduce O_3-mediated leaf injury. Numerous reports have shown O_3-induced increases in ethylene release (Mehlhorn et al., 1991; Langebartels et al., 1991; Bae et al., 1996; Wellburn and Wellburn, 1996). However, in all of these studies the exposures resulted in necrotic injury. There appears to have been no reports of O_3-induced stimulation of ethylene release in the absence of visible injury to the leaf tissue.

2.4.2 Plant responses to NO_2

2.4.2.1 Injury, growth and yield

Acute NO_X injury first appears as water-soaked lesions which later turn white, tan or bronze in color. The lesions may be marginal and tend to be near the leaf apex. Chronic effects are characterized by enhanced green color, followed by chlorosis and massive leaf drop. The overall symptomatology is influenced by a host of factors such as the genotype of the variety or cultivar, plant environment (light, temperature, water status, mineral nutrition, humidity), pollutant concentration, exposure duration, developmental stage, and others (Wellburn, 1990).

Examples of NO_2 injury or NO_2-induced reduction in growth and yield are few and largely of academic significance because of the extremely high concentrations applied. In short-term exposures, NO_2 concentrations greater than 3760ppb are needed to cause injury while 940ppb (10 times ambient) may cause visible injury in long-term exposure (Shriner et al., 1990). These thresholds for acute injury were based on the work of Middleton et al. (1958) on Pinto bean (*Phaseolus vulgaris*).

At the low end of the scale of exposures, treatment of Cherry Belle radish with 282ppb or 376ppb for 3h daily for up to 9 days produced no foliar injury nor changes in biomass accumulation nor partitioning (Reinert and Gray, 1981; Reinert and Sanders, 1982; Sanders and Reinert, 1982). Godzik

et al. (1985) reported not finding any effect on leaf or hypocotyl dry weight in radish exposed to 200ppb NO_2 for 20h. However, Sandhu and Gupta (1989) observed increased foliar N, overall growth and yield with long-term daily exposures to 'real world' levels. Decreased plant growth and leaf distortion, but increased total chlorophyll content were observed in pinto bean and tomato seedlings exposed to 500 ppb NO_2 for 10 to 22 days (Taylor and Eaton, 1966). In treatments with NO_2 concentrations ranging from 60 to 1000ppb applied for 35 days to navel orange trees, chlorosis and extensive defoliation occurred only at 500 and 1 000 ppb (Thompson et al., 1970). Greater injury has been reported from nighttime fumigation than fumigation during the day and Taylor (1968) found that 3000ppb NO_2 in darkness caused as much injury as 6 000ppb in light.

In contrast, several studies used concentrations as high as 250 000ppb (250ppm) and focused on injury and defoliation. In an early study, Benedict and Breen (1955) reported discoloration caused by cell collapse and necrosis in 10 annual perennial weeds exposed to the extremely high concentrations of 20 000 to 50 000ppb NO_2. However, in several species, young leaves were little affected. Other studies such as those of MacLean et al. (1968) and Czech and Nothdurft (1952) used concentrations as high as 250 000ppb (250ppm), at which most species were injured.

At low levels NO_2 can have a fertilizer effect on plants as confirmed by Zeevaart, (1976), Runeckles and Palmer (1987) and others. The presumption is that dissolved NO_2 is assimilated through the NO^-_3 assimilation pathway:

$$NO_X \rightarrow NO^-_3 \rightarrow NO^-_2 \rightarrow NH^-_4 \rightarrow \text{amino acids} \rightarrow \text{proteins}$$

(Malhotra and Khan, 1984), for which confirmation has been provided by Zeevaart (1976), Yoneyama and Sasakawa (1979) and Kaji et al. (1980). The outcome of exposure to NO_2 of a particular species appears to be a consequence of the beneficial effects of low concentrations being offset by the harmful effects of high concentrations (Wellburn, 1990).

2.4.2.2 Photosynthetic gas exchange

Photosynthesis is much less sensitive to the presence of nitrogen oxides than other pollutants. Hill and Bennett (1970), and Capron and Mansfield (1976) were only able to detect inhibition at the high levels of 500-700ppb NO_2 in short-term fumigation (<8hrs) or at 250ppb (over 20h). NO was reported to be four times as inhibitory as NO_2 at 1 000ppb in a four day fumigation of several greenhouse species (Saxe, 1986). The NO_2 threshold for inhibition of photosynthesis in alfalfa and oats was reported to be about 500 ppb (Hill and Bennett, 1970).

Decreased photosynthesis may be detected in the absence of visible injury in exposures to combinations of NO and NO_2 (Hill and Bennett, 1970; Capron and Mansfield, 1976). The two gases interact additively with the NO effect being faster than the NO_2 effect (Hill and Bennett, 1970). Recovery of photosynthesis was reported to occur on return to clean air (Hill and Bennett, 1970). Srivastava et al. (1975) also showed decreased apparent photosynthesis in bean exposed to 1 000-7 000ppb NO_2.

2.4.2.3 Stomatal response

Little is known about the effects of NO_2 on stomata. Yoneyama et al. (1979) and Kaji et al. (1980) observed that more labeled nitrogen dioxide ($^{15}NO_2$) was absorbed during the day than at night, and inferred that NO_2 uptake was dependent on stomatal aperture. Srivastava et al. (1975) reported NO_2-induced inhibition of transpiration supposedly resulting from partial stomatal closure. Sandhu and Gupta (1989) observed closure at concentrations as low as 25ppb (7h/d, 5d/wk for 3wks). Natori and Totsuka (1984) reported decreased transpiration in *Euonymus japonica* (100ppb for 2-3h). van Hove et al. (1992) reported closure in douglas fir (*Pseudotsuga menziesii*) while Sabaratnam et al. (1988b) observed a tendency for stomatal closure in soybean (*Glycine max* L.) (100ppb 7h/d for 5d). Carlson (1983) also observed closure with soybean although at somewhat higher exposure concentrations.

2.4.2.4 Other physiological processes

Increased NO_2 levels inhibited dark respiration in primary leaves of bean (Srivastava et al., 1975) and in soybean (Carlson, 1983) exposed to 600 ppb for up to 3h. However, at lower levels respiration was slightly stimulated in soybean (200ppb for 7h/day for 5 days) (Sabaratnam et al., 1988b) and in beans (100ppb 7h/day for 15days) (Sandhu and Gupta, 1989). A significant NO_2-induced increase in ethylene caused by short-term exposure (150ppb) has been observed in pea (*Pisum sativum* L.) without the appearance of foliar injury (Mehlhorn and Wellburn, 1987).

2.4.3 Plant response to NO_2-O_3 mixtures and combinations

Despite the fact that NO_2 and O_3 are both constituents of photochemical oxidant pollution, few studies have been made of plant response to their combined effects in sequential exposures although it is quite clear from these studies that the effects of the two gases in sequence may be very different from their individual effects.

Runeckles and Palmer, (1987) applied more realistic concentrations (100ppb) when they studied the effects of $NO_2 + O_3$ sequences on the growth of wheat (cv. Sun), bush bean (cv. Pure Gold Wax), and radish (cv. Cherry Belle). In wheat and radish, NO_2 applied from 09:00 to 12:00h sensitized the plants to 100ppb O_3 applied from 12:00h to 18:00h. NO_2 alone was stimulatory while the sequence decreased dry matter accumulation in leaves and roots even more than O_3 alone. There was a marked reduction in radish hypocotyl growth. In bean the responses were additive with the stimulation caused by NO_2 reducing the inhibition caused by O_3.

Bender et al. (1991) also working with bean in a field study found that NO_2 alone increased leaf dry weight and total shoot biomass until anthesis and NO_2-O_3 sequences resulted in stimulated total shoot biomass. Ozone alone tended to suppress growth during anthesis. In general, the effects of NO_2 were

counteracted by O_3 with the result that only the $NO_2 + O_3$ effect in stimulating biomass at anthesis was significant ($P<0.01$). Also in a field study, Adaros et al. (1991) reported that O_3 reduced but NO_2 had no effect on the growth of rape (*Brassica napus* L.). However, with sequences of NO_2 and O_3, the adverse effects of O_3 were largely countered by NO_2 and in some cases led to stimulations.

Goodyear and Ormrod (1988) reported that concurrent exposure to 210 ppb NO_2 and 80 ppb O_3 for 1 hour reduced leaf and stem fresh weight of tomato while individually they had no effect. Although the sequence NO_2-O_3 resulted in no significant differences in dry matter production from the control, the O_3-NO_2 sequence reduced leaf area and fresh weight, and stem fresh weight and dry weight.

Kress and Skelly (1982) studied several tree species and found that $O_3 + NO_2$ suppressed the growth (height) of virginia and loblolly pine, although individually the pollutants had no effect. In sweetgum (*Liquidambar Styraciflua*) accumulation of root and total dry weight was reduced. Ozone + NO_2 suppressed root and total dry weight in sweetgum and had an antagonistic effect on ash root dry weight.

2.5 Cellular mechanisms of response to O_3 and NO_2

2.5.1 Potential for free radical production from O_3

Ozone dissolves in water to give superoxide, peroxyl and hydroxyl radicals (Hoigne and Bader, 1975; Staehelin and Hoigne, 1982, 1985). The process is, however, slow at physiological pH. Calculations by Heath (1987a) indicated an O_3 rate loss of 0.015% min^{-1} at pH 7.0 in a saturated aqueous solution, yielding a steady-state superoxide anion radical concentration of only 8.75×10^{-15} M. Despite this, however, O_3 can react with substrates that yield hydrogen peroxide (H_2O_2) and lead to increased O_2^- levels and subsequent generation of the highly reactive hydroxyl radical ($\cdot OH$), through the Haber-Weiss reaction (R8) (Haber and Weiss 1934):

$$O_2^- + H_2O_2 \rightarrow \cdot OH + O_2 \qquad (R8)$$

Metal catalysts enhance the rate of this reaction as shown in reactions R9 and R10 (Saran et al., 1988).

$$Me^{n+} + O_2^- \rightarrow Me^{(n-1)+} + O_2 \qquad (R9)$$

$$Me^{(n-1)+} + H_2O_2 \rightarrow Me^{n+} + \cdot OH + OH^- \qquad (R10)$$

Given the lifetime and diffusion path length of 1.2 ns and 3.5 nm respectively for ·OH and intracellular dimensions of 5 nm for phosphoslipid bilayers, 7 nm for globular proteins and, 3 nm for the water thickness around biomolecules, Saran et al., (1988) concluded that ·OH radicals could only react with biomolecules close to their site of production. With a longer lifetime (1ms) superoxide, however, has the potential to travel several molecular distances to react with biomolecules well away from its site of production (Runeckles and Chevone, 1992).

Ozone also reacts with several organic molecules to produce free radical intermediates. According to Atkinson and Carter (1984) the most definitive mechanistic data have been accumulated for simple acyclic alkenes like ethylene, propylene, and the 2-butylenes. The initial reaction in the O_3-alkene reaction yields a "molozonide" which decomposes rapidly to a carbonyl and a biradical.

Apart from free radicals, O_3 can react directly with biomolecules to give singlet oxygen. At pH 7.0 the amount of singlet oxygen formed (mole of singlet oxygen/mole of O_3) with different reductants is: NADH, 0.95 ± 0.01; NADPH, 1.07 ±0.07; ascorbic acid, 0.96 ± 0.007; reduced glutathione, 0.33 ±0.02; methionine, 1.13±0.11; and cysteine, 0.49 ± 0.02 (Kanofsky and Sima, 1991). These are for a reaction of O_3 with an equivalent of reductant. Many other organic compunds can react with O_3 to yield singlet oxygen, e.g. sulphides, tertiary amines, sulphoxides, phosphites, sterically hindered olefins, some ethers and some aldehydes (Kanofsky and Sima, 1991). Since singlet oxygen is very reactive and capable of causing chain reactions of lipid peroxidation (Thompson et al., 1987), its production from the above reactions can also cause numerous perturbations within plant cells.

2.5.2 Potential reactions of O_3, oxyradicals and NO_2 with cell components

How O_3, its breakdown products, and NO_2 affect plants at the cellular level is still largely conjecture (Heath, 1994). Since O_3 is a powerful oxidant, it has the potential to react with virtually all cellular components. Indeed, historically O_3 was widely accepted to disrupt metabolic processes by peroxidation and/or ozonolysis of the plasma membrane lipids (Tomlinson and Rich, 1969). This would be followed by increased cell permeability and disruption of the chemiosmoregulatory processes (Chimiklis and Heath, 1975; Sutton and Ting, 1977). Such effects are now thought to represent reactions at extreme O_3 concentrations (>0.5ppm) (Runeckles and Chevone, 1992).

In reviews by Heath (1984) and Mudd (1982) the importance of the reactions of O_3 or its breakdown products with sulphydryl groups on membranes or proteins or oxidation of scavenging compounds was shown to be strong evidence against a primary attack on membrane lipids. *In vitro* experiments have documented the oxidation of peptides (e.g. glutathione), nucleic acids, purine and pyrimidine derivatives, numerous amino acids (especially cysteine, methionine, and tryptophan), lipids, and enzyme proteins (e.g. glyceraldehyde-3-phosphate dehydrogenase, catalase, peroxidase, papain, ribonuclease, and urease) (U.S. Environmental Protection Agency, 1986).

Runeckles and Chevone (1992) gave a summary of the rate constants for the reaction of O_3 with several biological compounds *in vitro*. The sulphydryl-containing tripeptide, glutathione (c-glutamylcysteinylglycine) and cysteine have the highest rate constants: $>1.0 \times 10^9$ $M^{-1}s^{-1}$ at pH7.0. This suggests the vulnerability of sulphydryl-rich peptides and proteins to oxidation by O_3 or its breakdown products. The rate constant of ascorbate is considerably higher (5.6×10^7 $M^{-1}s^{-1}$at pH7.0) than that of polyunsaturated fatty acids (1.0×10^6 $M^{-1}s^{-1}$). Among the least reactive compounds are alcohols, carboxylic acids, amines and amides.

NO_2 reacts with water to form a mixture of nitrous and nitric acids. According to Taylor et al. (1975) this is what probably happens when the gas comes into contact with the wet surface of the spongy parenchyma in leaves. The tissue may be injured if the acid exceeds a given threshold.

At the structural level, Runeckles and Chevone (1992) in reviewing the evidence for the penetration of O_3 rather than its reaction products into the cell, noted the rapidity with which structural changes such as the swelling of chloroplast thylakoids and shrinkage of mitochondria cristae could be observed.

Wellburn et al. (1972) in a study of the effects of NO_2 on the ultrastructure of chloroplasts in bean tissue, found that fumigations up to 3 000ppb NO_2 for 1h led to thylakoid swelling. This was reversible if the NO_2 was replaced by clean air. However, it should be noted that such changes were only observed with exposures to extremely high levels of the pollutant.

2.5.3 Photosynthetic electron transport

Photosynthetic electron transport involves the light-driven transfer of electrons from water through photosystems I and II (PSI and PSII) to $NADP^+$. The ultimate consequence of the process, which involves a large number of intermediate reactants and reactions, is to transform energy from light into a chemical form, which the organism can utilize to drive the synthesis of carbohydrates, nucleic acids, proteins and other biomolecules.

In the first step water is oxidized in the water-splitting/oxygen-evolving complex of PSII to O_2 with the release of four electrons:

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^-$$

The energy required comes from light absorbed by the light-harvesting complex (LHCII) of PSII. The electrons abstracted from water are passed through a series of intermediate electron acceptors via the cytochrome $\underline{b_6}$-$\underline{f}$ complex to PSI then eventually to $NADP^+$ in the so-called "Z" scheme (Fig. 3).

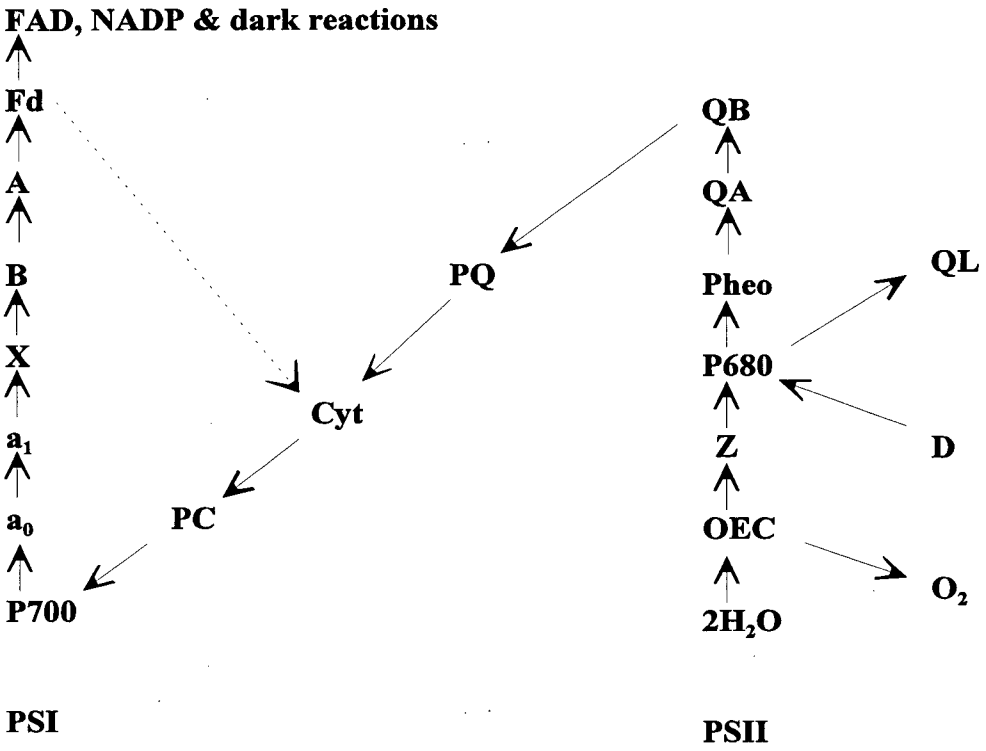


Fig.2.3 The Z scheme adapted from Hill and Bendall (1960).

PSI = Photosystem I; P700 = PSI reaction center chlorophyll a; a_0 and a_1 = unidentified electron acceptors; X = possibly an iron-sulphur electron acceptor; B and A = iron-sulphur centers; Fd = Ferredoxin; FAD = Flavin adenine dinucleotide; NADP = Nicotinamide adenine dinucleotide phosphate; PSII = Photosystem II; OEC = oxygen evolving complex; Z = immediate electron donor to P680; D = an auxillary electron donor to P680; P680 = PSII reaction center chlorophyll a; Pheo = pheophytin; QL = unidentified, maybe an electron acceptor; QA = iron-plastoquinone electron acceptor; QB = second iron-plastoquinone electron acceptor; cyt = cytochrome $\underline{b_6}$-$\underline{f}$ complex; PQ = plastoquinone, and PC = plastocyanin.

Inhibition by O_3 or NO_2 of some of the steps in the photosynthetic process has been observed by a number of workers. The techniques used involved exposure of isolated chloroplasts to pollutants bubbled through the suspension or analyses of chloroplasts isolated from plants pre-exposed to the pollutants.

Sugahara (1982) bubbled O_3 or NO_2 through chloroplast suspensions isolated from spinach (cv. New Asia) and lettuce (cv. Romaine) and measured photosynthetic electron transport by observing the rates of dichloroindophenol (DCIP) and NADP photoreduction and O_2 evolution. Photoreduction of DCIP and water to $DCIPH_2$ in illuminated chloroplasts together with release of O_2 indicates the functioning of PSII while photoreduction of NADP by $DCIPH_2$ indicates that PSI is functioning.

He found that O_3 at 100ppb did not suppress electron transport of either PSI or PSII. At 500ppb, however, the electron transport of both photosystems was inhibited confirming the earlier observations of Coulson and Heath (1974).

Murabayashi et al. (1981) and Suzuki et al. (1982) analyzed chloroplasts from O_3-fumigated spinach leaves and also bubbled O_3 through chloroplast suspensions. In both cases the electron transport of both photosystems was inhibited. NO_2 had little effect on electron transport even at very high concentrations. 4 000ppb NO_2 caused little inhibition of both photosystems after 10h fumigation and slight inhibition after 20h fumigation.

Wellburn et al. (1981) reported that exposure of ryegrass (*Lolium perenne*) to 255ppb NO_2 for 11 days did not affect electron transport in either photosystem but enhanced ATP production. Schreiber et al. (1978) carried out *in vivo* measurements of chlorophyll fluorescent transients in bean leaves exposed to low levels of O_3. They interpreted their results as indicating an O_3 effect culminating in a decrease in

water-splitting activity of the oxygen evolving complex. Shimazaki (1988) confirmed the results using spinach leaves. He also found inhibition of photosynthetic electron transport by high NO_2-fumigation (4 000ppb) during the dark and recovery in light. Barnes et al. (1988) showed a reduction in the electron flow from the water-splitting component of PSII by 75ppbv O_3 in pea leaves.

Studies using electron paramagnetic resonance (EPR) methods have also thrown some light on the effect of O_3 on photosynthetic electron transport. Rowlands et al. (1970) observed enhancement of the typical six-peak Mn^{2+} signal in soybean leaves exposed to 50ppb O_3. This is important considering that Mn plays a vital role in the 'oxygen clock' within the oxygen evolving complex (OEC) of PSII (Yocum, 1987).

EPR can detect the free radical signal I originating from photosystem I. This signal has been identified with the electron donor of PSI, $P700^+$ (Beinert et al., 1962), and resembles the Chl^+ signal (Weaver and Corker, 1977). It has a *g*-value* of 2.0025, a peak-to-peak width of 7.4G, a Gaussian shape and no hyperfine structure that can be revealed by simple EPR methods (Borg, 1976). It is formed rapidly in light and decays instantly in the dark.

Another signal detectable by EPR is signal II, attributable to tyrosine residues in PSII. The tyrosine residue Y_D (Tyr-160 of the D2 protein of PSII) can donate an electron to $P680^+$ and seems not to be involved directly in O_2 evolution (Debus et al., 1988 a and b, Vermass et al., 1988) although Nugent et al. (1987) suggested that it plays a role in stabilizing the OEC. The signal due to Y_D^+ is designated signal II_s (slow), because it decays slowly in the dark. Another tyrosine radical (Tyr-161 of the D1 protein of PSII) (Debus et al. 1988a and b; Vermaas et al., 1988) is Y_Z. P680+ oxidizes Y_Z (on a

*A brief description of electron paramagnetic resonance spectroscopy, in which the parameters used to define spectra are described, is presented in Appendix B.

nanosecond time scale) but the actual scale is S-state dependent (20-200ns) (Nugent et al., 1993). The manganese cluster then rereduces Y_Z^+. Y_Z^+ is responsible for signals II_f and signals II_{vf} (fast and very fast) which decay on a millisecond to microsecond time scale depending on the oxidation state of the manganese cluster (Dekker et al., 1984; Hoganson and Babcock, 1988). The EPR characteristics of Y_Z^+ make its detection difficult *in vivo* because of the persistent Y_D^+ radical which always overshadows it, since both yield identical EPR spectra. Both signals are produced during continuous illumination. In PSII membranes only 25% of the Y_D^+ is rereduced to Y_D in darkness. Thus even after dark adaptation 75% of the radical remains persistently detectable in PSII centers.

Vaartnou (1988) exposed attached Kentucky bluegrass (*Poa pratensis)* leaves to 100ppbv O_3 within the EPR spectrometer cavity and observed changes in some of the above signals. After 3h Signal 1 and Signal II_s disappeared to be replaced by a new signal (*g*-value 2.0041: peak –to-peak width 10 gauss). It is very possible that this indicates disruption of electron flow through PSII, possibly in the same manner that herbicides such as diuron and atrazine affect electron transport (Vermaas et al., 1987).

2.5.4 Photosynthetic carbon assimilation

Two key enzymes are required in the initial capture of CO_2. The enzymes are ribulose-1,5 bisphosphate carboxylase/oxygenase (rubisco) in C-3 plants and phosphenolpyruvate (PEP) carboxylase in C-4 plants. In C-4 species, PEP carboxylase is responsible for CO_2 capture in the mesophyll cells, followed by rubisco capture of the CO_2 or HCO_3^- produced by decarboxylation of C_4 acids exported from the mesophyll, in the bundle sheath cells. Ozone or its reaction products may affect both enzymes culminating in detrimental effects on glucose or starch production, among other processes.

Pell and Pearson (1983) reported O_3 -induced decreases in rubisco in alfalfa leaves. Similar decreases were observed in potato (Dann and Pell, 1989) and radish and poplar (Pell et al., 1992). Bahl and Kahl (1995) found that the mRNA levels for the gene encoding the small subunit of rubisco (rbcS) were rapidly reduced by low levels of O_3. However, they reported that no reduction in activity could be detected in less than 48-72h.

There appear to be no reports of the effects of O_3 on PEP carboxylase. In fact, other than several studies on corn and sorghum, little is known about the effects of O_3 on C-4 plants. However, since H_2O_2 has been shown to activate glucose-6-phosphatase in pea chloroplasts (Brennan and Anderson, 1980), O_3 may cause its activation as a result of H_2O_2 formation via the Haber-Weiss reaction. Koziol et al. (1988) have suggested that phosphofructokinase may be similarly activated. In contrast, glyceraldehyde-3-phosphate dehydrogenase is highly susceptible to O_3 (Mudd, 1982). Any of these changes would disturb the flow of CO_2 to assimilates.

Rubisco was not affected by NO_2 in pea (Horsman and Wellburn, 1975). Malhotra and Khan (1984) reported that *Pinus banksiana* and *Picea glauca* (White spruce) had increased rubisco activity on exposure to 200ppb to 1000ppb NO_2 for two weeks. Exposure to 2 000ppb NO_2 for 48h also increased rubisco activity and, more markedly, that of glycollate oxidase. Since glycollate oxidase is involved in photorespiration they inferred that this implied a drop in net photosynthesis.

Wellburn (1984) noted both increases and decreases in total ATP in leaf tissue from plants pretreated with O_3. Robinson and Wellburn (1983) supplied O_3 to a suspension of oat leaf thylakoid membrane preparations. They noted a progressive decrease in the pH gradient across the thylakoid membrane. This suggested a decrease in ATP synthesis as Heath's (1980) reinterpretation of earlier findings (Coulson and Heath, 1974) confirmed.

2.5.5 Nitrogen metabolism

There have been relatively few studies of the effects of O_3 on N-metabolism *per se* although there are numerous reports of effects on total content and the size of amino acid pools. The early observation that exposure of soybean to 250 ppb O_3 for 2h resulted in a rapid decline in nitrate reductase (NR) activity (Tingey et al., 1973b) was confirmed by later studies such as those of Purvis (1978) who reported that the effect was only observed in a cultivar that was sensitive to O_3. In a field study, Flagler et al. (1987) observed that O_3 exposures led to a modest decrease in NR activity. In broad bean, Agrawal and Agrawal (1990) observed that decreased NR activity could be observed after 10 daily exposures to 98 ppb for 2h, while decreased nitrite reductase (NiR) activity could be detected more rapidly. Tingey et al. (1973b) suggested that the observed loss of NR activity was probably attributable to reduction in the supply of NADPH needed for nitrate reductase.

Although Bender et al. (1991) observed no significant effects of O_3 on NR or NiR in bean, they found a small but significant increase in glutamate dehydrogenase (GDH) activity, while Manderschied et al. (1991) reported a decrease in glutamate synthetase (GS) activity as a result of comparable exposures. Since they and others have reported that O_3 accelerates protein degradation and increases the size of the free amino acid pool, they suggested that the changes reflected the increase in the photorespiratory N-cycle, previously suggested by Ito et al. (1985).

NO_2 has been found to affect many of the enzymes involved in N-metabolism (Wellburn, 1990) including NR, NiR and GS. The levels of NR activity in leaves are determined both by the supply of nitrate and by light.

Zeevaart (1976) demonstrated that exposure to very high NO_2 levels (12 000 ppb) could induce NR activity rapidly. Srivastava and Ormrod (1984) showed that large increases in NR activity in bean leaves only occurred when the supply of nitrate to the roots was low. However, in Scots pine, *Pinus*

silvestris, exposure to 85 ppb NO_2 for 7 days resulted in a more that 20-fold increase in NR, which appeared to be independent of the supply of nitrate from the roots (Wingsle et al. 1987). Evidence that NiR activity is induced by nitrite (the product of NR activity) was found in one tomato cultivar (Ailsa Craig) but not in another (Sonato) (Murray and Wellburn, (1985). Such differences in response to NO_2 have been reported in other species (Wellburn, 1990). However, NiR activity was found to increase significantly in *Lolium perenne* exposed to 250 ppb NO_2 for up to 15 days (Wellburn et al., 1981).

Increases in the levels of GS and in the related glutamine oxoglutarate aminotransferase (GS/GOGAT) following NO_2 exposures up to 500 ppb were found by Srivastava and Ormrod (1984) in bean independent of increases resulting from increased nitrate supply to the roots.

Glutamate can be reversibly deaminated through the activity of glutamate dehydrogenase (GDH), and NO_2 was reported to cause increases in GHD activity in *Lolium* (Wellburn et al., 1981) although Srivastava and Ormrod (1984) were unable to detect any such changes in bean. Wellburn (1990) has suggested that the GS/GOGAT pathway predominates but an excess of glutamate from NO_2-exposed tissue is removed by GDH.

2.5.6 Other metabolic processes

Although exposures to both NO_2 and O_3 have been reported to result in changes to other metabolic processes, most of these observations are inferred from changes in the sizes of the pools of metabolites, or of the levels of specific metabolites such as ascorbic acid, without reference to the intracellular location of such metabolites.

Exposures to NO_2 or O_3 lead to one striking difference. NO_2 tends to enhance the synthesis of chlorophyll while O_3 tends to destroy it. As a result plants exposed to NO_2 frequently appear greener, in contrast to the chlorotic yellowing observed with O_3, which results from the greater sensitivity of chlorophyll than carotenoids to destruction. For example, although Nash (1976) reported reduction in

chlorophyll content in lichens exposed to NO_2, Horsman and Wellburn (1976), reported increased (10%) chlorophyll content in pea exposed to 100ppp NO_2. However Zeevaart (1976) found that NO_2 inhibited pigment synthesis at concentrations above 4 000ppb in several species.

Ozone may also affect secondary metabolism. The effects of O_3 on phenolics have been reviewed by (Runeckles and Chevone, 1992). Howell (1970) reported increases in caffeic acid in bean showing O_3 injury symptoms. Keen and Taylor (1975) exposed soybean to 400-800 ppb O_3 for 2 hours and observed increased levels of the isoflavonoids, coumestrol, daidzein and sojagol. These observations have been inferred to indicate activity of polyphenoloxidases on phenolics released following membrane breakdown (Runeckles and Chevone, 1992).

3. RADISH STUDIES

3.1 Introduction

Ozone is the most important of the gaseous air pollutants phytotoxic to plants. NO_2 is a pollutant likely to be associated with it (Reinert and Gray, 1981) because of the role that NO_2 plays as a precursor of O_3 in polluted air. Since O_3 maxima typically occur during the early afternoon, while NO_2 maxima tend to occur in the morning and evening hours, the response of plants to exposures O_3 cannot be dissociated from daily sequences of exposure to both NO_2 and O_3 under field conditions. Although there are very few studies of the effects of such sequences, it has become clear that the two gases have complicated interactive effects on vegetation. Runeckles and Palmer (1987) showed that pretreatment with NO_2 reduced the impact of O_3 on leaf growth and retention in wheat (*Triticum aestivum* L.) and radish (*Raphanus sativus* L.). Kress and Skelly (1982) reported less than additive interactions between O_3 and NO_2 in sweet gum (*Liquidambar Styraciflua* L.) and white ash (*Fraxinus americana* L.). Goodyear and Ormrod (1988) observed reduced biomass caused by the sequence NO_2-O_3 and no effect of the O_3-NO_2 sequence in tomato (*Lycopersicon esculentum* Hill.).

Much of the early work on the effects of O_3 and NO_2 utilized "sudden" exposures to relatively high steady-state concentrations (>200 ppb) of either pollutant. More recent work has emphasized the importance of using exposures involving concentrations that increase to and decrease from a peak level (Bicak, 1978; Dann and Pell, 1989; Bahl and Kahl, 1995; Wellburn and Wellburn, 1996), since such exposure profiles more closely resemble those occurring in ambient air. Most studies involving open-top field exposure chambers have used exposures to fluctuating O_3 profiles (see Heagle et al., 1988).

If the daily cumulative exposures were equal, Musselman et al. (1986) found no differences in adverse

effects of O_3 on the growth of *Phaseolus vulgaris* L. between square-wave (steady-state) and fluctuating exposures with the same peak concentration. In this study, the daily fluctuating exposures lasted approximately twice as long as the steady-state exposure. However, if the duration of the daily exposure period and cumulative exposures were the same (achieved with peak levels in the fluctuating exposure double those in the square-wave) the fluctuating exposure led to greater adverse effects (Musselman et al., 1983). This work therefore points out the caution needed in making comparison among data obtained with different exposure profiles.

Although there is evidence of significant uptake of NO_2 by plant cuticles (Wellburn, 1990) foliar uptake of both O_3 and NO_2 is largely regulated by stomatal aperture (Neubert et al., 1993). Effects of either gas on diffusive resistance will therefore modify the fluxes of both gases into the leaf and thereby modify the magnitude of the phytotoxic stress attributable to either or both. Stomatal closure has frequently been reported as a consequence of exposure to O_3, but much of this information comes from short-term studies involving "sudden" exposures to relatively high levels of O_3 (>200ppb), rather than to repeated exposures which follow the diurnal rise and fall in O_3 levels typical of field situations. Of the few studies of the effect of NO_2 on stomata, most reported closure caused by high levels (>1000ppb). However stomatal closure with exposures at low levels has also been reported (Natori and Totsuka, 1984; Olszyk and Tingey, 1986; Salam and Soja, 1995; Barnes *et al.*, 1988; Barnes and Pfirrmann, 1992; Matyssek *et al*., 1991; Moldau *et al.*, 1990)

Chlorophyll fluorescence has been used as an indicator of O_3-induced stress following the initial work of Schreiber et al. (1978). Ozone has been reported to cause a decline in maximal fluorescence (Fm) (Schmidt et al., 1990), the ratio of variable to minimal fluorescence (Fv/Fo; Grimm and Fuhrer, 1992; Godde and Buchhold, 1992), the ratio of variable to maximal fluorescence (Fv/Fm; Farage et al., 1990; Salam amd Soja, 1995), non-photochemical quenching (qN), and increased photochemical

quenching (qP) (Godde and Buchhold, 1992). An NO_2-induced decline in Fv/Fo reported by Schmidt et al, (1990) was also a result of short-term "sudden" exposure to a high NO_2 concentration (5ppm for 4h). No information is available on the possible modification of chlorophyll fluorescence parameters resulting from NO_2-O_3 sequences.

Ethylene release has been reported as being stimulated by relatively high O_3 exposures, and its role in the appearance of visible symptoms of foliar injury has been proposed (Craker, 1971; Tingey et al., 1976; Mehlhorn and Wellburn, 1987). However, these effects were also obtained mostly with short-term "sudden" exposures and little to no information is available on the effect of either sequential, episodic exposures to O_3, or on the effects of NO_2 on ethylene release.

The specific objectives of this study were therefore to determine the overall effects of various sequential exposures to NO_2 and O_3 on plant growth, chlorophyll fluorescence (as an indicator of the functioning of the photosynthetic system) and endogenous ethylene production.

3.2 Methods and materials

3.2.1 Growth chamber exposures

Growth chambers (Model EF7, Conviron Ltd.) were modified to allow measurement of leaf diffusive resistance and chlorophyll fluorescence dynamics without removing the plants from the chambers. The modifications consisted of internal Plexiglas doors fitted with two long polyethylene sleeves, analogous to a glove-box. Plants were maintained in the growth chambers under the following conditions:

Photoperiod: 16.5/7.5h; the photoperiod started at 05:45h

Light intensity: 150μmol m^{-2} s^{-1}

Temperature (day): 24C; the thermoperiod started at 06:00 and ended at 22:00h.

Temperature (night): 18C

The chambers were supplied with air filtered through activated charcoal and Air Repair (alumina coated with $KMnO_4$) (Air Repair Products Inc.) to remove ambient O_3, NO_2 and NO. To simulate field conditions, exposures to NO_2 and O_3 both approximated sine waves, with <10ppb minimum and 120ppb maximum concentrations. A program written for a 21X Micrologger (Campbell Scientific Corp.) effected control of the exposure system. The program, which is presented in Appendix A, provided O_3 exposures between 10:00 and 18:00h and NO_2 exposures between 06:00 and 10:00h and between 18:00 and 22:00h in two chambers. Only one chamber was used for NO_2 and O_3 exposures in the present study; the second chamber was available in the event of equipment failure. A third chamber with filtered air was used as a control. The complete range of seven treatments was obtained by moving plants from chamber to chamber as required.

The sine waves were obtained by controlling the fraction of sequential 20s periods during which the solenoids admitting O_3 or NO_2 to the chambers were open. The value of the fraction was updated every 12min. The gases were admitted to the plenum in the treatment chamber to ensure rapid mixing.

Ozone and NO_2 levels in each chamber were monitored in sequence through a time-share system operated by the data logger (the program is also presented in Appendix A), which controlled input to a Model 1003-AH O_3 monitor (Dasibi Environmental Corp.) and a Model 14D/E NOx monitor (ThermoElectron Corp.). The time-share system switched among 8mm OD Teflon tubes of the same length from each chamber and from the ambient air (one line to each monitor). Each sample line was monitored for 3min, and the mean recorded from the last 2min of the sampling period. The NO_x monitor provided output of both NO_2 and NO levels. The sample lines were continuously purged with ambient or chamber air between the periods during which the sampling was switched to the monitors.

Ozone was generated by means of an ultraviolet source (Delzone Z0.300; Del Industries Ltd.) supplied with air from an oil-free diaphragm pump. NO_2 was supplied from a cylinder of compressed NO_2 in nitrogen (1%). Overall concentrations of NO_2 and O_3 in the chambers were adjusted by means of needle valve/flow meter combinations to produce the desired maximum concentrations (120ppb). The selection of 120ppb maxima for both pollutants was based in part on the 1-hour average concentrations that are defined in the Federal Air Quality Objectives for Canada, and the Air Quality Standards for the United States (Table 3.1) and in part on levels of pollution observed in the Fraser Valley of British Columbia over the past decade (Table 3.2).

The treatments were defined as follows (the symbols are used in all figures and tables of data):

Symbol	Treatment	Symbol	Treatment
C	control	N~N	early NO_2 + late NO_2
~O~	O_3 alone	NO~	early NO_2 + O_3
N~~	early NO_2 alone	~ON	O_3 + late NO_2
~~N	late NO_2 alone	NON	early NO_2 + O_3 + late NO_2

Typical exposure regimes for the seven treatments are shown in Fig.3.1. Typical levels of O_3, NO_2, and NO in the ambient air and the control chambers are presented in Fig. 3.2, which shows that the combination of charcoal and Air Repair effectively reduced the levels of each gas to <10ppb in the air supplied to the chambers. The curves in Fig. 3.2 were obtained with the distance weighted least squares (DWLS) smoothing function of SYGRAPH (SYSTAT Inc.) and show the typical daytime rise in ambient O_3, the tendency for NO_2 maxima to occur early and late in the day, and for NO to have a morning maximum.

The data logger also recorded chamber temperatures and light intensities to provide assurance of the temperature and light regimes.

Table 3.1 Federal Air Quality 1-hour Objectives for Canada for NO_2 and O_3, and National Ambient Air Quality Standards for the United States

	NO_2	O_3
Canada [1]	210 ppb	82 ppb
United States [2]	[53 ppb; annual average]	120 ppb

[1] Maximum Acceptable Objective levels, defined as providing adequate protection against effects on soil, water, vegetation, animals, visibility, personal comfort and well-being (Federal Clean Air Act).
[2] Secondary standard, defined as protecting the public welfare, which includes vegetation, crops, soils, water, animals, man-made materials, weather, climate, visibility, deterioration of property, hazards to transportation, economic values, personal comfort and well-being (U.S. Clean Air Act).

Table 3.2 Maximum 1-hour average concentrations (ppb) of NO_2 and O_3 measured at non-urban locations in the Fraser Valley of British Columbia.

Year	NO_2	O_3
1987	107	105
1988	90	154
1989	130	98
1990	76	117
1991	81	80
1992	84	101
1993	136	72
1994	80	101
1995	83	89

Source: Annual Reports of the Air Quality Department, Greater Vancouver Regional District, Burnaby, B.C.

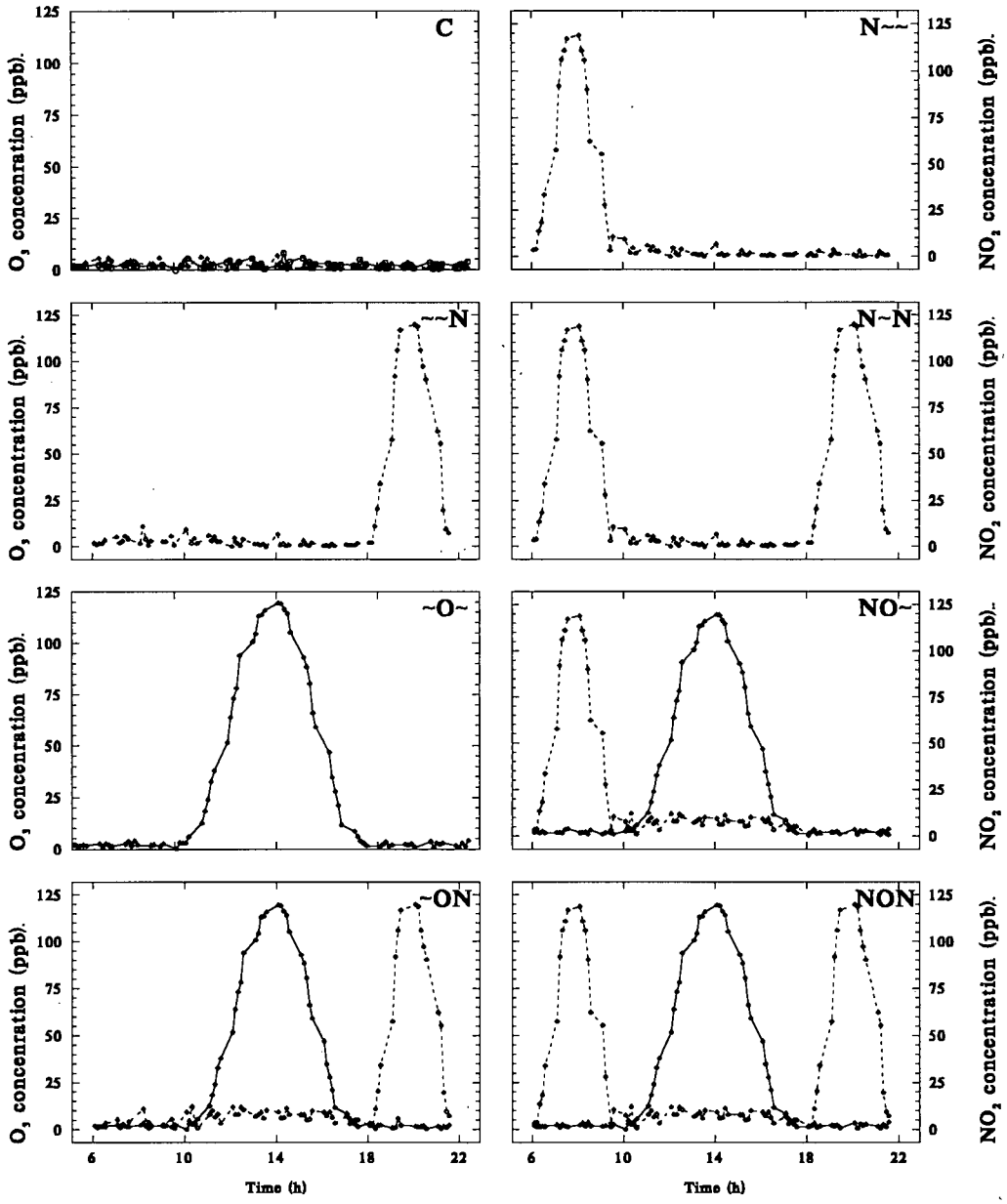


Fig. 3.1 The exposure regime for the NO_2/O_3 sequences. The sequences were: charcoal filtered air C. exposed to O_3 alone (~O~). early NO_2 (N~~). late NO_2 (~~N). both late and early NO_2 (N~N). early NO_2 plus O_3 (NO~). O_3 plus late NO_2 (~ON). and both late and early NO_2 plus O_3 (NON). The early NO_2 (broken line) was applied from 06:00 to 10:00h. late NO_2 was applied from 18:00 to 22:00h and O_3 (solid line) was applied from 10:00 to 18:00h.

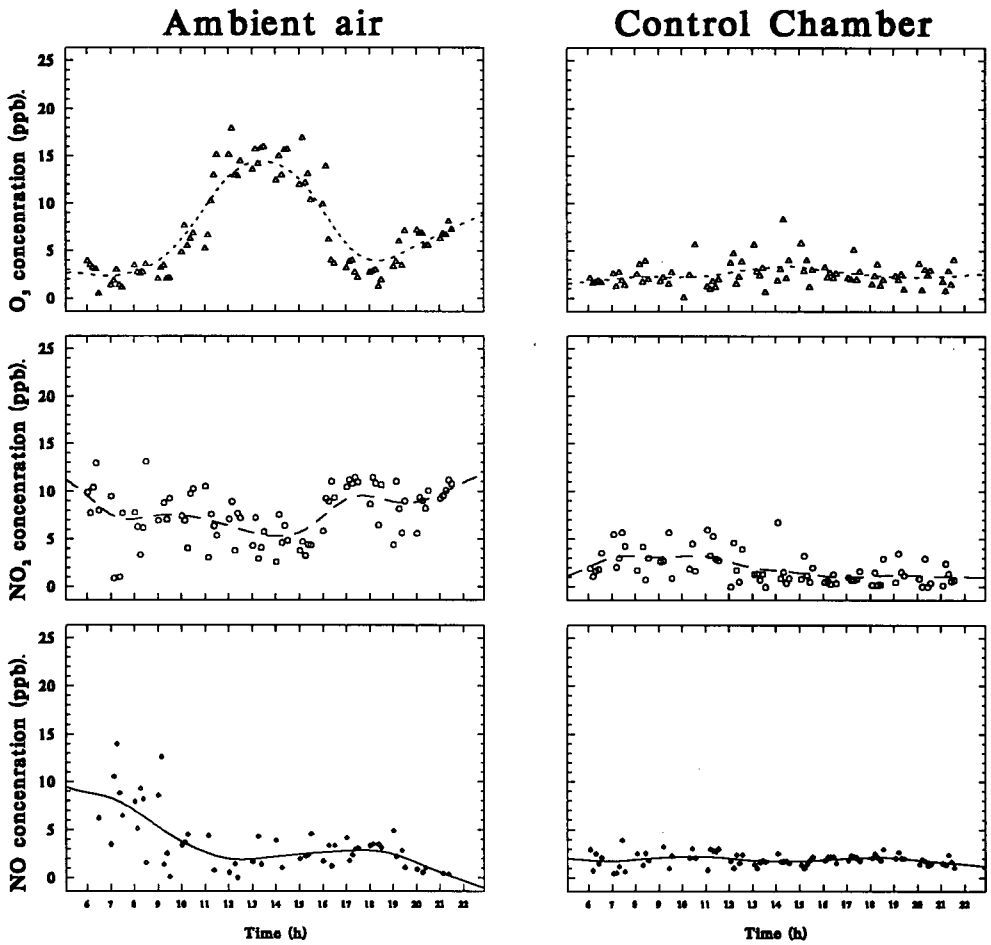


Fig. 3.2 Typical time-courses of the levels of O_3, NO_2 and NO in the control chamber and the ambient air illustrating the effectiveness of activated charcoal and Air Repair in reducing the gases in the air supplied to the chambers.

3.2.2 Plant materials

Four replicate experiments were carried out. Radish (*Raphanus sativus* L. cv Cherry Belle) plants were grown from seed in 5cm pots containing standard potting soil (85% loam; 15% peat) in a greenhouse. A slow release fertilizer with 14:14:14 NPK (Osmocote; Sierra Chemical Company) was applied to the standard potting soil. About 15 radish plants were sown per pot and these were thinned to 4 plants per pot 10 days after sowing. Soon after thinning they were transferred to the control growth chamber, where they acclimated for 3 days before the commencement of exposure (4 pots per treatment).

3.2.3 Growth measurements

After 21 days of exposure the plants were harvested and dried at 78C for 3 days prior to weighing. The design was a randomized complete block design (blocked 4 times in time) with 8 treatments (each treatment had 16 repeated observations per block).

3.2.4 Stomatal resistance measurements and estimation of pollutant fluxes

A Model LI-1600 (LI-COR Corp.) steady state porometer was used to measure leaf diffusive resistance. Measurements were made on the seventh day after the beginning of exposure to NO_2/O_3 combinations in each growth experiment. In each treatment, readings were obtained on the first true leaf of each of 4 plants per pot, at approximately hourly intervals from 07:00 to 22:00h. In order to analyze the data obtained for each treatment, the readings were segregated by hour (h) using the range: $h \pm 0.5$, and the mean time and mean diffusive resistance calculated using the data for each leaf and experiment (n=16).

The diffusive resistance data were normalized to the value at 14:00h in order to remove leaf to leaf variation. Fluxes of the gases were computed from the relationship:

$$F = \Delta C / r_d$$

where F is flux (g $m^{-2}s^{-1}$ units), ΔC is gas concentration gradient (g m^{-3} units), and r_d is diffusive resistance of the gas (s m^{-1} units), obtained by inverse proportionality of the molecular diffusivity of

the gas to that of water vapour (Runeckles, 1990).

3.2.5 Statistical analyses

SYSTAT/SYGRAPH (Systat Inc.) was used for all statistical analyses. Orthogonal contrasts were used to compare treatment means. The DWLS (distance weighted least squares smoothing) function of SYGRAPH was used throughout for curve fitting.

3.2.6 Fluorescence measurements

A Portable Fluorometer (Model PAM2000; H.Walz GmbH, Effeltrich, Germany) with integrated Poqet PQ-1024 computer was used to measure fluorescence parameters. The parameters determined were derived from the measurement of Fo, Fm, Fm', Ft, defined as in Table 3.3 (Schreiber et al., 1994). The relationships among the parameters in Table 3.3 is shown in Fig 3.3.

In the last three growth experiments (Section 3.2.2) the fluorescence parameters were measured on the first true leaf of one randomly selected plant from four pots for each treatment. On day seven of exposure to O_3-NO_2 sequences, plants were dark-adapted for 10 minutes (using a leaf clip supplied with the fluorometer) before the measurements were made. All the measurements were made while the plants were in the growth chambers with the lights on. At the end of dark adaptation the following measurements were taken:

1) Fo was measured using the measuring light (0.1μmol m^{-2} s^{-1}),

2) a saturating pulse (12 000μmol m^{-2} s^{-1}) was applied to measure Fm, and

3) the saturating pulse was applied every 20s for the next 5min 20s, to measure Ft and Fm'.

Since the last six data points were found to represent a steady state in fluorescence (a typical trace stored in the Poqet computer is shown in Fig. 3.4) the means of the last four readings of Y, qP, and qN

Table 3.3 Definition of chlorophyll nomenclature used in this study.

a. Fluorescence intensity indicators		
Ft	fluorescence intensity	fluorescence intensity at any time
Fo	minimal fluorescence (dark)	fluorescence intensity with all PSII reaction centers open i.e, dark or low light adapted; qP=1 and qN=0.
Fm	maximal fluorescence (dark)	fluorescence intensity with all PSII reaction centers closed (i.e. qP=0) after dark or low light adaptation and all non-photochemical quenching processes at a minimum (i.e. qN=0).
Fv	variable fluorescence (dark)	maximum variable fluorescence with all non-photochemical processes at a minimum; i.e. Fm-Fo.
Fm'	maximal fluorescence (light)	fluorescence intensity with all PSII reaction centers closed in a light-adapted state; qP=1 and qN≥0.
b. *Fluorescence quenching parameters*		
qP	photochemical quenching	**(Fm'-Ft)/(Fm'-Fo)**
qN	non-photochemical quenching	**(Fm-Fm')/(Fm-Fo)**, the coefficient of non-photochemical quenching correlated with 'thylakoid membrane energization'.
NPQ	non-photochemical quenching	**(Fm-Fm')/Fm'**, the coefficient of non-photochemical quenching reflecting heat-dissipation of excitation energy in the antenna system
Y	Fluorescence quantum yield	**(Fm'-Ft)/ Fm'**, the quantum yield of photochemical energy conversion reflecting the efficiency of the overall process.

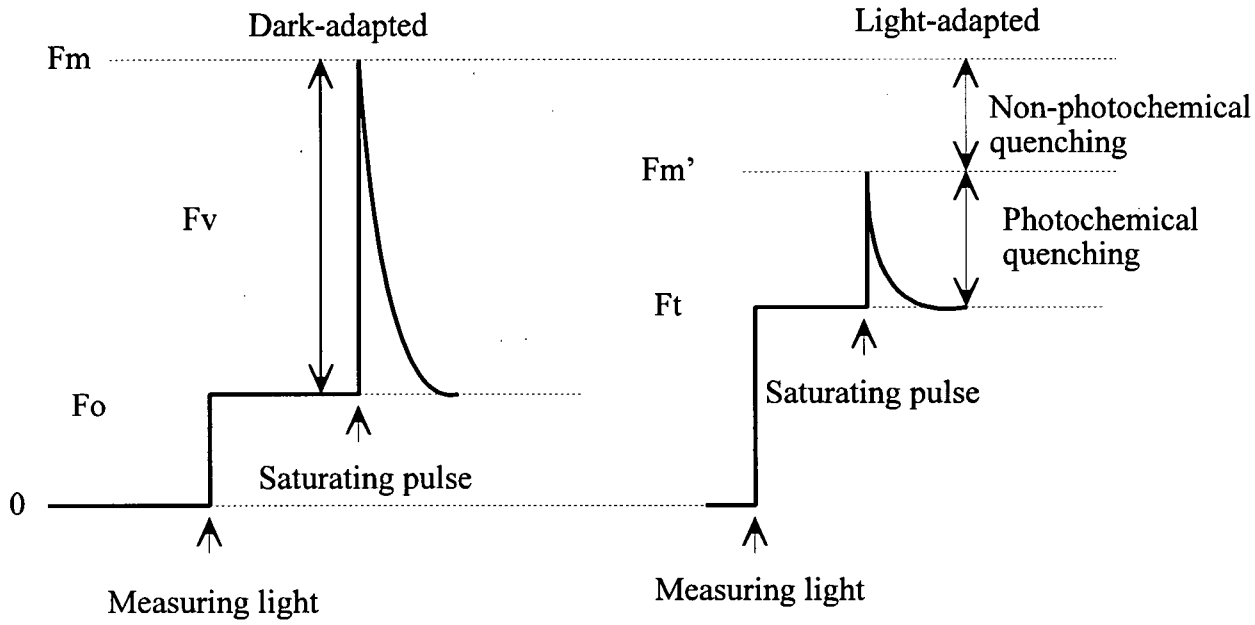


Fig 3.3. The relationship between the measured fluorescence parameters.

were used for data analysis (740 to 920s in the trace in Fig.3.4). NPQ data were manually calculated from the formula given in Table 3.3. Data were collected between 07:00 and 17:00h. The experiment was a randomized block design with 3 blocks (blocked in time).

3.2.7 Endogenous ethylene production measurements

In a separate experiment, in which plants were grown and handled as described in Section 3.2.2,

radish plants were exposed to O_3 for 5 days after 3 days' acclimation in the growth chambers. The shoots were harvested at the peak of the O_3 wave on day 5 (14:00h). Four plants (one plant from each of 4 pots per treatment) were randomly selected. The whole shoot system from the crown upwards was immediately transferred to 57 ml incubation tubes (two shoots per tube) holding 5 ml deionized distilled water. The tubes were held for 24h in subdued light (10μmol $m^{-2}s^{-1}$) at room temperature (24

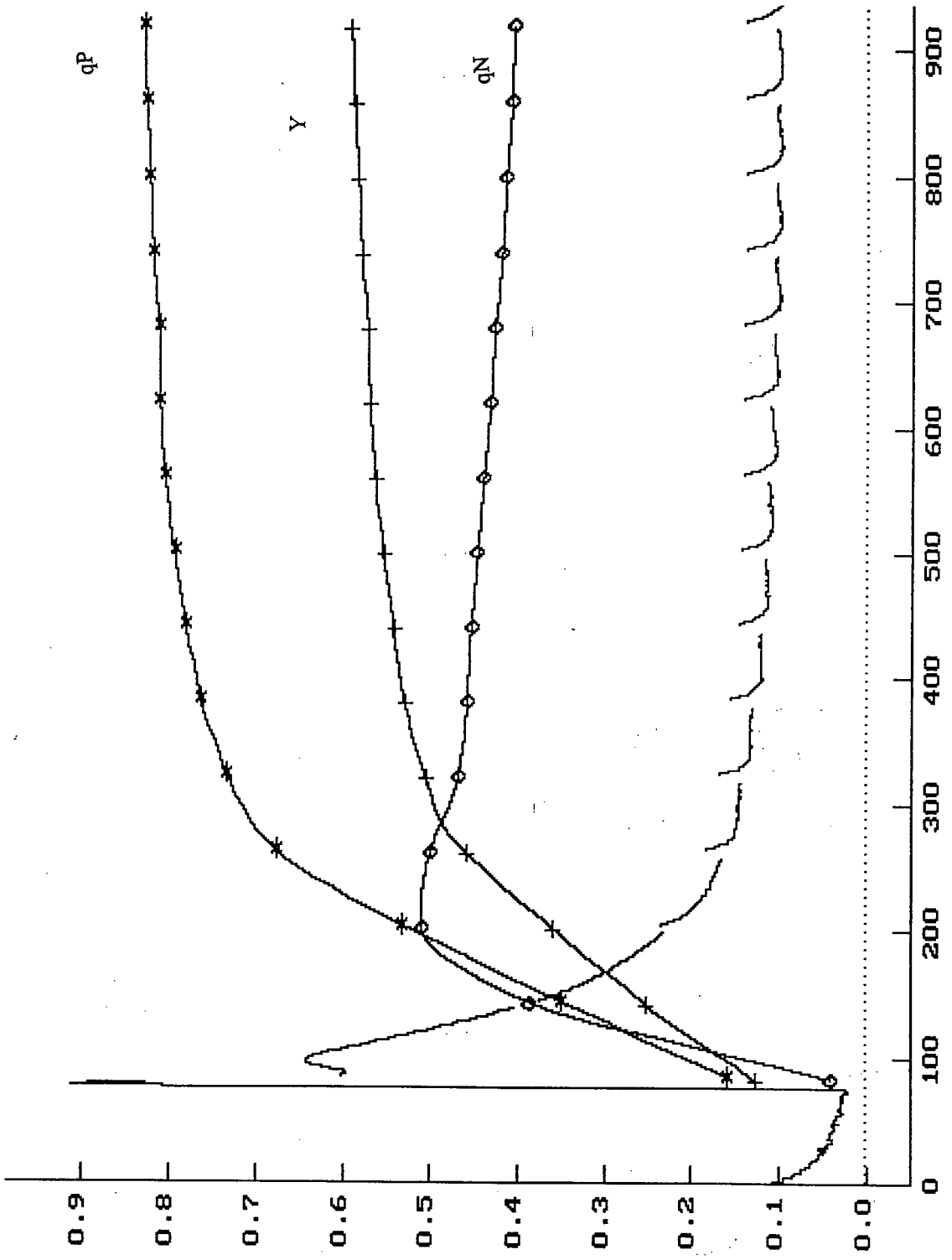


Fig 3.4 A typical trace of Y, qP, and qN, from which the last four data points were selected for data analysis.

±2C) before measuring the concentration of ethylene in the headspace by gas chromatography (HP 5830A Gas Chromatograph; Hewlett Packard Corp.). A 1 ml gas sample from each tube was injected onto a Porapak N 80/100 (column , injector and detector temperatures were 50, 150 and 200 respectively; carrier gas: N_2, carrier gas flow: 41ml/min; Column ID: NOV18/81D.C.; and detector: Flame Ionization Detector). The retention time for ethylene was approximately 1.6 min. The shoots were then dried at 78C for 3 days before their dry matter was measured. The data were expressed as nanomoles of ethylene gas per gram dry matter/hour.

3.3 Results

3.3.1 Effects of NO_2/O_3 sequences on growth

Early NO_2 significantly ($P<0.05$) stimulated total dry matter accumulation per plant relative to control plants in filtered air (cf. C vs. N~~; Fig. 3.5). The stimulation by early + late NO_2 was less significant (cf. C vs. N~N; $P<0.1$). The slight increase over the control due to late NO_2 was not significant, and late NO_2 had no effect on the response to early NO_2 (cf. N~N vs. N~~). Ozone alone had no significant effect on total dry matter accumulation (cf. C vs. ~O~), but significantly suppressed the stimulatory effects of early NO_2 (cf. N~~ vs. NO~) ($P<0.05$), late NO_2 (cf. ~~N vs. ~ON) ($P<0.1$) and early + late NO_2 (cf. NON vs. N~N) ($P<0.01$).

The effects of treatment on shoot dry matter production (Fig. 3.6) were similar to those on total dry matter. Early NO_2, late NO_2, and early + late NO_2 tended to stimulate shoot dry matter production. Late NO_2 had no effect on early NO_2 (~~N vs. N~N). Ozone alone had no effect on shoot dry matter accumulation. It significantly ($P<0.05$) suppressed the stimulatory effect of early + late NO_2 (cf. NON vs. N~N). It also suppressed ($P<0.1$) the slight stimulation of early NO_2 (cf. NO~ vs. N~~) or late NO_2 (cf. ~ON vs. ~~N).

Hypocotyl dry matter accumulation also showed similar effects of treatment to total dry matter. As shown in Fig. 3.7, early NO_2 and early + late NO_2 resulted in stimulation relative to the control (cf. C vs. N~~; $P<0.01$; C vs. N~N; $P<0.05$). Ozone alone had no effect, but it significantly reduced the stimulation caused by NO_2 (cf. NO~ vs. N~~; $P<0.01$; ~ON vs. ~~N; $P<0.1$; NON vs. N~N; $P<0.01$). As a result of the effects of treatment on the components of dry mass, shoot: root ratios relative to the control were marginally reduced by O_3 ($P<0.1$) while all the other treatments and treatment combinations were not significant (Fig. 3.8).

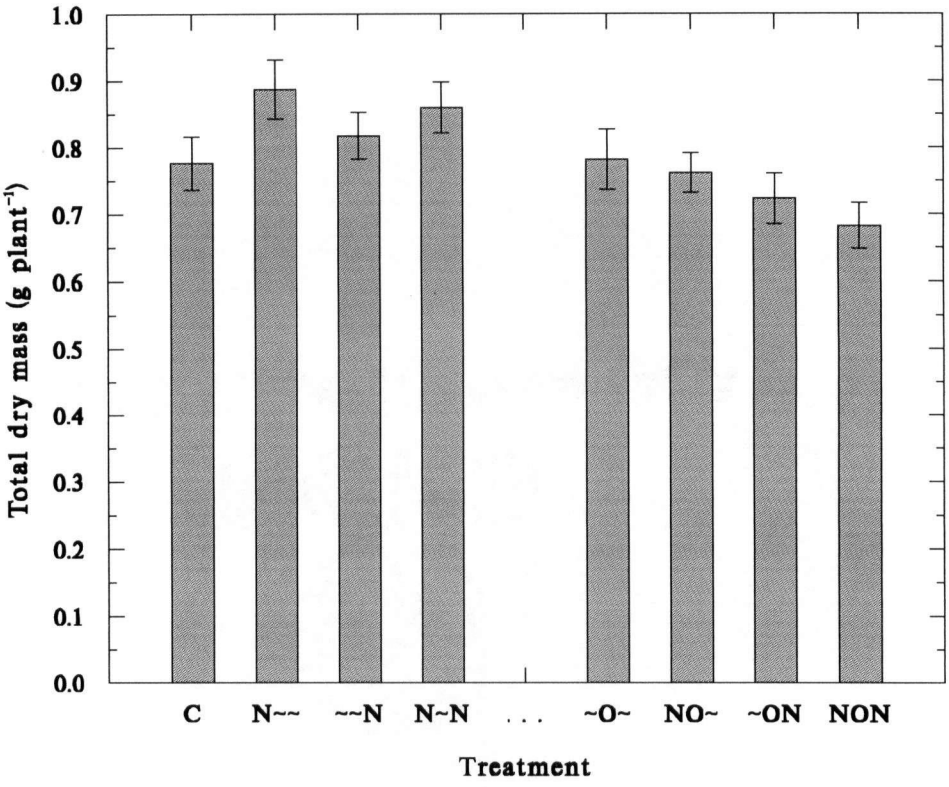


Fig. 3.5. Effects of treatment on total dry mass. Error bars are standard errors (n=64).

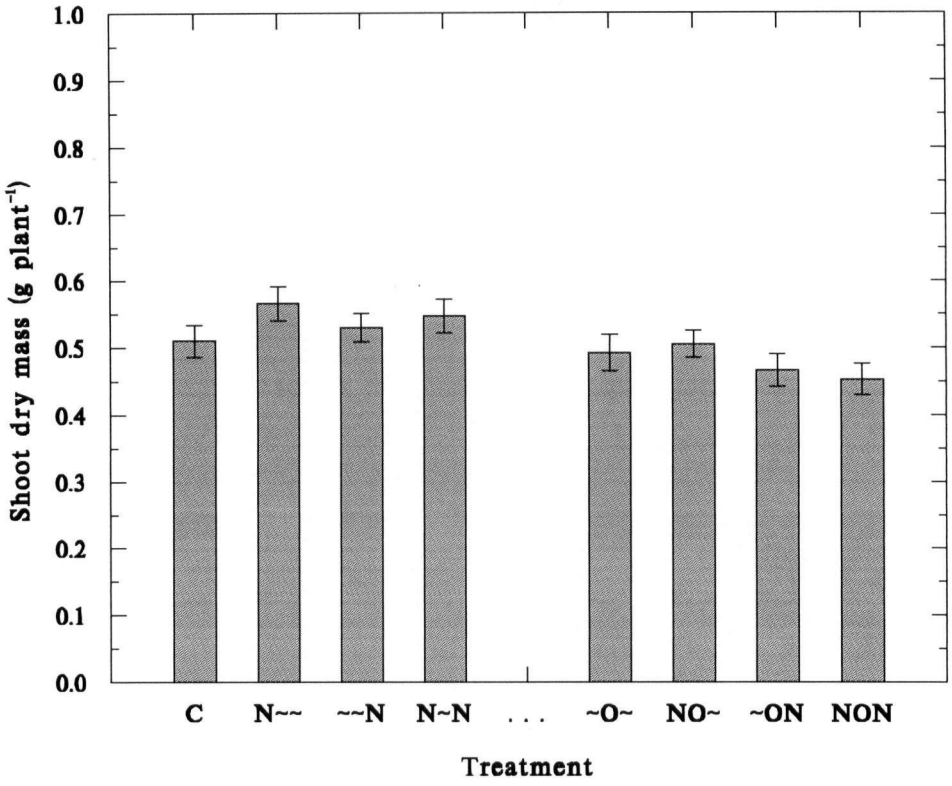


Fig. 3.6. Effects of treatment on shoot dry mass. Error bars are standard errors (n=64).

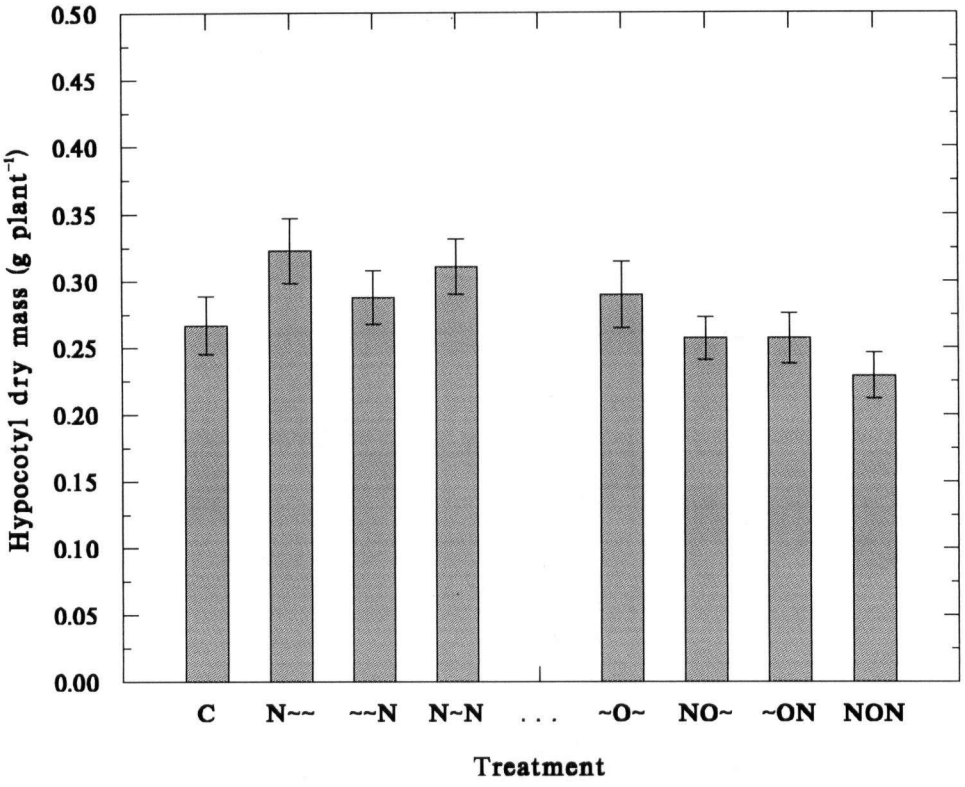


Fig. 3.7. Effects of treatment on hypocotyl dry mass. Error bars are standard errors (n=64).

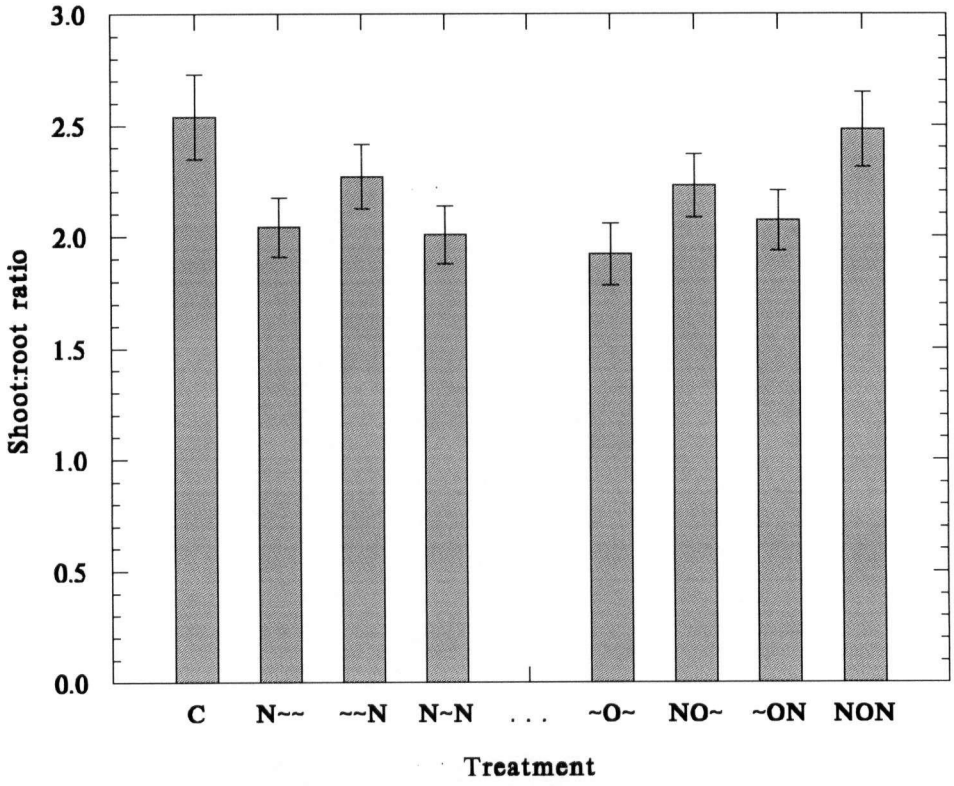


Fig. 3.8. Effects of treatment on shoot:root ratio. Error bars are standard errors (n=64).

3.3.2 Stomatal Conductance and Flux

Fig. 3.9 presents a consolidation of the data obtained during the seventh day of treatment in each experiment, i.e. for plants having received six daily cycles of treatment before the measurements were made. Repeated exposure to early NO_2 tended to enhance early morning stomatal closure (cf. N~~ vs. C; Fig. 3.9), late NO_2 accelerated closure late in the day (cf. ~~N vs. C) and the combination of two daily exposures to NO_2 tended to increase the late afternoon resistance which persisted through the following morning (cf. N~N vs. C). Thus, the combination of early + late NO_2 enhanced the early and late stomatal closures due to either early or late NO_2 alone (cf. N~N vs. N~~, N~N vs. ~~N).

In addition to suppressing the normal morning and evening closure of stomata in the control, exposure to O_3 tended to overcome both early and late closure of stomata induced by late NO_2 (cf. ~ON vs. ~~N) and to a lesser extent, the early closure caused by early NO_2 (cf. N~~ vs. NO~) or early + late NO_2 (cf. NON vs. N~N). Conversely, late NO_2 had no effect on the course of diffusive resistance caused by early NO_2 and O_3 (cf. NON vs. NO~), while early NO_2 tended to overcome the suppression of early stomatal closure caused by O_3 (cf. NON vs. ~O~).

The estimated fluxes of both gases are shown in Fig. 3.10. The NO_2 flux reached a maximum of 0.5 $\mu g\ m^{-2}s^{-1}$ in the absence of O_3. However, in the presence of O_3 the NO_2 fluxes approximately doubled (cf. NO~, ~ON, and NON) because of the low morning and evening resistances caused by O_3. The O_3 flux was not affected by exposure to NO_2 and reached maxima of about 1.5 $\mu g\ m^{-2}s^{-1}$

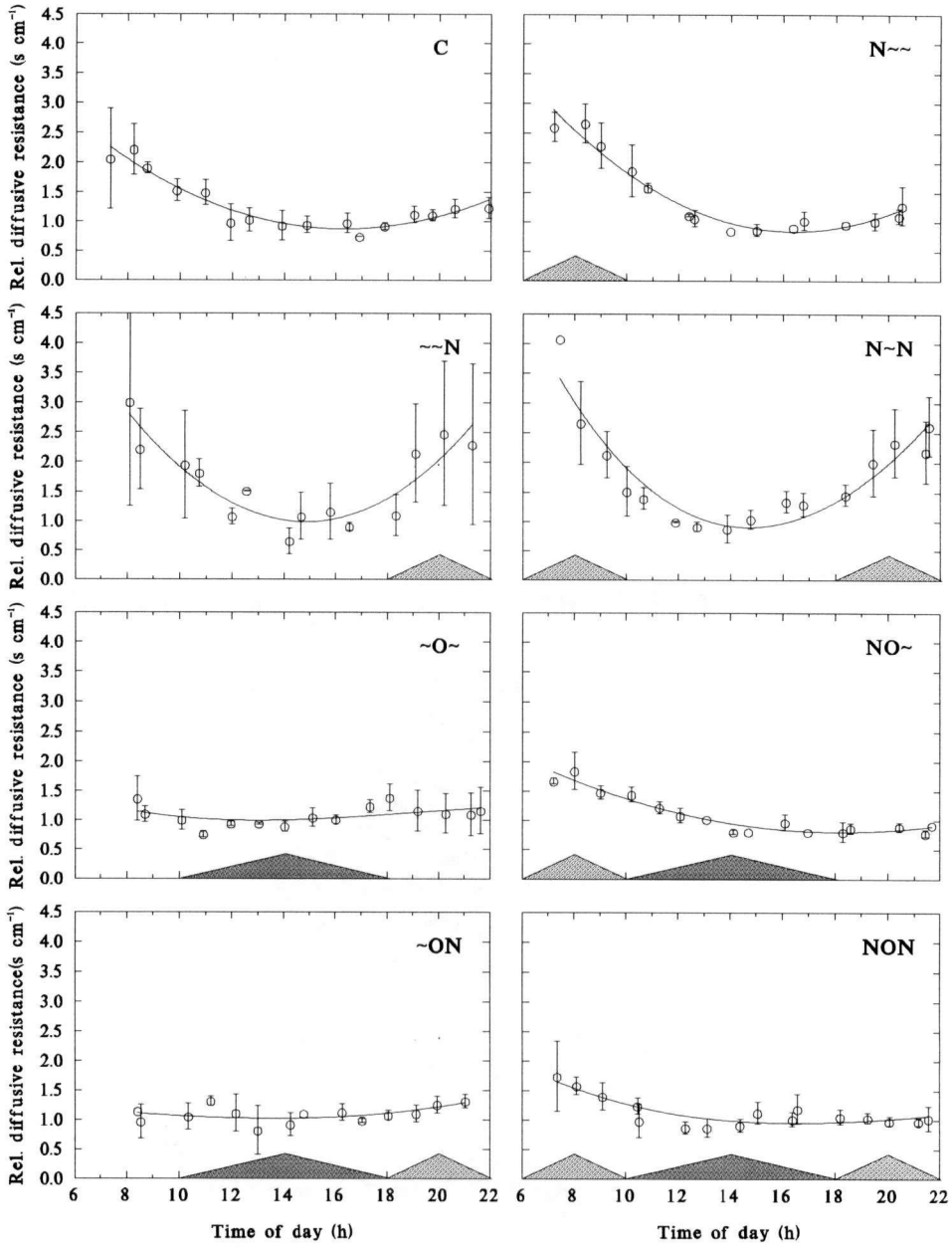


Fig. 3.9 Time course of relative diffussive resistance by treatment. Error bars are standard erros (n=16). The shaded triangles indicate the periods of exposure to NO_2 (light shading) and O_3 (dark shading).

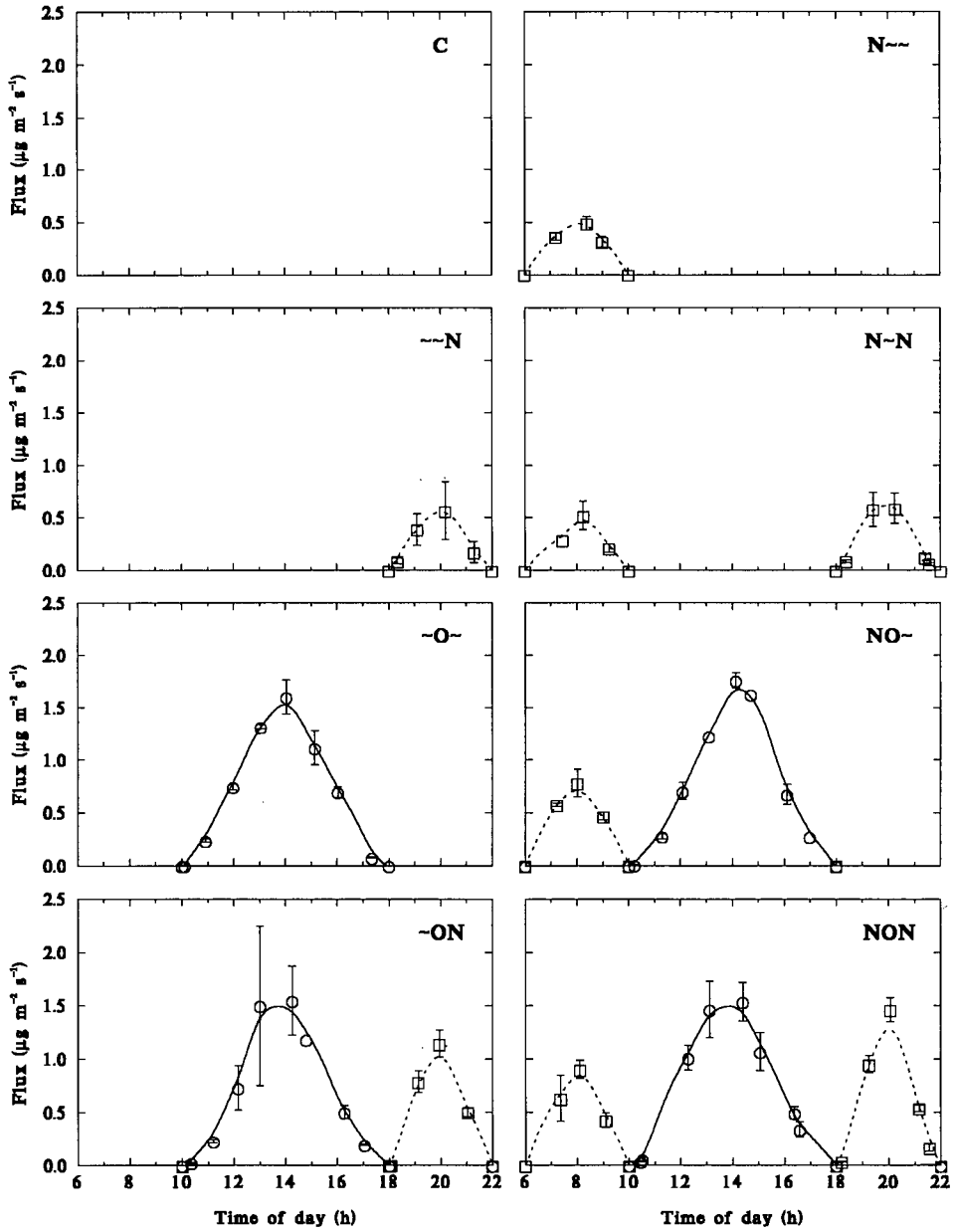


Fig. 3.10 Time course of estimated fluxes of NO_2 (broken line) and O_3 (solid line) by treatment. Error bars are standard errors (n=16); for data without error bars, N=1.

3.3.3 Fluorescence

As shown in Fig. 3.11, there was little difference in mean minimal fluorescence (Fo) due to treatment in dark-adapted leaves, recorded at the onset of data collection at about 07:00h over one hour after the start of the photoperiod. Since the data were collected on the seventh day of exposure, this shows that exposures received during the previous six days had had no cumulative effect on Fo, with the possible exception of the early NO_2+O_3 combination.

With regard to mean maximal fluorescence (Fm)(Fig 3.11), O_3 alone and early NO_2 resulted in slight but not significant ($P>0.05$) stimulation and the combination of early + late NO_2 had no effect relative to the control. Ozone suppressed the effect of early NO_2 (cf. N~~ vs. NO~; $P<0.1$) but had no effect on late NO_2 or late + early NO_2. The mean fluorescence quantum yield (Y) (Fig. 3.12) calculated from (Fm-Fo)/Fm, however, showed no distinct treatment effects, other than stimulation with early NO_2 indicating that this parameter had less variability even when Fm was variable. Values for Y were in the range (0.75 to 0.85) of values reported in many studies (Bolher-Nordenkampf et al., 1989).

In light-adapted leaves, mean Ft and mean Fm' showed greater variability among treatments (Fig. 3.13). Mean Ft was lower than the control in all treatments except ~~N and NON. The time course of Ft

(Fig. 3.14) showed little variation over time for any treatment, although significant ($P<0.05$) negative slopes for the linear regressions were found for the control and all NO_2 treatments.

As is also shown in Fig. 3.13, in all treatments with O_3, Fm' was significantly increased relative to the control. Mean Fm' was also increased by early NO_2 (cf.. N~~ vs. C; Fig. 3.13). Fig. 3.15 shows the time course of Fm' for each treatment. Other than initially higher values in early NO_2 ($P<0.06$ for the slope of the linear regression over time), the trends in the other treatments with NO_2 alone were similar

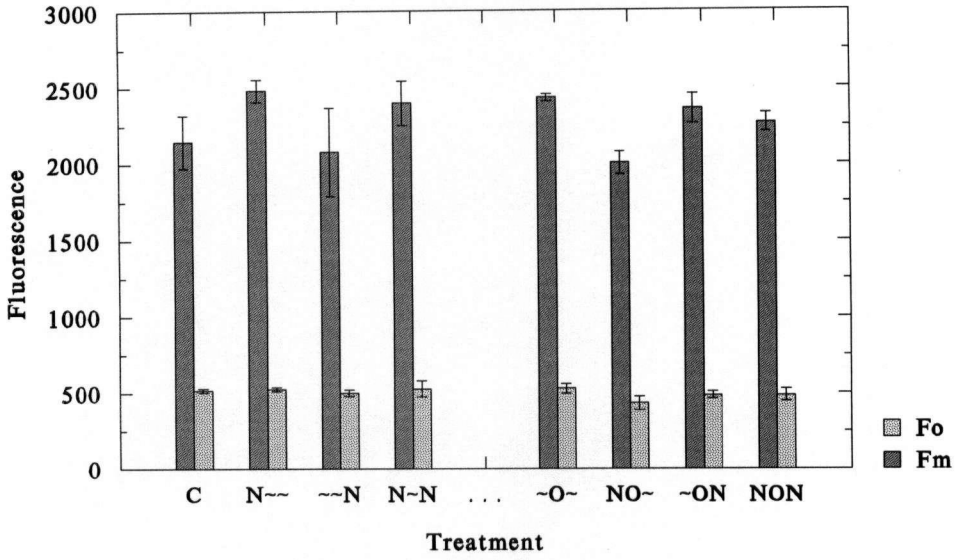


Fig. 3.11 Fm and Fo vs. treatments for radish. Error bars are standard errors (N=3).

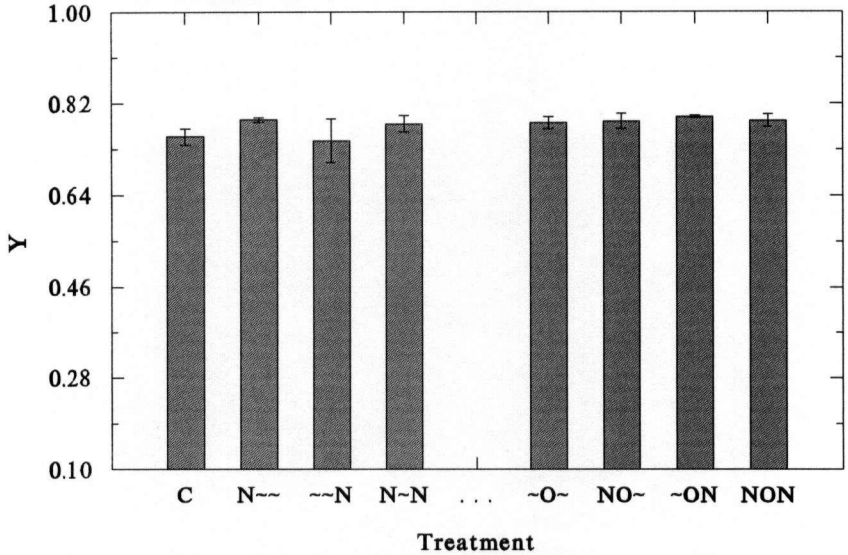


Fig. 3.12 Fluorescence quantum yield (Y) vs. treatments for radish. Error bars are standard errors (N=3).

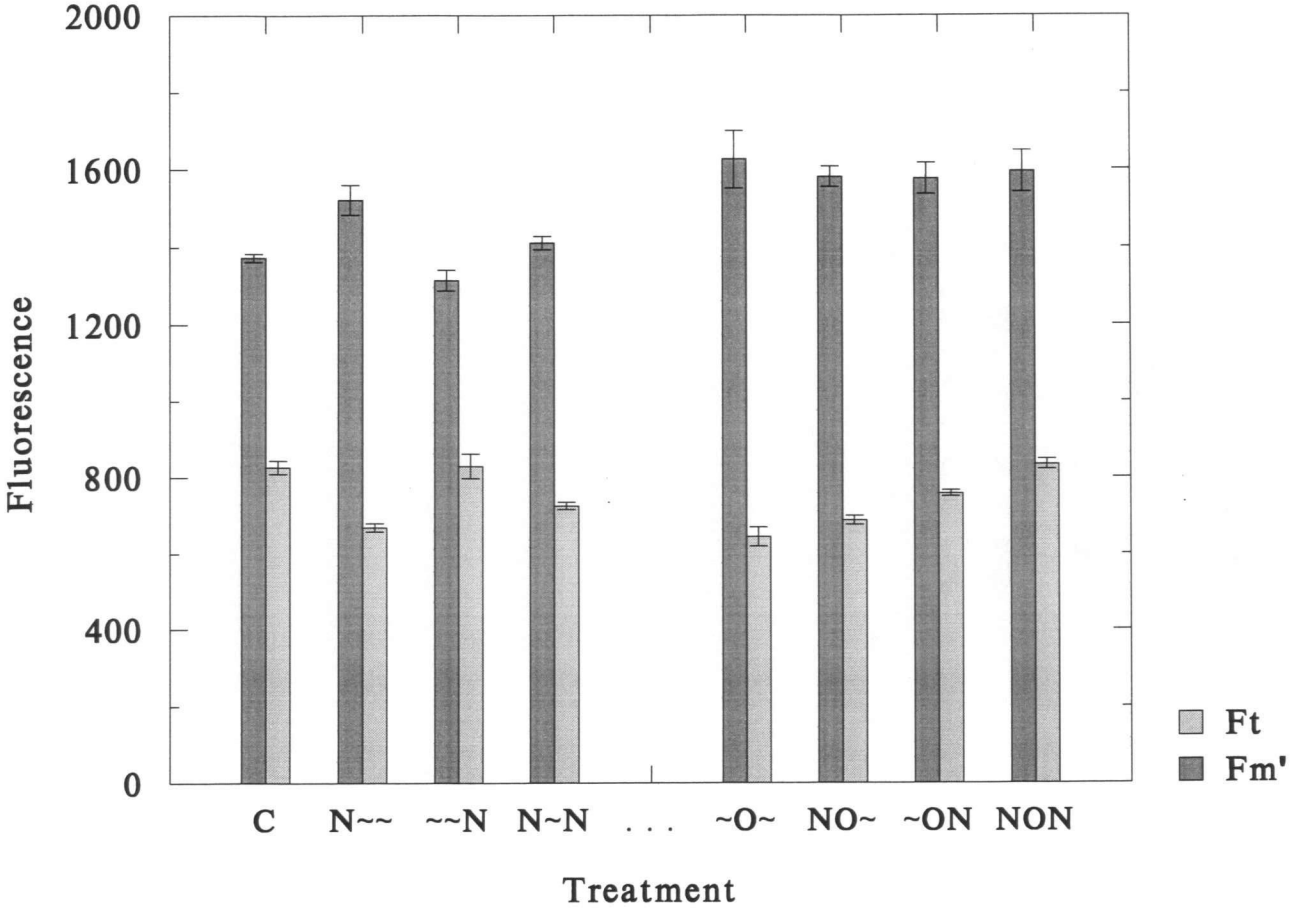


Fig. 3.13. Effects of treatment on Fm' and Ft. Error bars are standard errors (n=3)

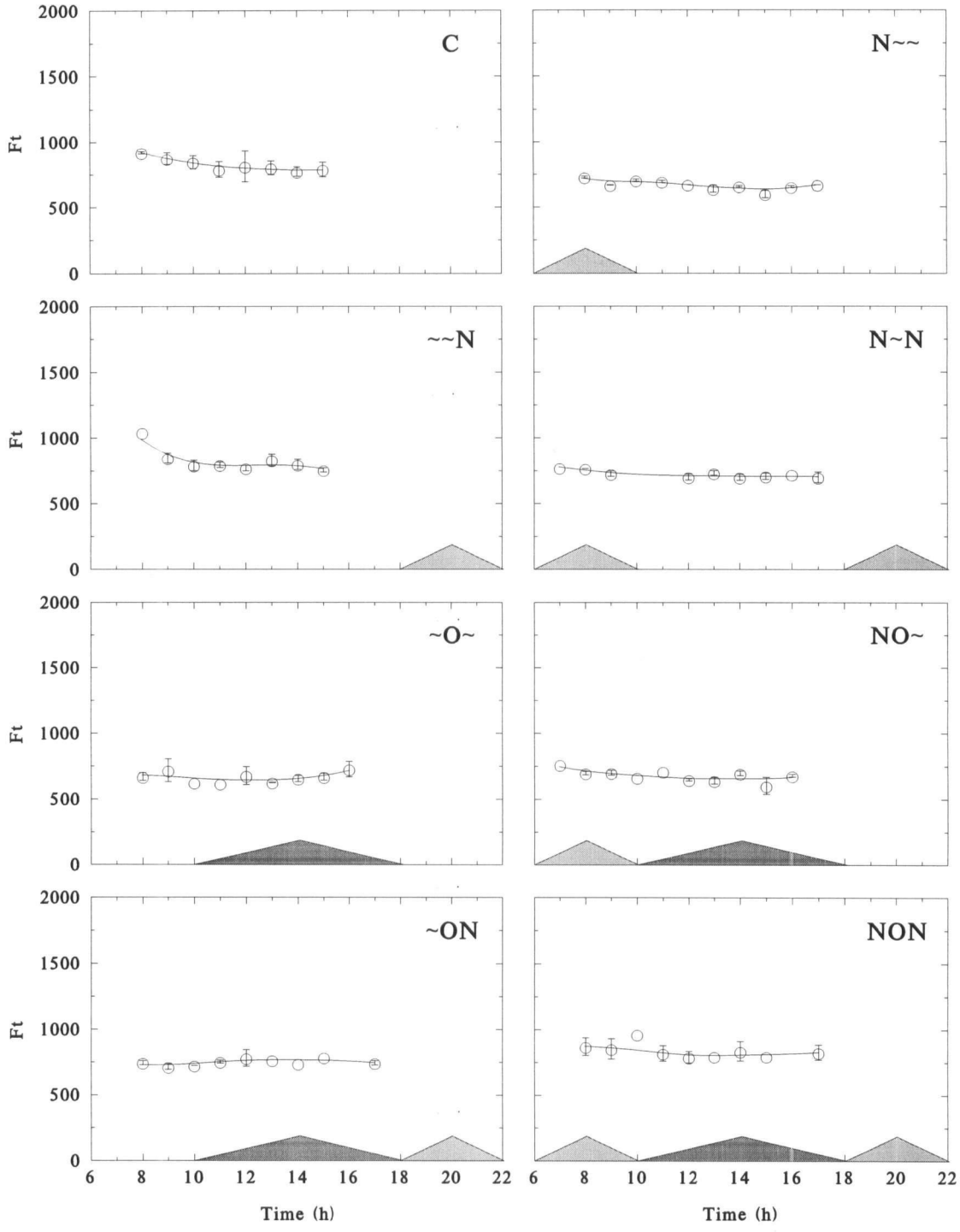


Fig. 3.14 Time course of fluorescence (Ft) by treatment. Error bars are standard errors (n=3); for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

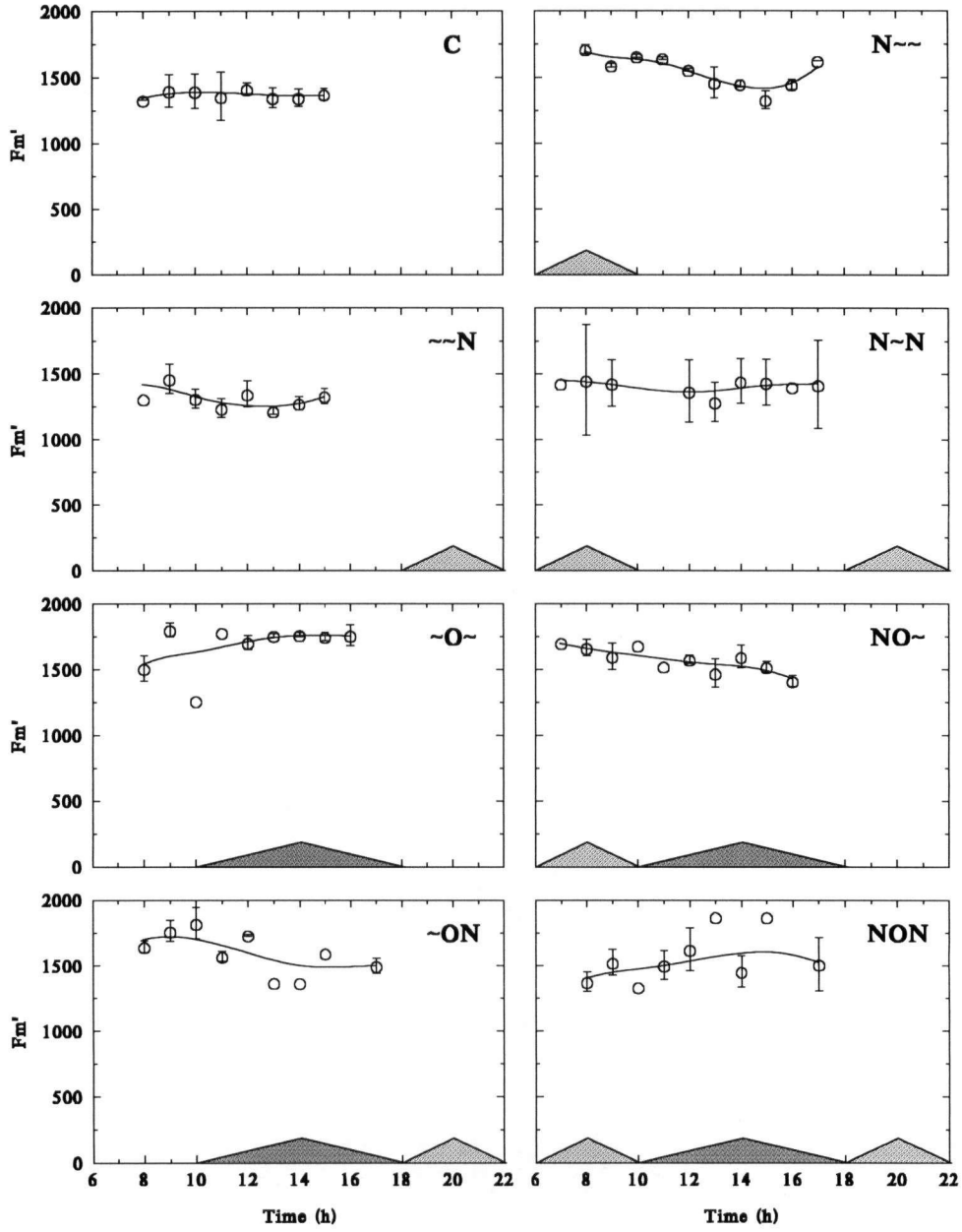


Fig. 3.15 Time course of maximal fluorescence (light adapted: Fm') by treatment. Error bars are standard errors (n=3): for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

to the control (cf. C vs. N~~, ~~N, and N~N; Fig. 3.15). Although Fm' tended to increase during exposure to O_3 alone (cf. C vs. ~O~; the linear regression has a positive slope, $P<0.02$), it declined if the plants were also exposed to early or late NO_2 ($P<0.01$ and $P=0.01$, respectively for the slopes of the linear regressions over time).

Quantum yield (Y; derived from Fm' and Ft) showed that Fm' was the overriding factor in determining yield values (cf. Fig 3.16 vs. Fig. 3.13). Early NO_2 increased yield while the level in late NO_2 did not differ from the control. The combination of early + late NO_2 led to an intermediate stimulation. Higher quantum yields also resulted from exposure to O_3 alone or in combination with early or late NO_2 (cf. C vs. ~O~, NO~, ~ON; Fig. 3.16), although the increase with early NO_2 was not additive (cf. ~O~ and N~~ vs. NO~). Late NO_2 decreased the O_3-induced stimulation (cf. ~O~ and ~~N vs. ~ON), and with the early + late NO_2 combination, the O_3-induced stimulation was completely suppressed (cf. ~O~ and N~N vs. NON). The differences due to treatment and the changes in photochemical quenching (qP) over time were virtually identical to those of quantum yield (cf. Fig. 3.18 vs. Fig. 3.17). This was not surprising in view of the lack of treatment effect on Fo (Fig. 3.11).

With regard to non-photochemical quenching (qN), Fig. 3.16 shows that regardless of sequence, early NO_2 had no effect on the overall level, relative to the control, but late NO_2 alone increased qN. Exposure to O_3 alone and in all combinations with NO_2 reduced qN (cf. C vs. ~O~, NO~, ~ON, and NON; Fig. 3.16). A greater overall reduction occurred with early NO_2 and O_3 (NO~). Non-photochemical quenching determined as the NPQ parameter showed similar responses to treatment as qN (Fig. 3.16).

The time courses of the change in these fluorescence parameters are shown in Figs. 3.17 to Fig.3.20. None of the parameters changed markedly over time, although significant slopes of linear regressions

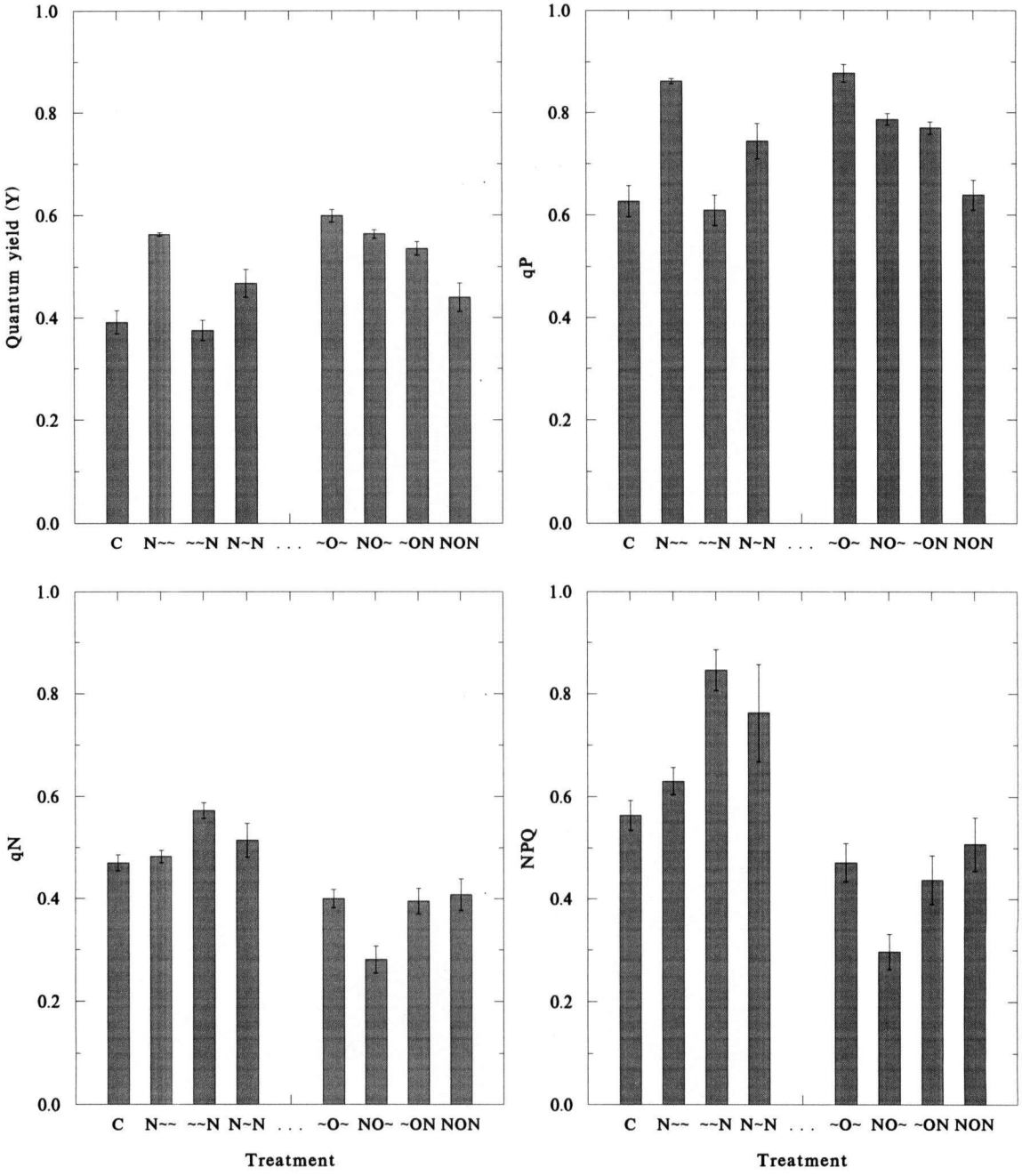


Fig. 3.16. Effects of treatment on mean quantum yield (Y), qP, qN and NPQ. Error bars are standard errors (n=16).

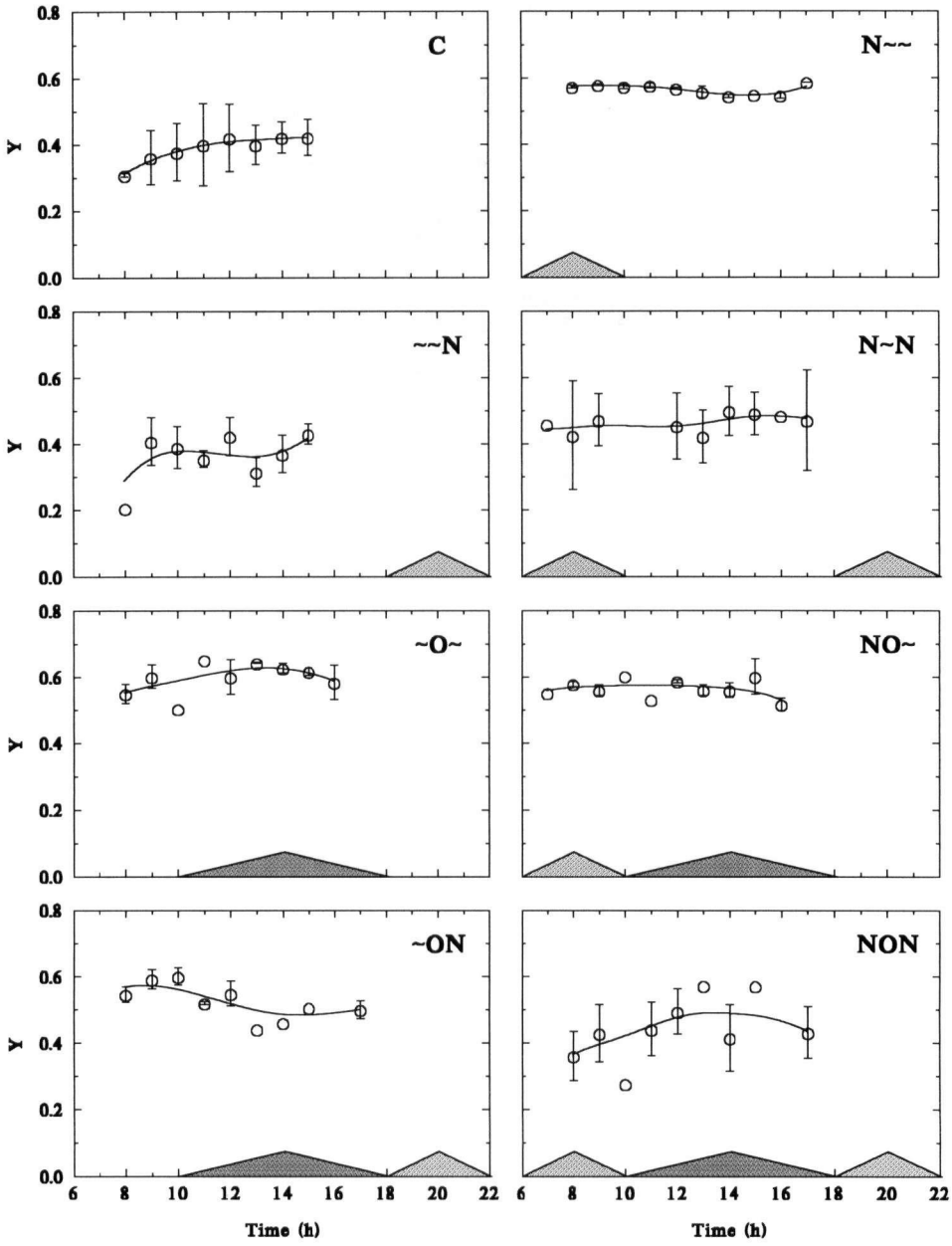


Fig. 3.17 Time course of fluorescence quantum yield (Y) by treatment. Error bars are standard errors (n=3); for data points wthout error bars. n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

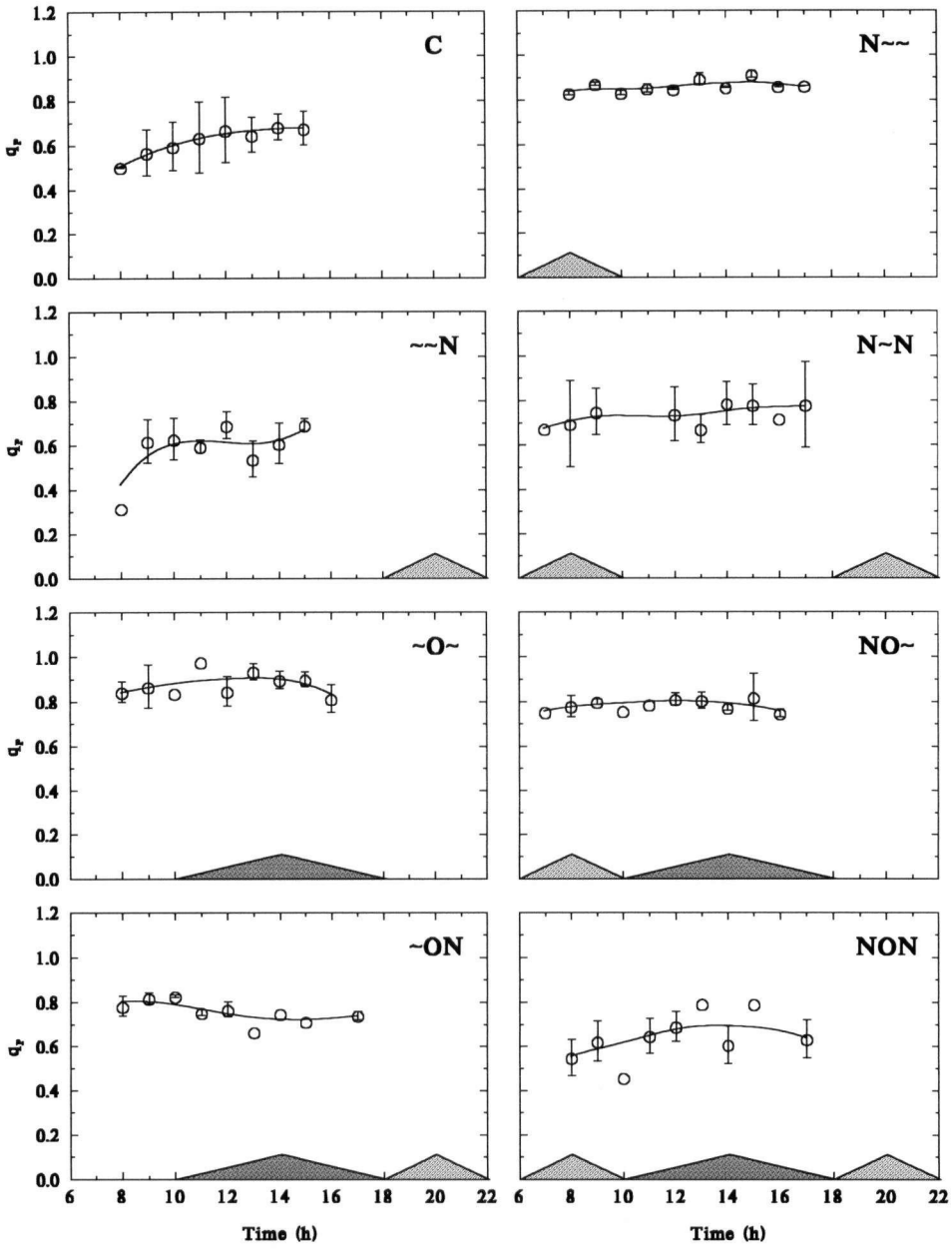


Fig. 3.18 Time course of photochemical fluorescence quenching (qP) by treatment. Error bars are standard errors (n=3); for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

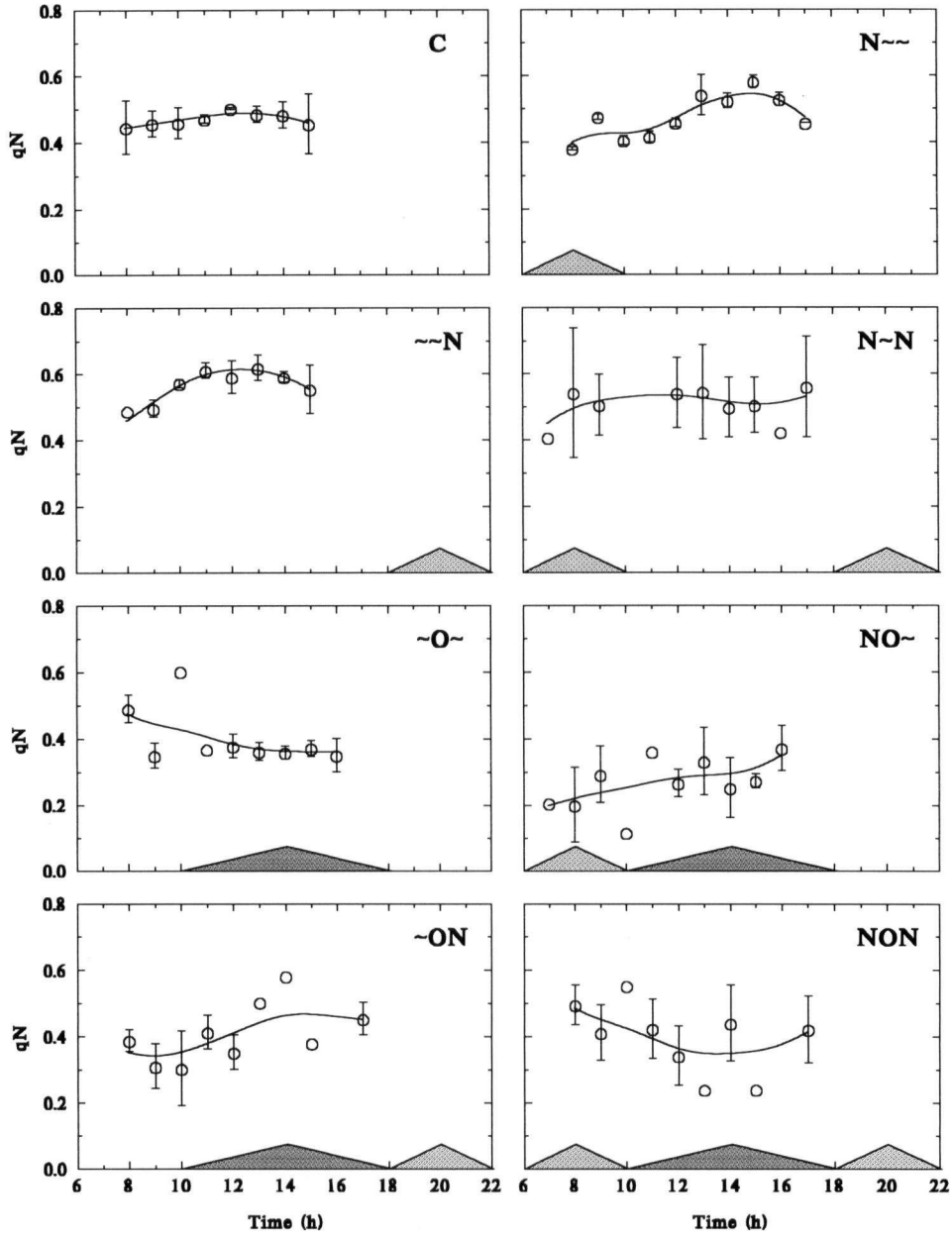


Fig. 3.19 Time course of non-photochemical fluorescence quenching (qN) by treatment. Error bars are standard errors (n=3); for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

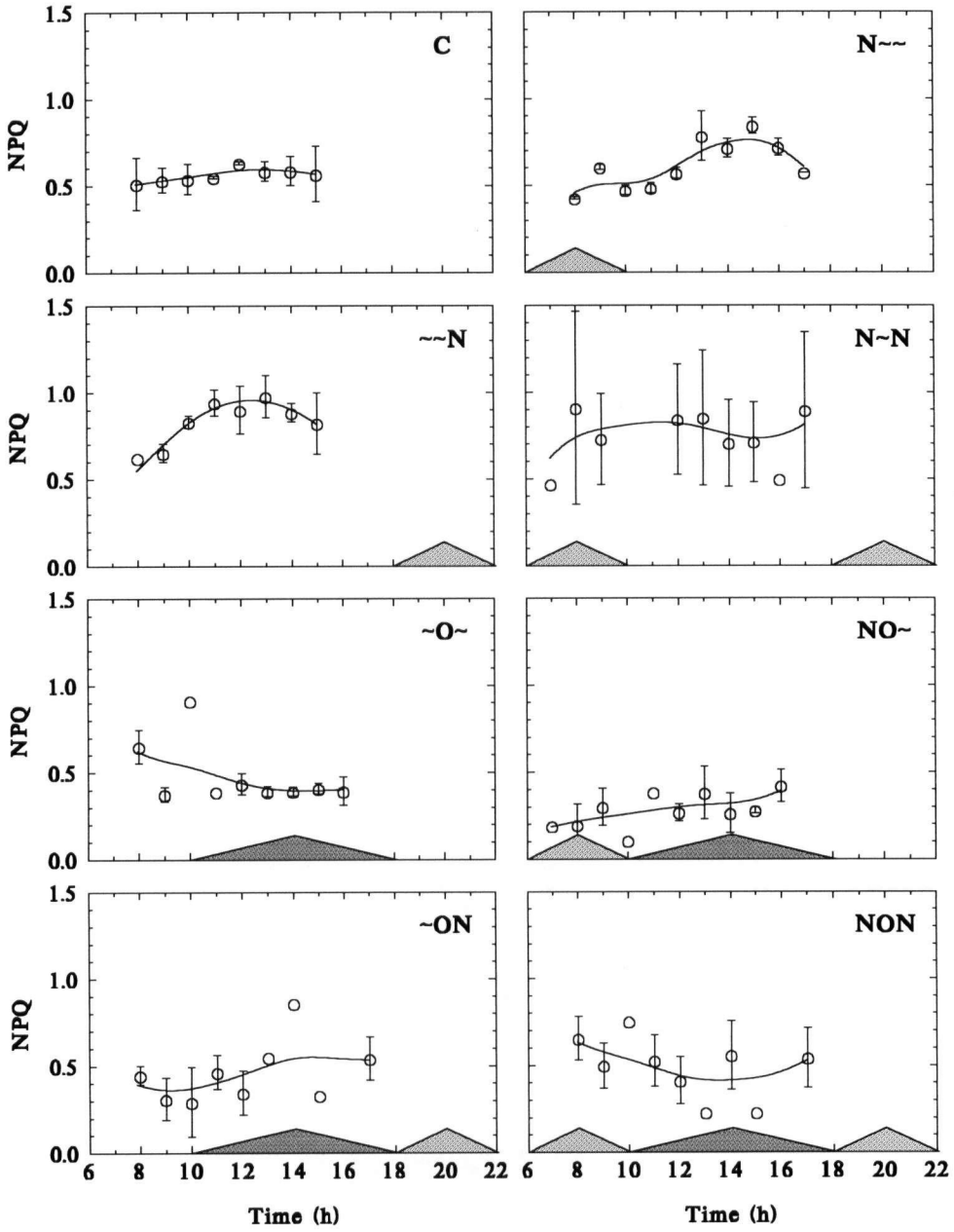


Fig. 3.20 Time course of non-photochemical fluorescence quenching (NPQ) by treatment. Error bars are standard errors (n=3); for data points without error bars, n=1. The shaded triangles indicate the period of exposure to NO_2 (light shading) and O_3 (dark shading).

of qP, qN, NPQ or Y vs. time for the different treatments were occasionally found. In the control, no significant changes over time were observed. Early NO_2 resulted in a significant linear decrease in quantum yield over time (Fig. 3.17) ($P<0.01$), but significant linear increases occurred in qP (Fig. 3.18), qN (Fig.3.19) and NPQ (Fig. 3.20) ($P<0.05$, $P<0.001$ and $P<0.001$, respectively). Neither late NO_2 nor early + late NO_2 resulted in significant changes over time. An O_3-induced linear increase in quantum yield was significant (Fig. 3.17; $P<0.05$), and the decreases in qN (Fig. 3.19) and NPQ (Fig. 3.20) were significant at $P<0.01$ and $P<0.01$, respectively. Treatments with late NO_2 (NO~), early + late NO_2 (N~N) or early + late NO_2+O_3 (NON) showed no significant linear trends for any parameter in quantum yield (Fig. 3.17), qP (Fig. 3.18), qN (Fig. 3.19) or NPQ (Fig. 3.20). Ozone + late NO_2 (~ON) showed significant negative linear trends in yield (Fig. 3.17; $P<0.01$) and qP (Fig. 3.18; $P<0.05$), a positive trend in qN (Fig.3.19; $p<0.05$) but no significant trend in NPQ.

3.3.4 Effects on ethylene production

As shown in Fig 3.21, O_3 alone slightly suppressed ethylene production relative to the control (cf. C vs. ~O~; $P<0.1$) and in the presence of late NO_2 (cf. ~ON vs. ~~N; $P<0.05$). No other single treatment yielded significant differences, although early NO_2 tended to reduce ethylene release while late NO_2 tended to stimulate it relative to the control. These trends cancelled out in the early + late NO_2 treatment. However, when compared with O_3 alone, NO_2 + O_3 combinations tended to result in greater ethylene release, the increase becoming marginally significant when both early and late NO_2 were applied (cf. NON vs. ~O~; $P<0.1$).

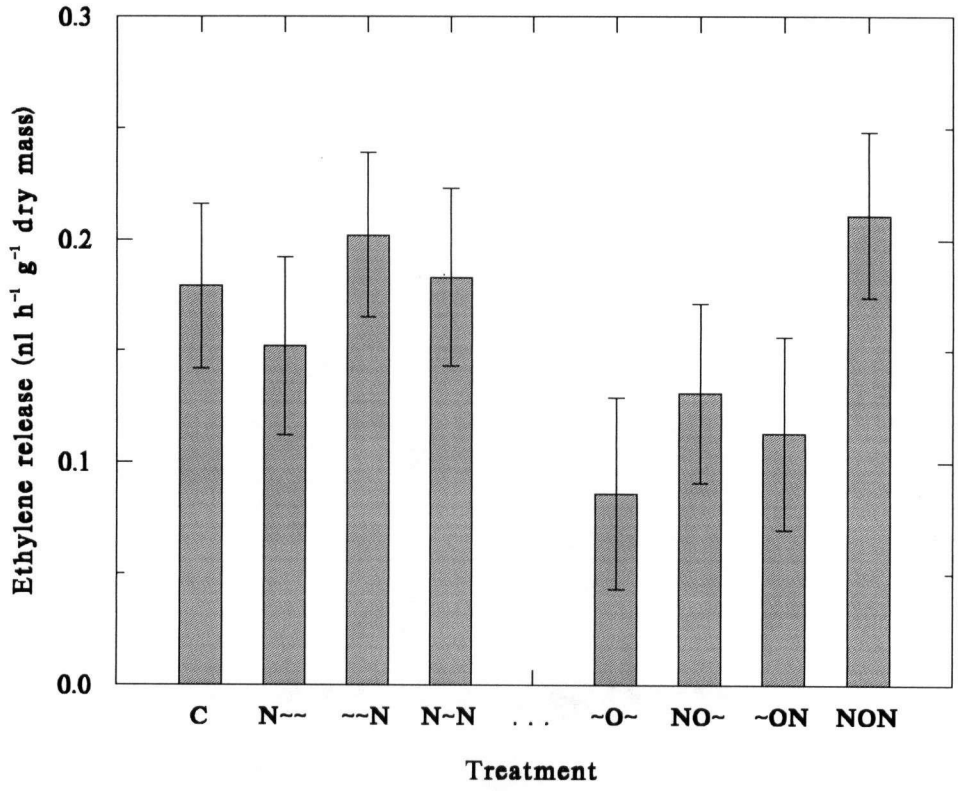


Fig. 3.21. Effects of treatment on ethylene release. Error bars are standard errors (n=8).

3.4 Discussion

The present study employed fluctuating exposures, to both NO_2 and O_3, in which the maximum concentration (120±5ppb) was maintained for approximately one hour at the middle of the exposure period (4h for NO_2; 8h for O_3). The sine-wave time course used resulted in total exposures to either pollutant that amounted to one half of those that would have occurred with "square-wave" exposures in which 120ppb was maintained throughout the exposure periods. The exposures used in the present study were within the ranges of the maxima for NO_2 and O_3 experienced in the region (Table 3.2, Section 3.2.1). 120ppb NO_2 is below the Canadian Maximum Acceptable 1-hour objective, while 120ppb O_3 is about 50% greater than the Canadian 1-hour objective, and equal to the U.S. standard (Table 3.1, Section 3.2.1). Since these levels were adopted as being sufficient to protect vegetation from significant adverse effects, the exposures used in the present study would be expected to be close to the thresholds for adverse effects.

In spite of the low exposures used, repeated exposures to NO_2 regardless of the time of day were found to increase significantly (or tend to increase) total, shoot, and hypocotyl dry matter accumulation (Figs. 3.5-3.7) without significantly affecting the shoot/root ratio (Fig. 3.8). No visible symptoms of foliar injury were observed, which is in keeping with the reports that such NO_2-induced injury is uncommon in radish (see review by Kostka-Rick and Manning, 1992) even when "sudden" and relatively high concentrations are applied as square waves (200-400ppb: Reinert and Gray, 1981; 400ppb: Reinert and Heck, 1982; 3000: Sanders and Reinert, 1982; 200ppb: Godzik et al., 1985). In all these studies the tendency was for NO_2 to cause increased biomass. Using relatively lower early exposure concentrations (80-100ppb for 3h) daily for 40 days, Runeckles and Palmer (1987) also found that NO_2 increased the biomass of radish and bean (*Phaseolus vulgaris*). The observations in the present study thus confirm previous work and suggest that NO_2 was probably used as an additional source of N as was suggested in spinach (*Spinacia oleracea*) (Yoneyama and Sasakawa, 1979).

In contrast, repeated exposure to waves of O_3 alone had no significant effect on any of the components of dry matter nor did the O_3 exposures result in any visible injury to the leaves. The lack of growth inhibition by O_3 observed in this study was unexpected since radish is ranked as 'sensitive' relative to other crops (Tingey et al., 1973a; Ormrod et al., 1984) and the cultivar used, Cherry Belle, is considered a sensitive cultivar (Wellburn and Wellburn, 1996). Ozone-induced reduced growth in radish has been widely documented (Reinert and Heck, 1982; Reinert et al., 1972; Tingey et al,. 1973a, Reinert et al., 1982; Reinert and Sanders, 1982; Kostka-Rick and Manning, 1992). These studies, however, also tended to involve higher concentrations of O_3 (>200ppb) than those used in the present investigation. In some cases where concentrations in the range 150-370ppb were applied, no biomass changes were observed despite 13-26% leaf injury (Tingey and Reinert, 1975; Ormrod et al., 1983; Reinert and Gray, 1981). The present results therefore suggest that, because of the type of exposure profile used and the maximum concentration achieved, the actual uptake of O_3 was insufficient to exceed the threshold for adverse effects on radish dry matter production, in spite of the effects on stomatal conductance and photosynthesis discussed below.

The combinations of O_3 and NO_2, irrespective of sequence, caused no visible injury but either significantly suppressed or tended to suppress the stimulatory effect of NO_2 and confirmed the previous reports for radish exposed to the NO_2-O_3 sequence (Runeckles and Palmer, 1987) or to NO_2+O_3 mixtures (Reinert and Sanders, 1982; Sanders and Reinert, 1982; Reinert et al., 1982). Suppression of NO_2-induced stimulation of growth by O_3 was also reported in wheat and bean (Runeckles and Palmer, 1987).

Diffusive resistance measured after 6 days of exposure was increased by NO_2 regardless of sequence (Fig. 3.9). NO_2-induced stomatal closure has been reported for other species such as soybean, (*Glycine max*, Carlson, 1983), and *Euonyous japonica* (Natori and Totsuka, 1984), although Carlson (1983) used higher exposure levels (up to 600ppb) than those used in the present study. However, Sandhu and

Gupta (1989) reported decreased stomatal resistance in bean (*Phaseolus vulgaris*) exposed daily to 100 NO_2 ppb for 7h per day for 3 weeks.

Ozone suppressed stomatal closure and disrupted the apparent diurnal cycle of stomatal activity. This response of radish to O_3 was contrary to the generally held view that O_3 induces stomatal closure even at relatively low concentrations (e.g. Beckerson and Hofstra, 1979; 150ppb; Barnes and Pfirrman, 1992; 75ppb). However, the review by Darrall (1989) lists O_3-induced stomatal closure in four species (one of which was radish), opening in two, and no effect in one, at concentrations <200ppb. In the present study, the repeated daily exposure to daily maxima of 120ppb O_3 apparently caused the stomata to lose their ability to respond to the daily photoperiod with closure early and late in the day. This confirms the findings by Hassan et al. (1994) who recently reported stomatal opening in radish caused by 80ppb O_3 and those of Runeckles and Rosen (1977) who found that repeated exposure to 50ppb caused stomatal response in bean (*Phaseolus vulgaris*) to be sluggish with suppressed diurnal opening-closing cycles.

In the sequences of NO_2 and O_3, repeated exposures to O_3 countered the NO_2-induced stomatal closure, leading to diffusive resistances during the morning and evening that were intermediate between those in the control and those exposed to O_3 alone. As a result of these effects on diffusive resistance, the estimated flux of NO_2 was increased when followed by O_3 (during the previous daily cycles) or preceded by O_3 (Fig. 3.10). Hence although O_3 alone had no significant effect on growth, it suppressed the growth stimulation induced by NO_2, in spite of an approximate doubling of the amounts of NO_2 taken up by the leaves. Conversely, since the effects of NO_2 were to increase diffusive resistance early and late in the day, NO_2 exposures had no apparent effect on O_3 flux.

The observed effects on growth and diffusive resistance lead to several conclusions. First, despite the fact that NO_2 alone caused increased stomatal closure early and late in the day, relative to the control, the increases in dry matter production must have resulted from increased rates of CO_2 assimilation.

Stimulations of apparent photosynthesis by low levels (100 ppb) of NO_2, based on CO_2 uptake, have been reported by Sabaratnam et al. (1988b) and Sandhu and Gupta (1989) although Carlson (1983) reported no effects with exposure as high as 600 ppb. Inhibition of net photosynthesis by high exposure levels has been reported by several workers (see review by Wellburn, 1990), and occasionally for low levels (50 ppb; Bull and Mansfield, 1974).

Second, the O_3-induced suppression of the normal pattern of stomatal closure suggests that CO_2 assimilation would not be limited by any stomatal-dependent reduced CO_2 flux throughout the whole photoperiod with constant illumination and hence might be greater than in the controls in filtered air if O_3 did not inhibit the photosynthetic process. However, O_3-induced inhibition of net CO_2 assimilation has been widely reported, as has O_3-induced stimulation of dark respiration although responses of dark respiration are varied, with several reports of O_3-induced inhibitions (see review by Runeckles and Chevone, 1992). Amthor (1988) and Amthor and Cumming (1988) partitioned respiration into its growth and maintenance components and reported significant O_3-induced increases in maintenance respiration in bean (*Phaseolus vulgaris*). Hence, under the exposure conditions used in the present study, any potential for increased CO_2 assimilation resulting from the constant illumination photoperiod and reduced stomatal closure may have been negated either by direct inhibition of photosynthetic CO_2-fixation or by increased diversion of assimilation to maintenance, or both.

Third, if no NO_2-induced stimulation of CO_2 assimilation persisted in the presence of O_3, the tendency for the stomata to remain open in the NO_2-O_3 sequences in contrast to exposures to NO_2 alone suggests that dry matter accumulation should have been further stimulated. Conversely, the stomatal closure induced by NO_2 relative to O_3 suggests that, if the reduction in CO_2 assimilation or increase in assimilate utilization for repair resulting from O_3 alone were maintained then growth should have been further reduced. The observations that growth in NO_2-O_3 sequences was consistently less than in NO_2 alone therefore suggests that the inhibition of CO_2 assimilation or stimulation of assimilate utilization

for repair induced by O_3 overcame the NO_2-induced stimulation of CO_2 assimilation.

The observations of the effect of treatment on chlorophyll fluorescence parameters provide additional information on the mechanisms involved. First, comparison of the data sets with or without O_3 exposure for growth (Fig. 3.5), quantum yield and photochemical quenching (Fig. 3.16) show similar patterns of change resulting from NO_2 exposure. The changes in quantum yield and qP are virtually identical since Fo was not affected by treatment (Fig. 3.11). Relative to the control, early and early + late NO_2 resulted in stimulations with late NO_2 resulting in little or no change. In contrast, relative to O_3 alone, early, late and early + late NO_2 in combination with O_3 resulted in progressive reductions in these variables. However, comparison of the $\pm O_3$ sets shows that, since growth in the control and O_3 alone treatment were the same, the combinations of O_3 with NO_2 led to reduced growth relative to the control, in contrast to the stimulations of quantum yield and photochemical quenching observed in all of the combinations with O_3, relative to the control. Stimulation of quantum yield and photochemical quenching were reported by Godde and Buchhold (1992) in *Picea abies* following several days exposure to 200ppb O_3.

Based on the kinetics of fluorescence rise in dark-adapted bean leaves, Schreiber et al. (1978) attributed adverse effects of O_3 on decreased water-splitting in photosystem II. However, these effects were found using square-wave exposures to higher concentrations (250-500 ppb). Although no comparable measurements of fast kinetics were made in the present study, preventing an analysis such as that conducted by Schreiber et al., the lack of O_3 effects on dark-adapted quantum yield (Fig. 3.12) or Fo (Fig. 3.11) observed before the start of the O_3 exposure suggest that the lower O_3 levels used in the present study, did not lead to any residual effects on water-splitting activity.

The overall effect of O_3 treatment observed was to stimulate quantum yield and qP, both of which are indicative of increased photochemistry (Genty et al., 1989). Since photochemical quantum yield and the quantum yield of CO_2-fixation have been found to be linearly related (Genty et al. 1989) the

stimulation of photochemical quantum yield would be expected to result in increased photosynthesis and growth.

Because of the separation of photochemistry from the dark reactions of photosynthesis, increased photochemistry could well result in increased O_2-evolution, independent of any effects on CO_2-fixation. The effects of O_3 on photosynthetic O_2-evolution have rarely been studied, but Rowland-Bamford et al. (1989) reported stimulation in O_2-evolution in barley exposed for 12 days (6h per day) to steady 50ppb O_3, although inhibitions were observed with exposures to concentrations of 100 and 150ppb. They also report that quantum yield based on O_2 release was decreased by 50ppb O_3, but close inspection of their data (their Figure 6) suggests that the O_2-based quantum yields they report for the control and 50ppb treatments were interposed, and that an increase due to 50ppb existed, in agreement with that suggested in the present study, although decreases were caused by higher exposure levels. Rowland-Bamford et al. (1989) also observed a non-significant increase in shoot dry mass in 50ppb O_3, but CO_2 exchange rate was slightly inhibited.

Several studies have shown that the activity of rubisco (ribulose *bis*phosphate carboxylase/oxygenease) is slowly but significantly inhibited by low levels of O_3 (Dann and Pell, 1989; Pell et al., 1992). Bahl and Kahl (1995) observed that O_3 rapidly reduced the mRNA levels for the gene encoding the small subunit of rubisco (rbcS), although reduction in rubisco activity was only reported to occur after 48-72h. However, the significant decrease reported by Pell et al. (1992) in radish only appeared after 21 days, the age at which the fluorescence measurements were made in the present study, and hence may not be germane. Indeed, in one year of their study, they observed a slight stimulation of rubisco activity on day 22 (14 days after the start of daily O_3 exposure). However, their observations of inhibited net photosynthesis only commenced five days later.

In view of the lack of the effect of O_3 on growth observed in the present study, the only conclusion possible therefore appears to be that increased CO_2-assimilation (suggested by changes in

photochemical quantum yield and qP) was nullified by increased maintenance/repair respiration. There is no reason to beleive that the products of the apparently increased CO_2-fixation due to O_3 exposure were utilized in other processes such as N-assimilation.

With the NO_2 exposures, the increases in growth, quantum yield and qP are compatible with the suggestion of increased CO_2-assimilation, resulting from the uptake of NO_2 early in the photoperiod, in spite of reduced stomatal conductance early and late in the day (Fig. 3.9). However, it is not possible from these data alone to elucidate the mechanisms involved. Although Holmes et al. (1989) showed that in N-limited cultures of the alga, *Selenastrum minutum*, provision of NO_3^- effectively caused cessation of CO_2-fixation until the supply of NO_3^- was exhausted, such a mechanism appears unlikely in the present studies in which the plants were well supplied with N.

The effects of NO_2 alone, both on growth (Fig. 3.5) and fluorescence parameters (Fig. 3.12), show that late NO_2 exposures caused no significant effects relative to the control, although the estimated flux of NO_2 was comparable to those observed in other NO_2 treatments (Fig. 3.10). The NO_2 was taken up during the exposure in the light, and would result in the formation of nitrate and nitrite on dissolution in water. Since the plants had been maintained in light up to and during this exposure the levels of nitrate and nitrite reductases should have been close to maximal, up to the end of the photoperiod. However, late NO_2 failed to result in a significant stimulation of photochemistry in the chloroplast but caused significant residual increases in non-photochemical quenching (both qN and NPQ; Fig. 3.16) observed during the middle of the day, responses which imply decreased efficiency of the photochemical processes that might account for the lack of growth effects. Such increases may have resulted from perturbation of the pH status of the thylakoid membrane caused by the accumulation of nitric and nitrous acid (Wellburn, 1990).

In spinach leaves, Schmidt et al. (1990) reported that effects of exposures to massive (5 000ppb) concentrations of NO_2 lasting up to 24h on chlorophyll fluorescence could only be observed if the

exposures occurred in darkness. They attributed the lack of responses in light to the effective removal of NO_2^- by nitrite reductase.

Whatever the mechanism by which NO_2 alone may have stimulated photosynthesis, it was suppressed by the mechanism involved in the suggested O_3-induced effect. If the effects of O_3 alone on growth were the result of both increased CO_2-fixation and respiration then the tendency for additional exposures to NO_2 to reduce growth and the fluorescence parameters relative to O_3 alone suggest that the effects were on CO_2-assimilation rather than on maintenance/repair respiration.

Ethylene release was not affected by NO_2 although early NO_2 tended to reduce and late NO_2 tended to increase it. A significant NO_2-induced increase in ethylene caused by short-term exposure (150ppb) has been observed in pea (*Pisum sativum* L.) without the appearance of foliar injury (Mehlhorn and Wellburn, 1987).

On the other hand, O_3 alone significantly decreased ethylene release relative to the control. Exposure to O_3 has usually been associated with increased ethylene release in radish (Wellburn and Wellburn, 1996), tomato (*Lycopersicon esculentum*, Bae et al,. 1996), tobacco (*Nicotiana tabacum*, Langebartels et al., 1991), Pinto beans and tobacco (*Phaseolus vulgaris* and *Nicotiana tabacum*, Mehlhorn et al., 1991) and pea (*Pisum sativum*: Mehlhorn and Wellburn, 1987). All of these increases were observed following one or more days of exposures to steady-state concentrations (>150 ppb) of plants that had previously been kept in filtered air. These exposures led to significant necrotic injury to the leaves. However, following 3-week exposures to O_3, decreased ethylene release was observed in pea (Melhorn and Wellburn; 1987). In this study no foliar injury was observed following these exposures, although severe injury resulted from single exposures of plants of the same age previously maintained in filtered air.

The possibility that the low release of ethylene observed in O_3-treated plants in the present study was

the result of the reaction of ethylene with residual O_3 is unlikely. The O_3 supply to the chambers was turned off and the plants removed to the greenhouse bench before the tops were removed. This procedure took sevseral minutes, during which any O_3 in the intercellular spaces of the leaf would have disappeared.

In the present study repeated exposure to NO_2-O_3 sequences tended to counter the O_3-induced inhibition of ethylene release relative to the control. A similar response was observed in pea, in which, although long-term exposure to O_3 decreased ethylene release, short-term exposure to mixtures of NO_2 and O_3 (50, 100 or 150ppb, each) resulted in stimulations of ethylene release relative to filtered air controls (Mehlhorn and Wellburn, 1987). However, such mixed exposures also resulted in significant foliar injury. Since none of the exposure sequences used in the present study resulted in any visible foliar injury, the ethylene responses observed appear to be effects on "healthy" leaf tissue, suggesting that the reported O_3-induced stimulations may be the consequence of actual or incipient necrosis.

In summary, the exposures to NO_2 and O_3 sequences used in the present study led to effects on growth, diffusive resistance and chlorophyll fluorescence that suggest increased CO_2-assimilation that was balanced by increased maintenance/repair respiration resulting from O_3 exposures. Although increased CO_2-assimilation could account for increased growth resulting from NO_2 exposures, O_3 reduced the NO_2-induced stimulation of CO_2-assimilation. The observed reduction in ethylene release resulting from O_3 and its stimulation by NO_2 appear to be responses attributable to healthy leaf tissue, since no treatment resulted in foliar injury. Although no effects of O_3 alone could be demonstrated on radish growth, the effects of the different sequences of exposures to NO_2 and O_3 show clearly that observations made on individual pollutants may not reflect effects occurring in exposure situations that more closely resemble those likely to occur in polluted ambient air.

4. GRASS STUDIES.

4.1 Introduction

Ozone-induced effects on plants range from clear injury symptoms such as chlorosis or necrosis to subtle metabolic changes (Heath, 1988) resulting in reduced photosynthesis leading to reduced growth and yield (see review by Runeckles and Chevone, 1992). Based mainly on theoretical grounds toxic oxyradicals such as superoxide anion and hydroxyl have been implicated in plant response to O_3 exposure because of the possibilities of their formation during reactions of O_3 with cell constituents and their high reactivity and potential for membrane perturbation (Mudd, 1982).

Superoxide is produced normally in the photosynthetic electron transport system, but is scavenged by superoxide dismutase (SOD) and other antioxidants such as ascorbic acid, peroxidase, glutathione and α-tocopherol (Asada and Takahashi, 1987) thereby reducing its potential toxicity. Hence superoxide-induced adverse effects are thought to be a result of reduced scavenging, elevated production or both.

Until recently, evidence for the involvement of superoxide in O_3 phytotoxicity has been circumstantial and mainly based on observations of the activity of SOD. Increased SOD activity as a result of exposure to O_3 was reported by Lee and Bennett (1982) in bean (*Phaseolus vulgaris*) and Decleire et al. (1984) in spinach (*Spinacia oleracea*) leaves. Reports of decreased SOD levels also exist (*Spinacia oleracea*; Sakaki et al., 1983), and Chanway and Runeckles (1984) only detected increased SOD in bean leaves at the onset of necrosis. However, Runeckles and Vaartnou (1997) recently presented evidence for transient superoxide formation in grass and radish leaves exposed to 100ppb O_3, based on electron paramagnetic resonance (EPR) spectroscopic evidence.

EPR provides a unique means of direct detection and characterization of free radical spectra. Two free radical signals associated with the photosynthetic apparatus can usually be detected in intact leaves *in situ* (Runeckles and Vaartnou, 1992). One, which is relatively stable and persists in darkness (Signal II) and is attributable to tyrosine residues on the D1 and D2 peptides of photosystem II (Barry and Babcock, 1987). A second distinct Signal (I) appears upon illumination with white light, and is usually enhanced in far-red light (>700nm). Signal I is thought to be produced by oxidized chlorophyll P700 in photosystem I (Beinert et al., 1962), and disappears rapidly when illumination ceases. In 710nm illumination, Signal I is indicative of cyclic electron flow in photosystem I.

Spin traps are compounds which form relatively stable radical adducts. The use of selective spin traps allows the detection and identification of some free radicals in circumstances where their concentration is too low or reactivity too great for normal EPR detection. For the detection of hydroxyl and superoxide radicals *in vitro*, extensive use has been made of the nitrone spin traps, DMPO (5,5-dimethyl-1-1-pyrroline-1-oxide) and PBN (phenyl-N-*tert*-butylnitrone) or its analogue, 4-POBN (α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone). The adducts formed with hydroxyl or superoxide anion radicals and many carbon-centered radicals have characteristics (*g*-values, hypefine splitting constants) that permit their identification. Using DMPO, Harbour and Bolton (1975) reported evidence for superoxide adduct formation in illuminated spinach chloroplasts. However, Grimes et al. (1983) could only find evidence for the DMPO-OH· adduct when an *in vitro* buffered system containing phenol or substituted cinnamic acids was treated with O_3. Furthermore, several workers have pointed out the likelihood of the DMPO adduct with superoxide decomposing to form DMPO-OH· (Kaur and Halliwell, 1994). Although these and other spin traps have been widely used in *in vitro* investigations of radical formation in organelles and fragments of plant and animal cells, reports of their use in whole plant tissue seem to be limited to the single study by Mehlhorn et al. (1990).

Chlorophyll fluorescence measurements have been used by several workers studying the effects of O_3 on a range of species following the early report of Schreiber et al. (1978). Since changes in such fluorescence are attributable to changes in photosystem II, the combination of EPR and fluorescence measurements permits the simultaneous observation of effects of O_3 on both photosystems.

The objectives of this study were therefore to elucidate the effects of O_3 on photosystem functioning in intact leaf tissue by means of EPR measurements of free radical intermediates in Photosystems I and II, and chlorophyll fluorescence transient kinetics, and to use selective infiltrated spin traps to attempt to detect the presence of O_3-induced free radicals. Since it is impossible to follow changes in EPR signals in excised leaf tissue over several hours because of the appearance of a large signal attributable to tissue wounding that appears after about one hour (Runeckles and Vaartnou, 1992), the studies were confined to intact, attached grass leaves. Little information is available on the differences in response to O_3 of

C-3 and C-4 species. Hence the studies were conducted on species representative of each type, Kentucky bluegrass (C-3) and green foxtail (C-4), respectively.

4.2 Materials and methods

4.2.1 Plant material and growing condition

Kentucky bluegrass (*Poa pratensis* L.) and green foxtail (*Setaria viridis* Beauv.) plants were grown from seed in 5cm pots containing standard potting soil as described in Section 3.2.2. About 50 seeds were sown per pot and these were thinned to 4 plants per pot, 5 days after sowing. The plantings were staggered in order to provide plants of the same age for the experiments. The plants were grown on the greenhouse bench at 25 ±3C and maximum illumination of 500 $\mu mol\ m^{-2}\ s^{-1}$ (PAR). Green foxtail plants were exposed to O_3 at 4 weeks and Kentucky bluegrass at 6 weeks after sowing.

4.2.2 Exposure to O_3

A similar exposure regime was used to that described for O_3 in Section 3.2.1. The exposures were carried out within the quartz dewar insert in the cavity of the EPR spectrometer (see Section 4.2.3). Ozone levels approximated sine waves and were controlled by a 21X Micrologger (Campbell Scientific Inc) using the program as described in Section 3.2.1. Exposure commenced at 10:00h and the peak (120 ppb) occurred at 14:00h. To maintain a moist airflow over the leaf, compressed air from a cylinder of medical grade air was bubbled through distilled water. The flow rate was approximately 2 l min^{-1} through the quartz insert. Ozone was generated in the air stream by means of an ultraviolet source (Delzone Z0.300; Del Industries) and was monitored by sampling through a 3mm OD Teflon tube feeding to a Model 1003-AH O_3 monitor (Dasibi Environmental Corp.).

Illumination was supplied from a slide projector to the leaf through the slits in the cavity. Total white light intensity within the cavity was estimated to be 530 μmol $m^{-2}s^{-1}$(PAR), obtained by halving the measured intensity at the surface of the cavity as per manufacturers's specifications. For far red light, obtained by using an optical bandpass filter (PTR Optics Ltd.), with 85% transmission at 710nm, the total intensity was estimated to be 10μmol $m^{-2}s^{-1}$. The cavity was at room temperature (24±3C).

4.2.3 Electron paramagnetic resonance (EPR)

EPR spectra were obtained using a Varian E-line X-band spectrometer (Varian). A model 5256A frequency converter (8-18GHz) (Hewlett.Packard) was used to measure frequency and a gaussmeter (Varian) to measure magnetic field strength. An E102 microwave bridge (Varian) generated microwaves. The microwave generator was connected to an E238 TM110 cavity (EPR cavity) via a 3 cm wide waveguide. An overview of the cavity and insert is shown in Fig. 4.1.

Although preliminary observations were made using a Hewlett-Packard computer for data processing, malfunctions required that it be replaced. The improved configuration used an XT computer

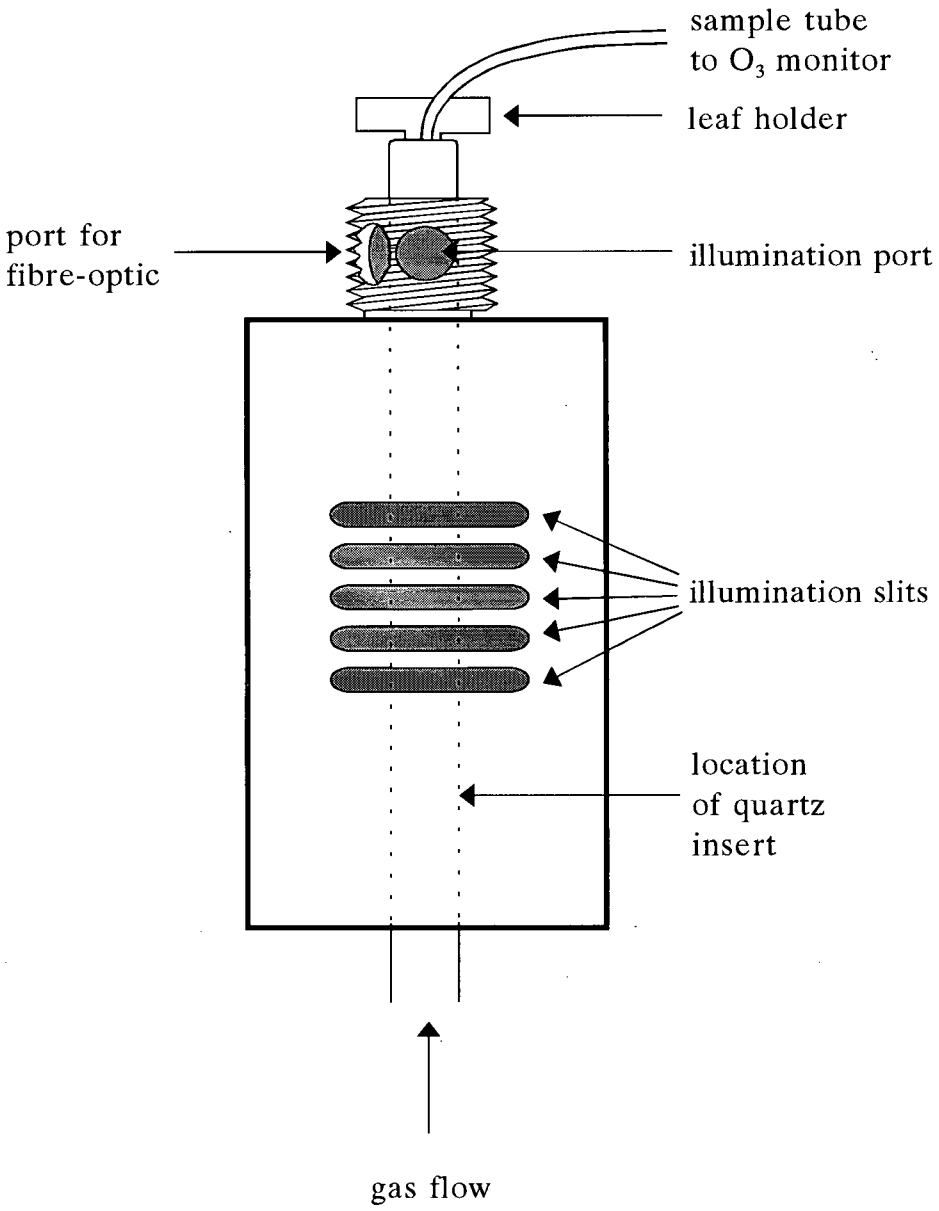


Fig. 4.1 The microwave cavity showing the adaptation of the threaded collar to accommodate the fibre-optic cable from the fluorometer, and provide an additional illumination port.

(PB8810; Packard Bell) and computer EPR interface (UBC Chemistry Dept.). Dr. Paul Phillips (Okanagan University College) developed the EPR software. The new system allowed accurate measurement of microwave frequency and magnetic field strength. The data collected were stored in the computer for analysis (see section 4.2.5).

The arrangement for positioning the end of an attached leaf within the quartz insert was described by Runeckles and Vaartnou (1992) and involved the loose attachment of the leaf to a T-shaped, cellulose acetate holder by means of cellulose adhesive tape with the lower leaf surface against the holder and the upper surface facing the cavity slits as shown in Fig. 4.2. At each hour the following EPR signals were recorded: first the signal in white light, then the signal in 710nm light and lastly the signal in darkness. Between each spectrum a 2 min period was allowed for adaptation. Each spectral measurement took 4min. Spectra were recorded between 11:00 and 16:00h. The leaves were maintained in white light between measurements. For spectral recording the EPR conditions employed were:

Sweep width:	250G,
Scan time:	4min,
Time Constant:	0.128s,
Power:	10mW
Detector current:	100μA,
Gain:	$5x10^{-4}$

4.2.4 Infiltration of spin traps into leaves

The spin traps, DMPO and 4-POBN (Sigma) were vacuum-infiltrated into leaves by dipping almost all of the attached leaf into a vial of spin trap (20mM in distilled water). The vial plus the attached leaf was placed in a 13 L desiccator and a vacuum applied using an aspirator. The pressure was reduced

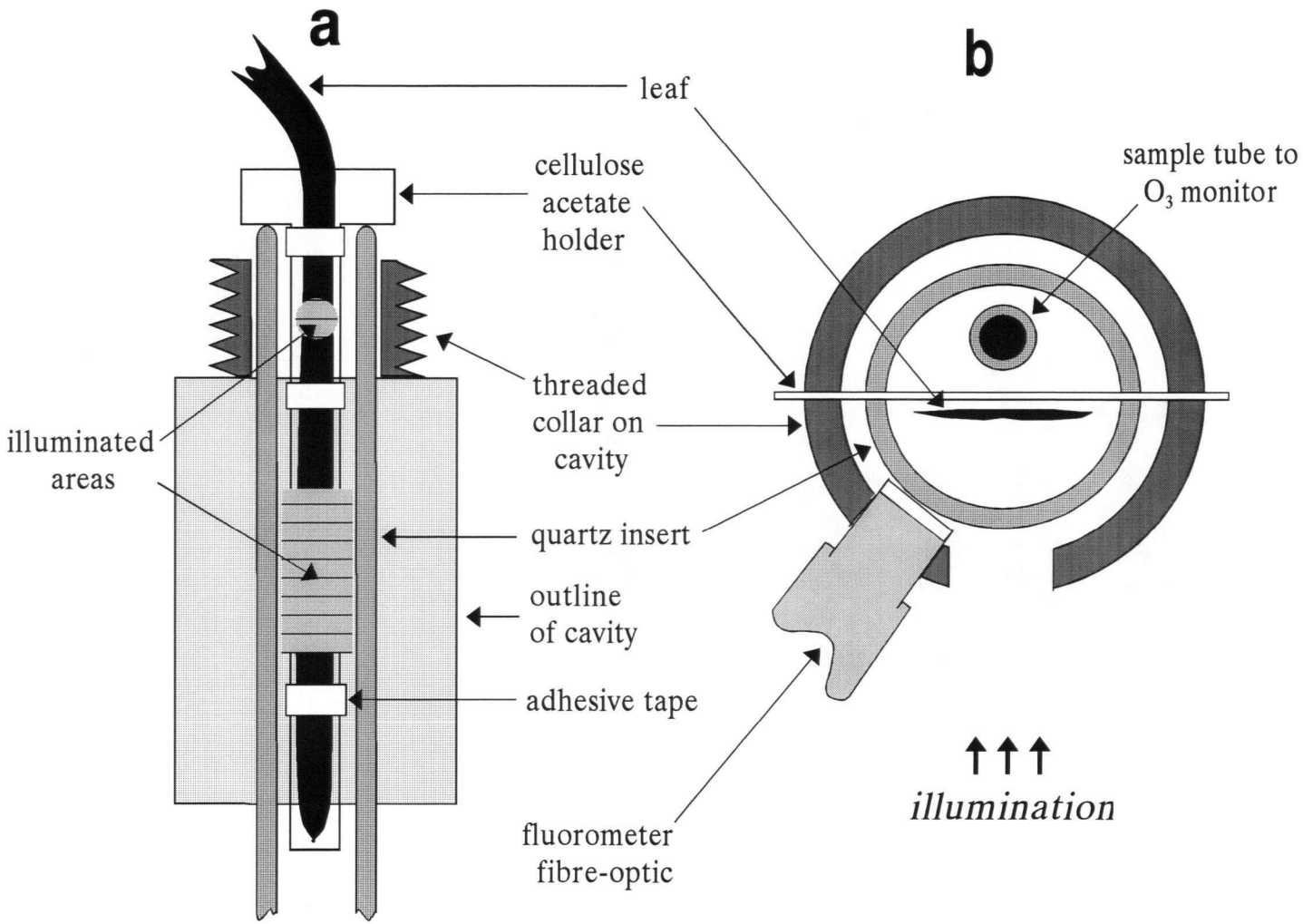


Fig. 4.2 Longitudinal (a) and cross-sectional (b) views of the cavity, showing the arrangement of the leaf and leaf holder,the ozone sampling tube, the fluorometer fibre-opitic, and the illuminationed areas of the leaf.

from 0 to -100kPa in 15 min, and was maintained at -100kPa for a further 20 min. The gauge pressure then was slowly released over 25 min, and the plants were held for a further 30min before the treated leaf was mounted in the EPR cavity. All these steps were done in room light. Control plants were vacuum-infiltrated with deionized distilled water.

4.2.5 Experimental design, spectra data handling, normalization and statistical analyses

Four experiments were conducted with each species. In each experiment, the four treatments were, water-infiltrated ± O_3, and spin trap-infiltrated ± O_3. Hence for the water-infiltrated control and O_3 treatments n = 8, while n = 4 for each spin trap and spin trap + O_3 treatment, for each species.

Typical first-derivative EPR spectra in Kentucky bluegrass are shown in Fig. 4.3, illustrating the measurement of signal heights and showing *g*-values. In order to remove leaf-to-leaf variability the EPR signal height data were normalized to the value at 1100h (i.e.1h after the start of the O_3 exposure wave). Additional experiments were conducted to examine the kinetics of Signal I changes resulting from changing light conditions. In these experiments, the leaves were mounted in the cavity at 10:00h and maintained in white light until the traces were recorded at 14:00h. Since the O_3 exposure wave started at 10:00h these traces were recorded at the time of the peak (120ppb). Traces like the one shown in Fig. 4.4 were collected with the system locked onto the low-field maximum of Signal I and the leaf in darkness or illuminated with 710nm or white light in various sequences. Since the low field maximum of the first derivative of Signal I occurs at approximately the same field strength as that at which the first derivative of Signal II is close to zero, i.e. the maximum of the Signal II absorption spectrum, the effect of any changes in signal II on the amplitude of the first derivative of Signal I is minimal (Blumenfeld et al., 1974). Each of the traces for a specific treatment (± O_3; ± spin trap) were obtained using different leaves and hence were subject to leaf-to-leaf variability. No normalization of

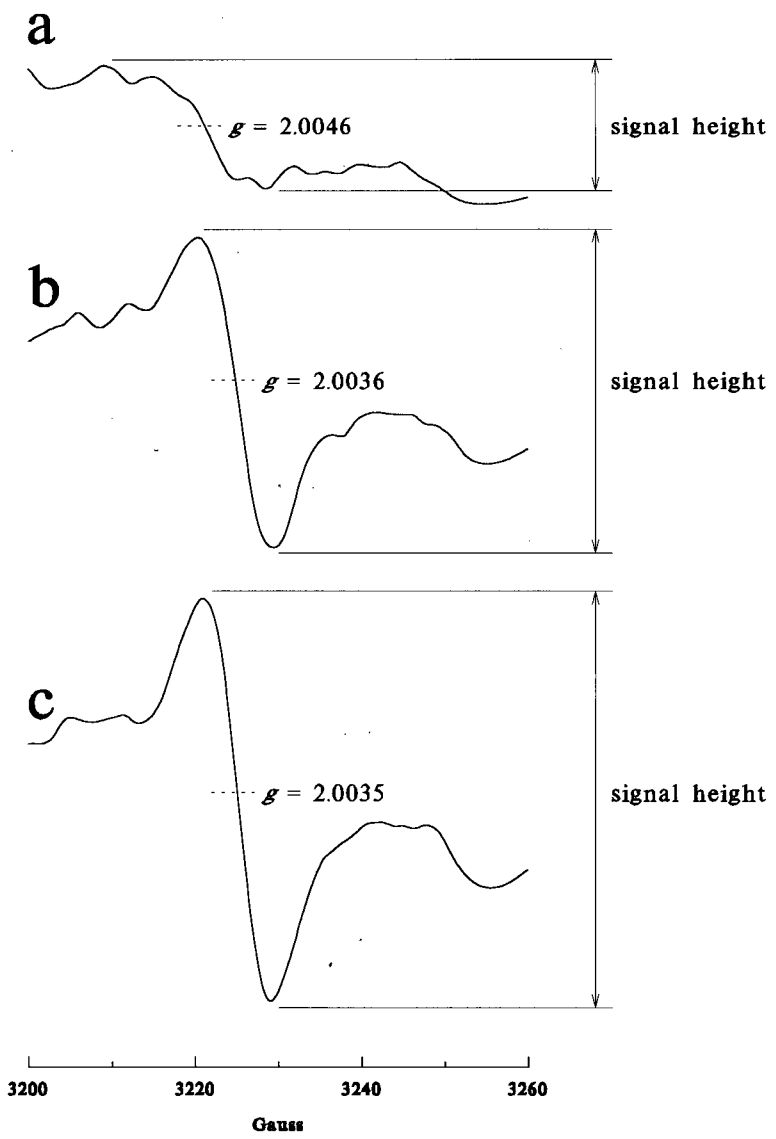


Fig. 4.3. Typical first derivative signals in a bluegrass leaf in a, darkness; b, white light; c, 710nm light, illustrating signal height measurements and *g* values (±0.0002).

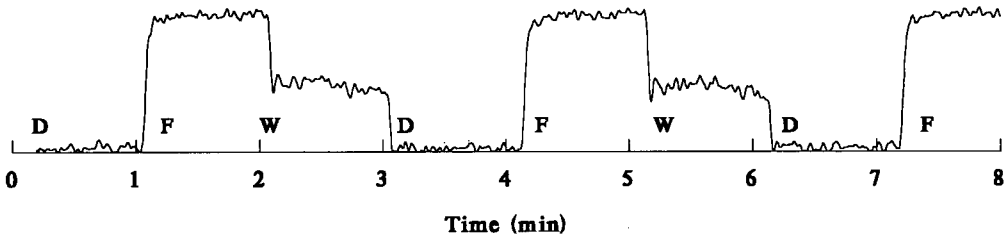


Fig. 4.4 Trace of Signal I lowfield maxumum in a bluegrass leaf in changing light conditions. D = darkness; F = 710nm light; W = white light.

these was possible, to remove this variability. As a result, between-treatment comparisons of signal heights in specific traces may not appear to show the same effects as those observed from the normalized means.

SYSTAT/SYGRAPH (Systat Inc.) was used for all statistical analyses and graphics. The DWLS (distance weighted least squares) function of SYGRAPH was used for curve-fitting to depict EPR and fluorescence data. Unless otherwise stated the trends depicted were established by polynomial contrasts.

4.2.6 Fluorescence measurements

Fluorescence measurements were made on leaves mounted within the EPR cavity using the PAM2000 Fluorometer as described in Section 3.2.6. Since it was not possible to modify the cavity itself, the threaded section at the top of the cavity was adapted to accommodate the fibre optic cable of the fluorometer (Figs. 4.1 and 4.2). A port for the illumination of the part of leaf viewed by the fibre-optic was provided to ensure that the same light intensity was used for both fluorescence and EPR measurements. The fluorescence parameters were measured as previously described in Section 3.2.6. These measurements were taken at the same time as EPR measurements at hourly intervals from 11:00 to 16:00h.

In order to remove leaf-to-leaf variability the fluorescence parameters were normalized to the value at 11:00h, as described for EPR spectral data in Section 4.2.5. An example of the normalization is shown in Fig. 4.5.

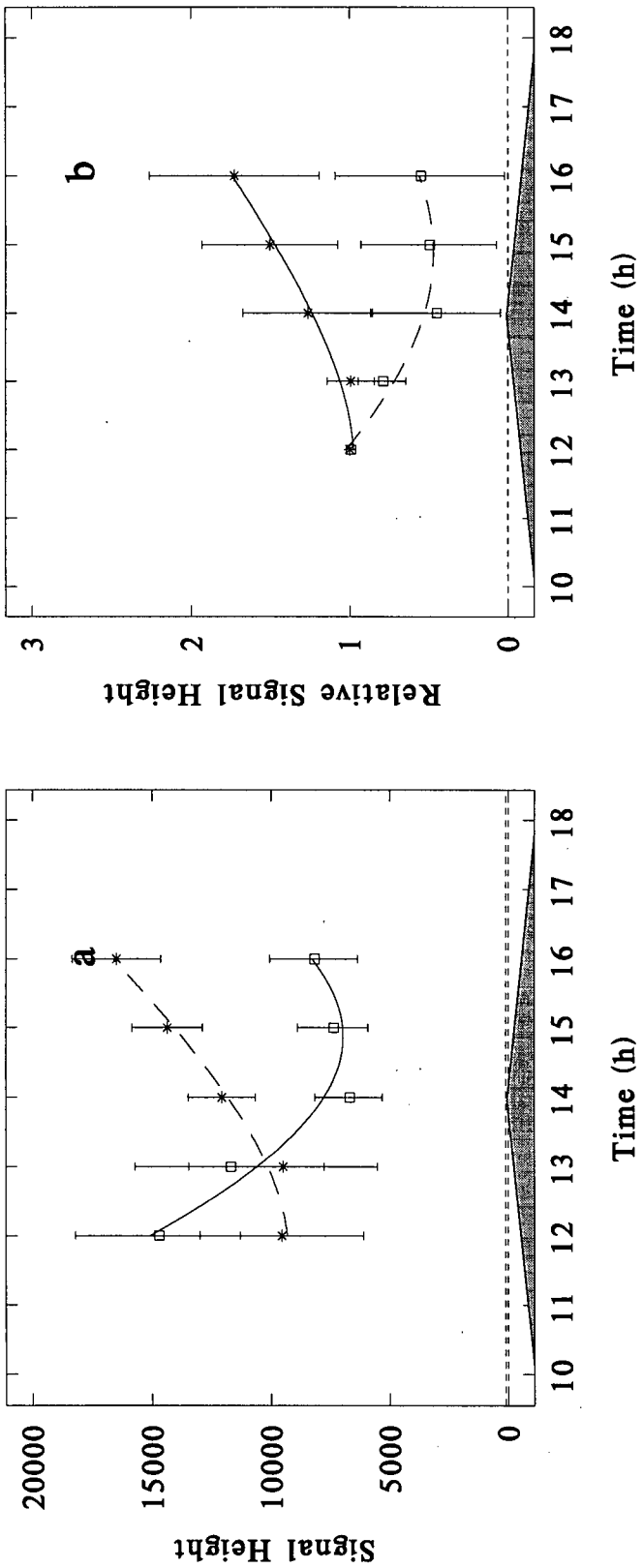


Fig. 4.5 The signal height for bluegrass vacuum-infiltrated with 20mM 4-POBN (squares) and exposed to O_3 (stars): a. before and b. after normalization to 11:00h. The shaded triangle indicates the O_3 exposure period. Error bars are standard errors (n=4).

4.3 Results

4.3.1 Kentucky bluegrass

4.3.1.1 Effects of O_3 on photosynthetic radicals

Ozone led to distinct increases in the relative height of the signals obtained in darkness (Signal II), white light (Signal I + II) and 710nm light (Signal I + II) (Fig. 4.6). In the control the signals tended to decrease slightly with time. Two hours after the maximum O_3 concentration was reached (14:00h) the relative heights of the signals in white light and darkness were approximately 3 times the control. The signal in 710nm light behaved similarly but O_3 only resulted in a doubling of the relative height. It should be noted that the absolute signal heights in darkness were much less than in either white or far-red light. While the absolute heights of the white light signals were only about one half of the absolute heights of the 710nm light signals in the control, in O_3 their absolute heights were approximately equal and both were significantly greater than the 710nm light signals in the control.

The general relationship between signal heights in white and 710nm light is shown in the traces obtained in kinetic studies of Signal I observed at 14:00h illustrating the effects of switching among darkness (D) and illumination in white (W) or 710nm (F) light (Fig. 4.7). A summary of the observations in four replicate experiments is presented in Table 4.1. As expected, in Kentucky bluegrass the signal in 710nm light (F) was larger than the signal in white light (W) in the control. In the control, W caused a rapid increase in Signal I, which dropped back to a lower level within 10s, from which it tended to decline slowly. The "overshoot" of the signal at the start of W occurred after D. Ozone stimulated the signal in W so that the two signals were approximately equal (Fig. 4.7 and Table 4.1). The saturation of the signal in W was slowed by O_3 (Fig. 4.7 and Table 4.1) following a rapid initial rise to a level comparable to the control. Ozone caused a slower decay of the signal on switching from F to D relative to the control, after an initial rapid decline. In contrast, switching from W to D in O_3 resulted in a rapid decline to the baseline signal.

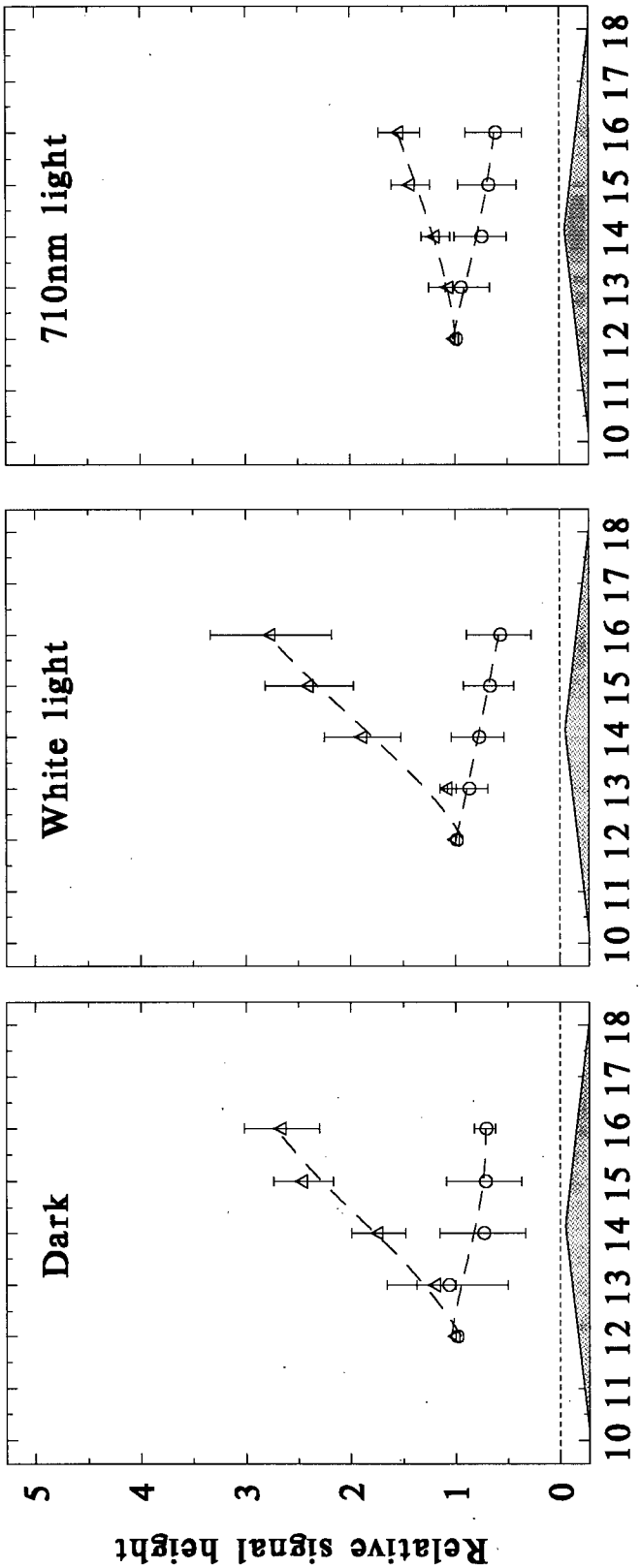


Fig. 4.6 Effects of O_3 on relative signal height in the dark or in white and 710nmlight for bluegrass vacuum-infiltrated with distilled water. (○). control: (△). O_3. Error bars are based on individual means for each hour. n=8.

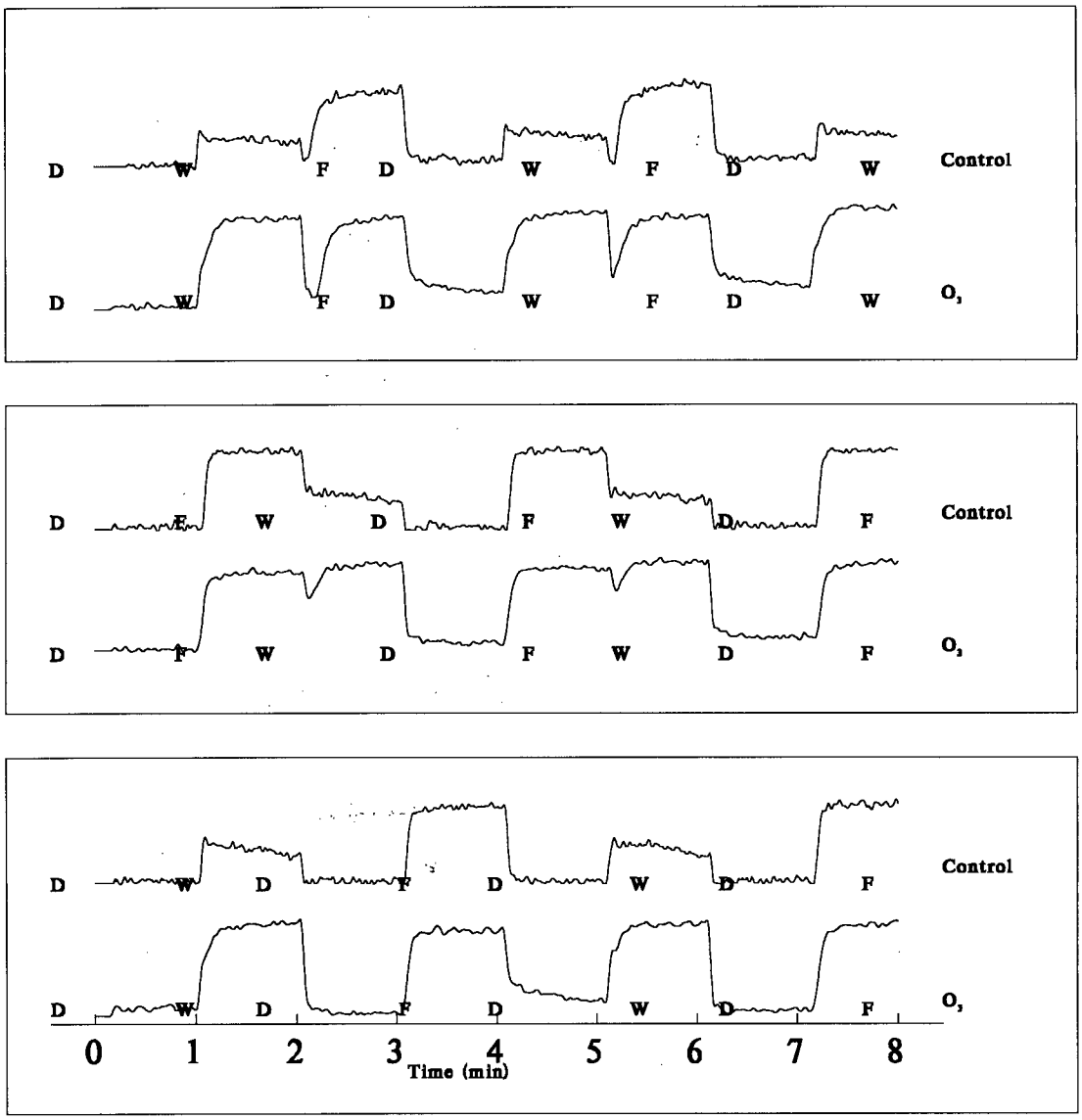


Fig. 4.7 Typical EPR kinetic Signal I traces for bluegrass leaves (after infiltration with water) in medical air or during exposure to 120 ppb O_3 with different sequences of illumination. D = Dark, W = White light, F = 710nm light.

The O_3-induced slow decay of signal I in D after F was independent of the increased relative heights of signal II observed in D shown in Fig. 4.6 since the latter were observed after 2min acclimation to darkness.

Table 4.1 Effects of 120 ppb O_3 on kinetics of signal in Kentucky bluegrass subjected to different sequences of illumination.

Observation	Frequency*
1. Signal in F> signal in W in controls	4/4
2. Signal in F ~signal in W in O_3	4/4
3. Saturates fast in W and then declines in controls.	4/4
4. Saturates slowly in W in O_3.	4/4
5. Saturates more slowly in F in controls after W than after D.	4/4
6. Slower saturation rates in F after D or W in O_3.	4/4
7. Signal in F decays rapidly in D in controls.	4/4
8. Signal in F decays slowly in D in O_3, in two phases.	4/4

*Frequency of observations in 4 replicate experiments.

4.3.1.2 Effects of O_3 on photosynthetic radicals in the presence of spin traps

In darkness, O_3 alone or with DMPO produced relative signal II levels that increased markedly with time (Fig. 4.8). The control and DMPO alone were virtually the same. In white light, marked increases in the combination of Signal I + II also occurred with O_3 alone and particularly in combination with the spin trap. The 710nm light signal followed the same trends, although the increases in relative signal size were smaller than in darkness or white light.

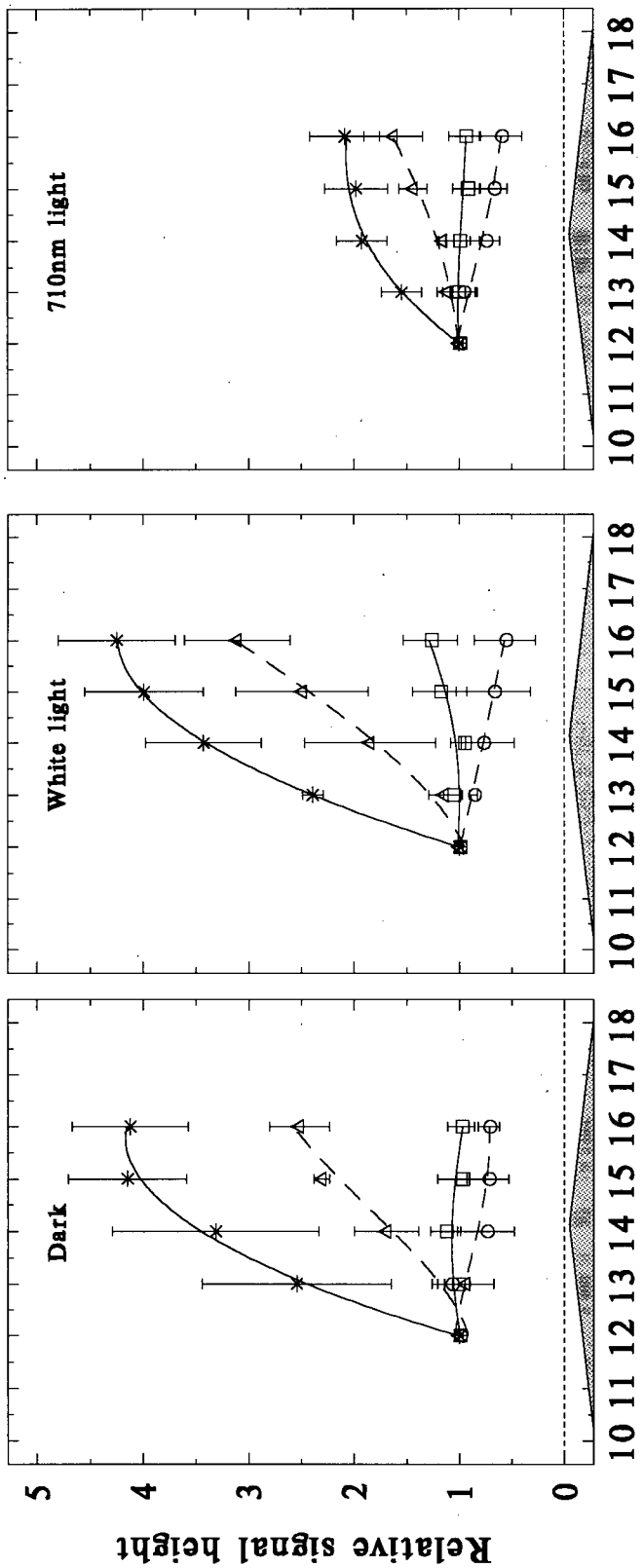


Fig. 4.8 Effects of O_2 on relative signal height in the dark or in white and 710nmlight for bluegrass vacuum-infiltrated with distilled water or 20mM DMPO. (○). control: (△). O_2: (□). DMPO: (✱). DMPO + O_2. Error bars are based onindividual means for each hour. n=4.

A few traces of the kinetics of Signal I changes on switching illumination were obtained in the presence of DMPO (data not shown), which resembled those obtained with 4-POBN (see below).

No dark signal could be reliably determined with the spin trap, 4-POBN. However, the white light signals (I + II) in the control and 4-POBN showed little change over time (Fig. 4.9), and did not differ significantly from each other. The signal in white light increased similarly with O_3 exposure over time in the presence or absence of 4-POBN. The 710nm signal behaved somewhat differently. Whereas the signals in the control and 4-POBN were similar and only began to differ towards the end of the exposure, (Fig. 4.9), exposure to O_3 in the presence of 4-POBN produced a larger signal relative to the signal in O_3 alone which itself became significantly greater than the control. Further, the O_3 + 4-POBN signal tended to reach a maximum at the time of maximal O_3 exposure and remained steady thereafter.

No kinetic studies were done with DMPO but the effects of 4-POBN on the kinetics of Signal I are shown in the typical traces in Fig. 4.10. A summary of the observations in four replicate experiments is presented in Table 4.2. Comparison of the traces in Fig. 4.7 and Fig. 4.10 show that the spin trap had no effect on the size of signal I in F and W relative to the control (i.e. F>W), and that O_3 stimulated the signal in W (i.e. F~W) in both cases. Ozone also caused the signal in F to decay slower in darkness than in the 4-POBN control. The saturation of the signal in W was slowed by O_3 + 4-POBN (Table 4.2; Fig. 4.10). In contrast to the situation in the absence of 4-POBN (Fig. 4.7), however, O_3 also markedly slowed the saturation of Signal I in F light (Fig. 4.10).

Comparison of Tables 4.1 and 4.2 shows that the presence of 4-POBN did not affect the kinetics of Signal I changes induced by different sequences of illumination with the possible exception of increased sluggishness in the rise in Signal I following the onset of F light, which appeared to be

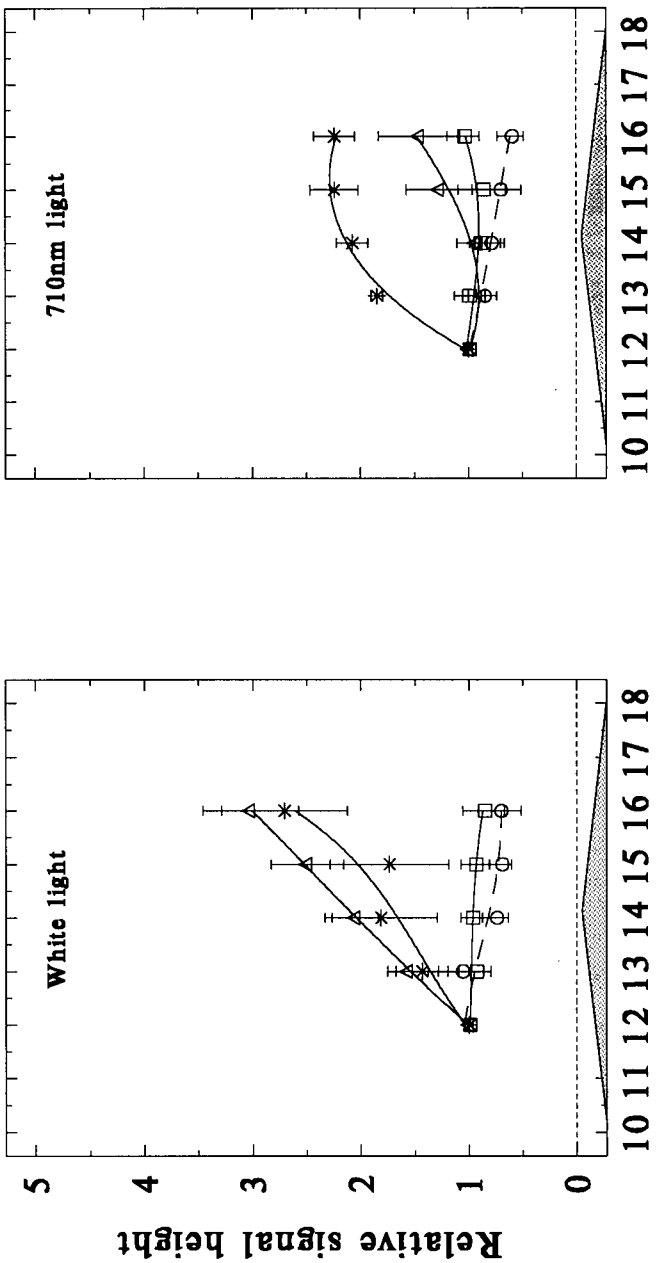


Fig. 4.9 Effects of O_2 on relative signal height in white and 710nmlight for bluegrass vacuum-infiltrated with distilled water or 20mM 4-POBN. (○). control: (△). O_2; (□). 4-POBN; (✱). 4-POBN + O_2. Bars are standard errors. n=4.

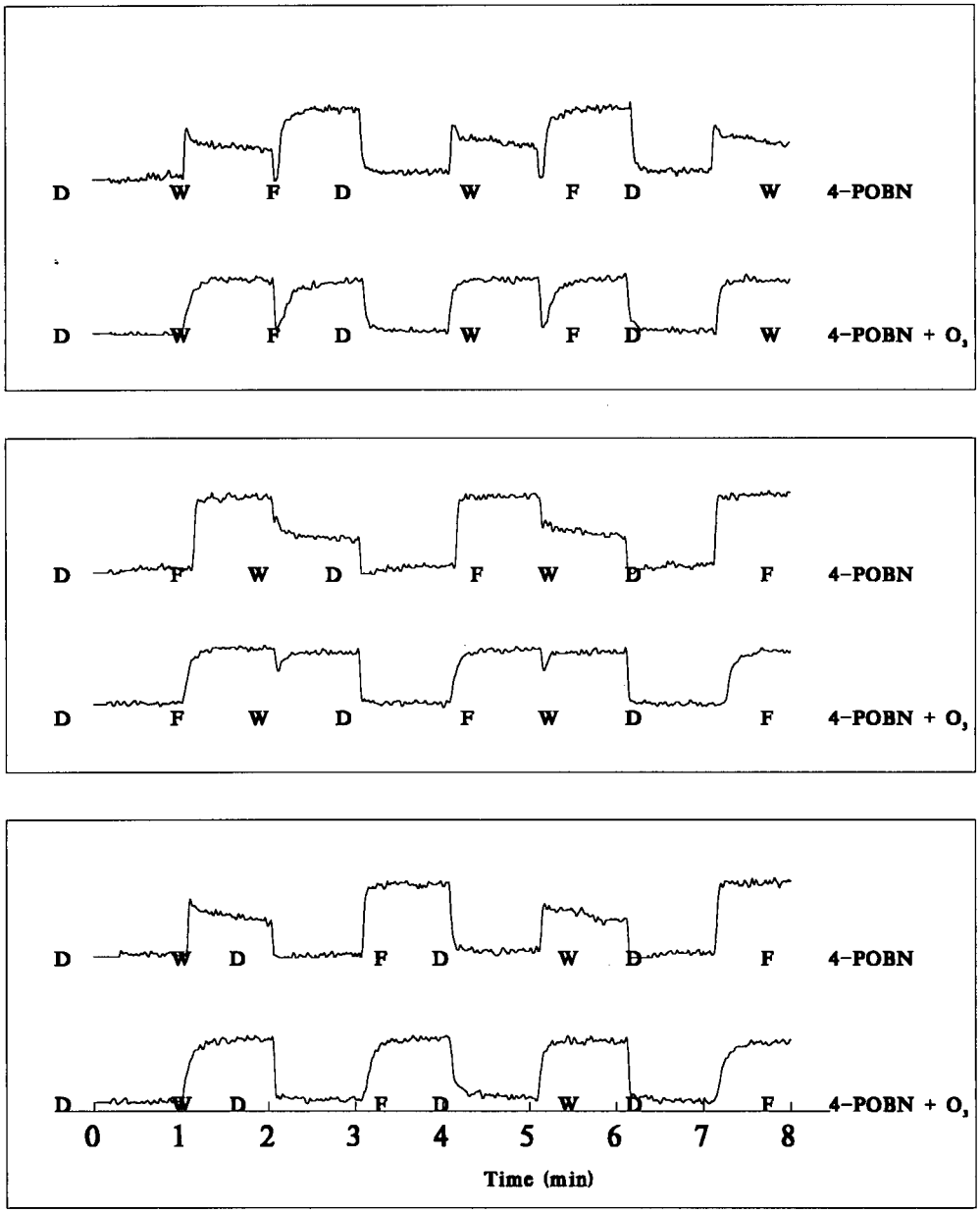


Fig. 4.10 Typical EPR kinetic Signal I traces for bluegrass leaves (after infiltration with 20 mM 4-POBN) in ambient air or during exposure to 120 ppb O_3 with different sequences of illumination. D = dark, W = white light, F = 710nm light.

increased by 4-POBN. The effects observed on Signal I kinetics are in agreement with the relative changes in the heights of Signal I observed at 14:00h in W and F light (Fig. 4.8).

Table 4.2 Effects of 120 ppb O_3 on signal kinetics in Kentucky bluegrass infiltrated with 4-POBN, subjected to different sequences of illumination.

Observation	Frequency*
1. F> W in 4-POBN	4/4
2. F ≃ W in 4-POBN+O_3	4/4
3. Saturates fast in W and then declines in 4-POBN	3/4
4. Saturates slowly in W in 4-POBN+O_3, in two phases.	3/4
5. Saturates more slowly in F in 4-POBN after W than after D.	4/4
6. Slower saturation rates in F after D or W in 4-POBN+O_3.	4/4
7. Signals in F decays rapidly in D in 4-POBN	4/4
8. Signals in F decays slowly in D in 4-POBN+O_3, in two phases.	4/4

*Frequency of observations in 4 replicate experiments.

4.3.1.3 Effects of O_3 on chlorophyll fluorescence

In the control, there were slight but significant increases in relative quantum yield (Y) and photochemical quenching (qP) over time to 13:00h, after which they remained virtually constant (Fig. 4.11 a and b). In O_3 neither Y nor qP changed over time, leading to significant decreases relative to the control. Both expressions of non-photochemical quenching (qN and NPQ) showed initial relative declines over time in the controls, while in O_3 they were constant (Fig. 4.11 c and d, respectively). As a result, both qN and NPQ increased relative to the controls during the O_3 exposure period, with the maximum differences coinciding with the peak of O_3 exposure wave.

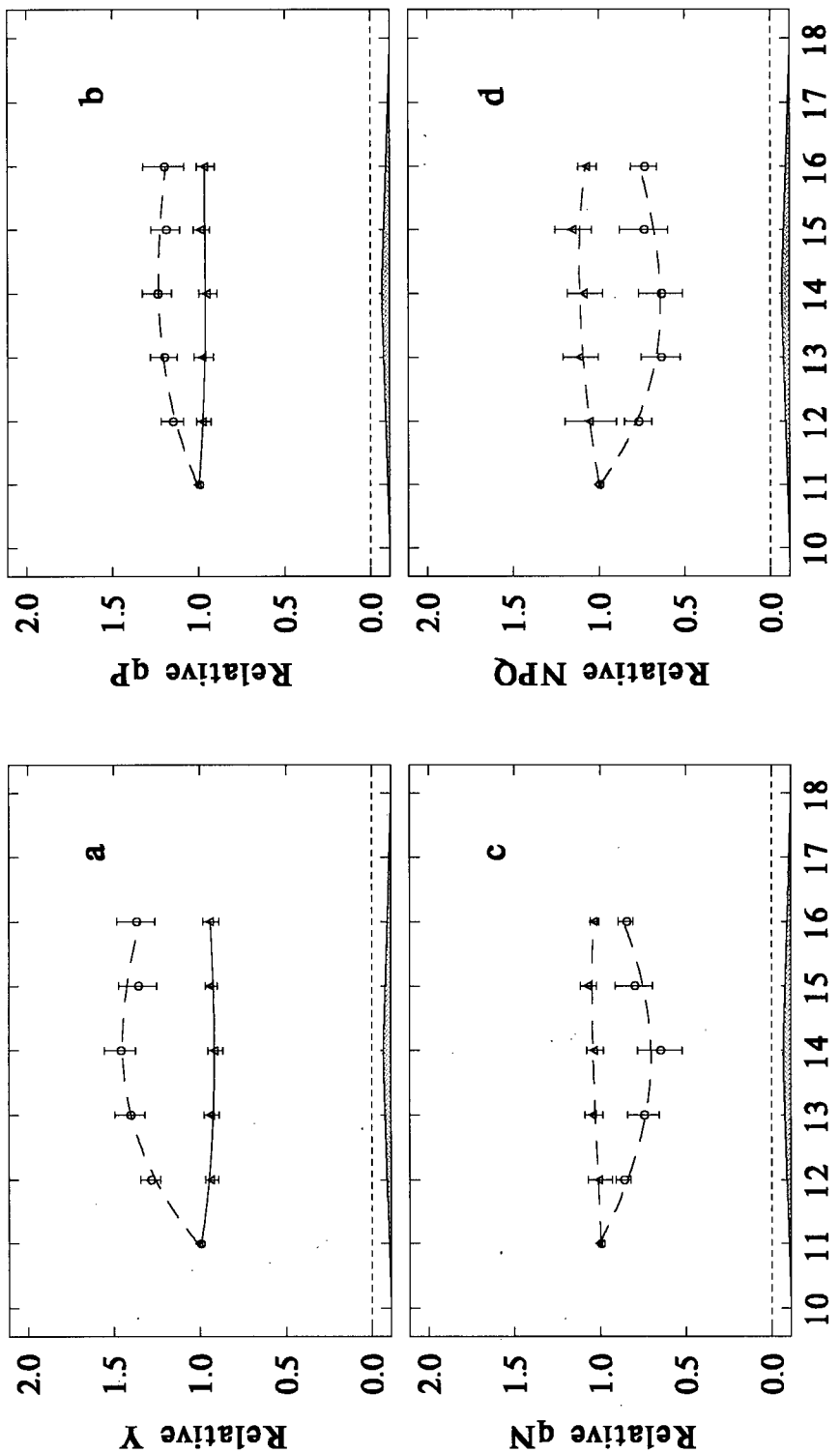


Fig. 4.11 Effects of O_3 on a. quantum yield (Y); b. photochemical quenching (qP);and non-photochemical quenching c. qN and d. NPQ in bluegrass. (○). control; (△). O_3. Bars are standard errors. n=8.

4.3.1.4 Effects of O_3 on chlorophyll fluorescence in the presence of spin traps

With O_3, DMPO and DMPO + O_3 the trends for relative quantum yield of fluorescence (Y) and photochemical quenching (qP) to increase over time in the controls were significantly suppressed (Fig. 4.12). In the control, both relative Y and qP increased to maxima at about 14:00h. Non-photochemical quenching (qN) was not affected by O_3, DMPO or DMPO + O_3 relative to the control (Fig. 4.12c) while NPQ remained constant in all treatments rather than declining as in the control (Fig. 4.12d).

In contrast to DMPO, when 4-POBN was used as a spin trap, it suppressed the rises in quantum yield (Y) and photochemical quenching (qP) observed in the control over time (Fig. 4.13a). Although O_3 alone also suppressed the increase in the control yield, in the presence of 4-POBN the increase over time was maintained. On the other hand, the effects of $O_3 \pm$ 4-POBN on qP showed no significant changes from the control.

Both parameters of non-photochemical quenching (qN and NPQ) showed similar interactions with O_3 and spin trap. Although O_3 and 4-POBN individually maintained the levels of both parameters relative to the declines observed in the controls, O_3 in the presence of 4-POBN resulted in declines that were virtually identical to the controls (Fig. 4.13 c and d).

Hence, although both spin traps resulted in generally similar effects on both EPR signals and chlorophyll fluorescence, they showed some difference in their interactions with O_3.

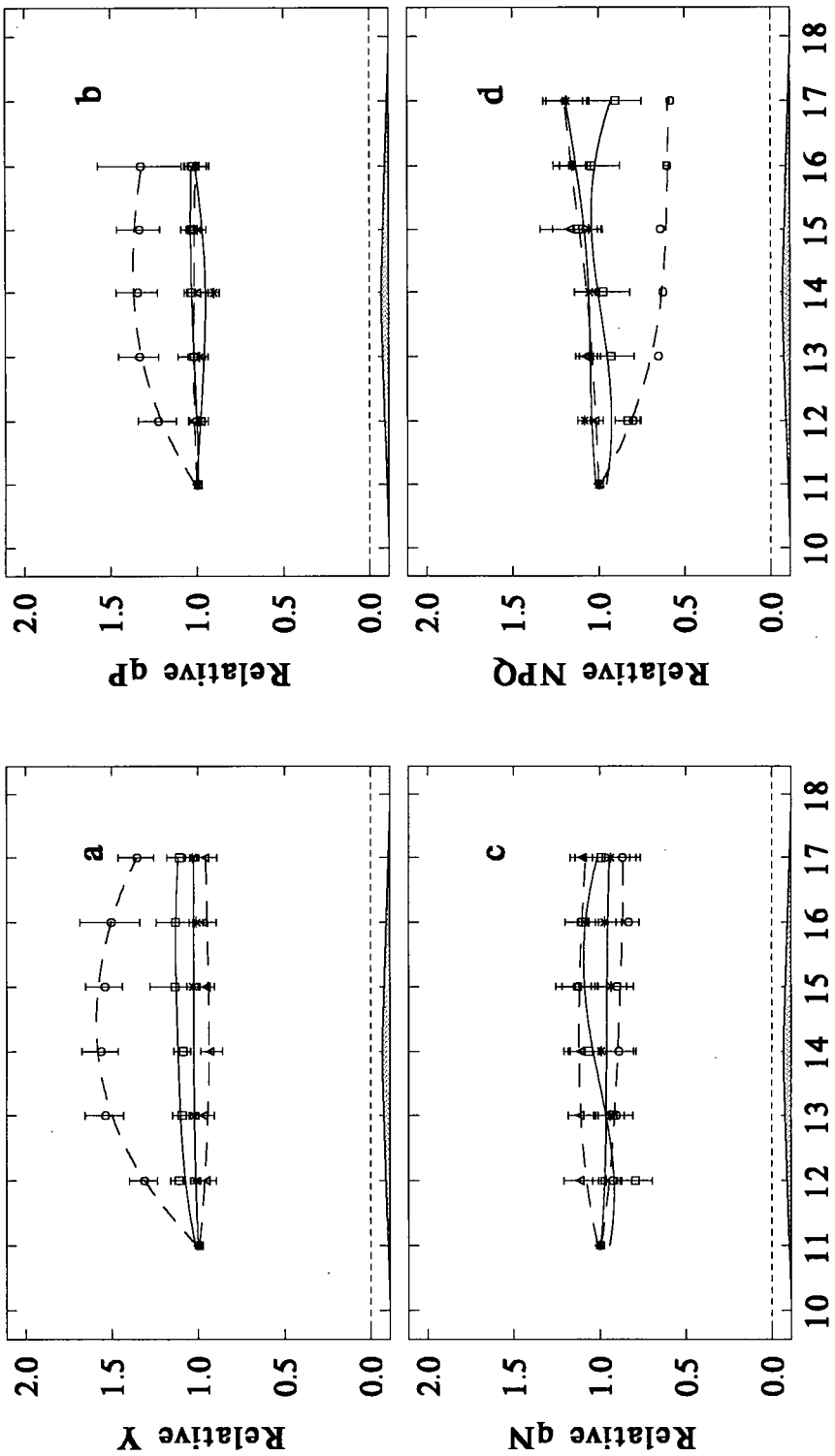


Fig. 4.12 Effects of DMPO on the time course of a. quantum yield (Y); b. photochemical quenching(qP) and non-photochemical quenching c. and d. in bluegrass. (○), control; (△), O_2^-; (□), DMPO; (✱), DMPO+O_2^-. Bars are standard errors, n=4.

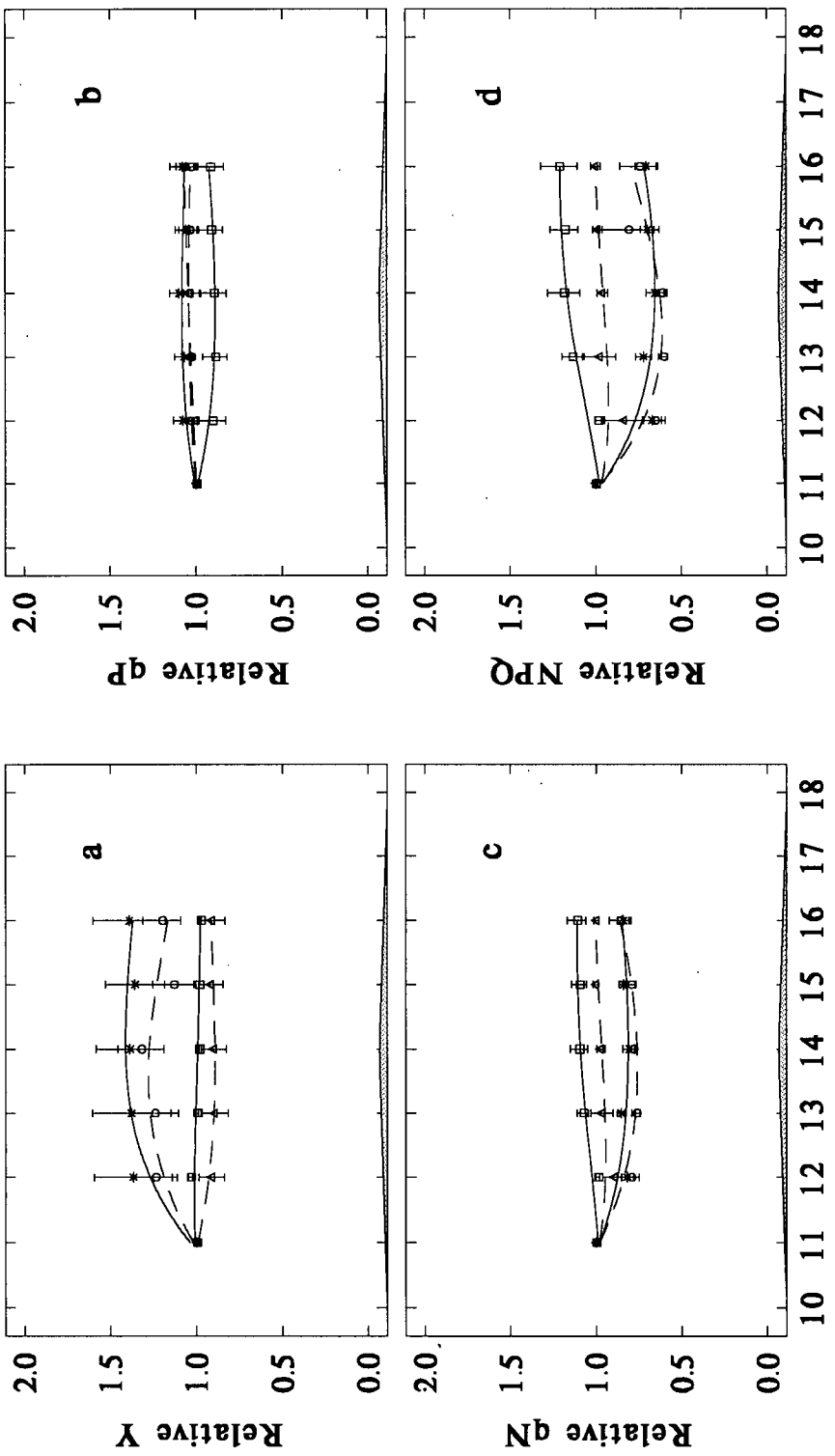


Fig. 4.13 Effects of 4-POBN on the time course of a. quantum yield (Y); b. photochemical quenching (qP); and non-photochemical quenching c. qN and d. NPQ in bluegrass. (○). control; (△), O_2; (□). 4POBN; (✱). 4POBN+O_2,Error bars are standard errors. n=4.

4.3.2 Foxtail

4.3.1.1 Effects of O_3 on photosynthetic radicals

The EPR signals obtained with foxtail leaves were much smaller than those observed with Kentucky bluegrass although comparable areas of leaf area were exposed within the cavity. As a result, the signal/noise ratios were less and the results with foxtail showed greater inherent variability and fewer clearly significant effects of treatment. The white light signal (Signal I + II) increased slowly with time during O_3 exposure (Fig. 4.14) and it was significantly larger than the control after 15:00h. However, the signal in 710nm light showed more variability over time and although the signal in O_3 was consistently higher than the control, the differences were only marginally significant (Fig. 4.14). No dark Signal II could be reliably detected. In terms of absolute signal heights, those in white light were consistently greater than those in far-red light, regardless of exposure to O_3.

Table 4.3 shows a summary of the EPR data on signal I kinetics in foxtail and Fig. 4.15 typical traces of the signals from which the summary data were derived. Unlike in Kentucky bluegrass, the signal in white light (W) was larger than that in 710nm light (F) regardless of exposure to O_3 (Table 4.3). After exposure to W, switching to F resulted in a slow rise in Signal I in contrast to the rapid rise following D. Ozone, however, caused a slight 2-phase lag in the saturation of the signal in W after D. Although Signal I in F rose faster after D than after W in the control, the low levels of Signal I in F make it impossible to determine whether similar differences in saturation rates occurred in the presence of O_3.

4.3.1.2 Effects of O_3 on photosynthetic radicals in the presence of spin traps

In the experiment with DMPO, the control white light signal (I and II) remained virtually unchanged over time (Fig. 4.16), but because of the variability in the data the only clear difference in white light was between the control and the greater level in the DMPO + O_3 treatment at 16:00h. This same

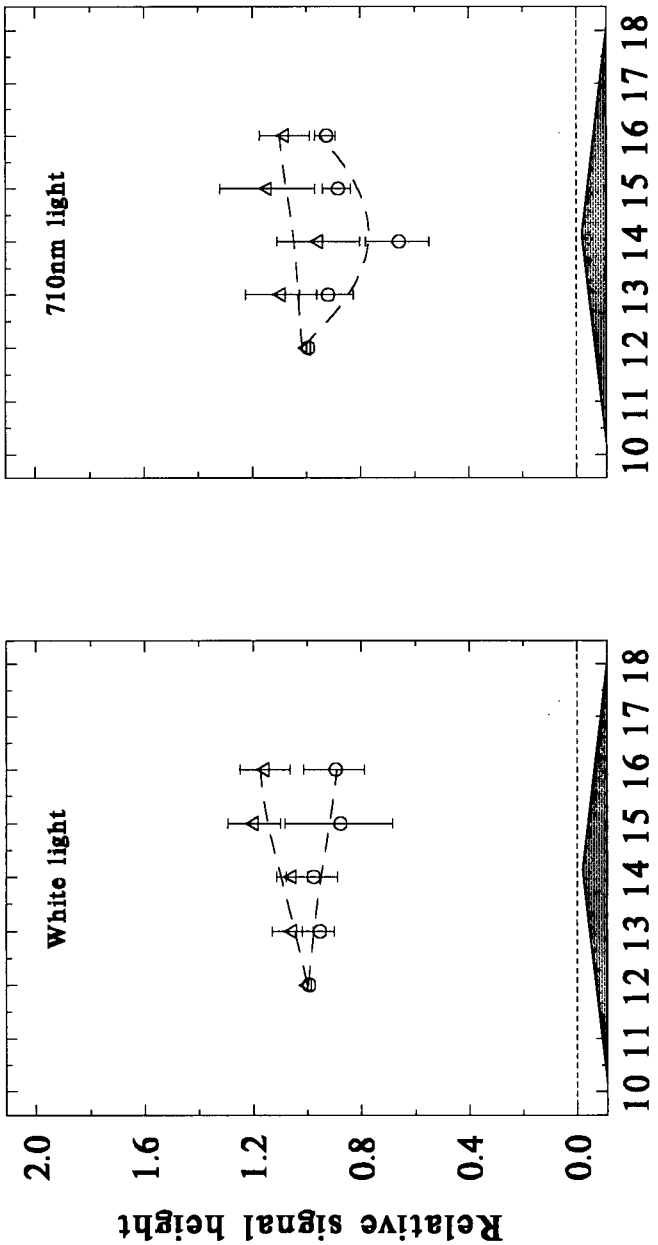


Fig. 4.14 Effects of O_3 on relative signal height in white and 710nmlight for foxtail vacuum-infiltrated with distilled water. (○). control: (△). O_3. Error bars are based on individual means for each hour. n=8.

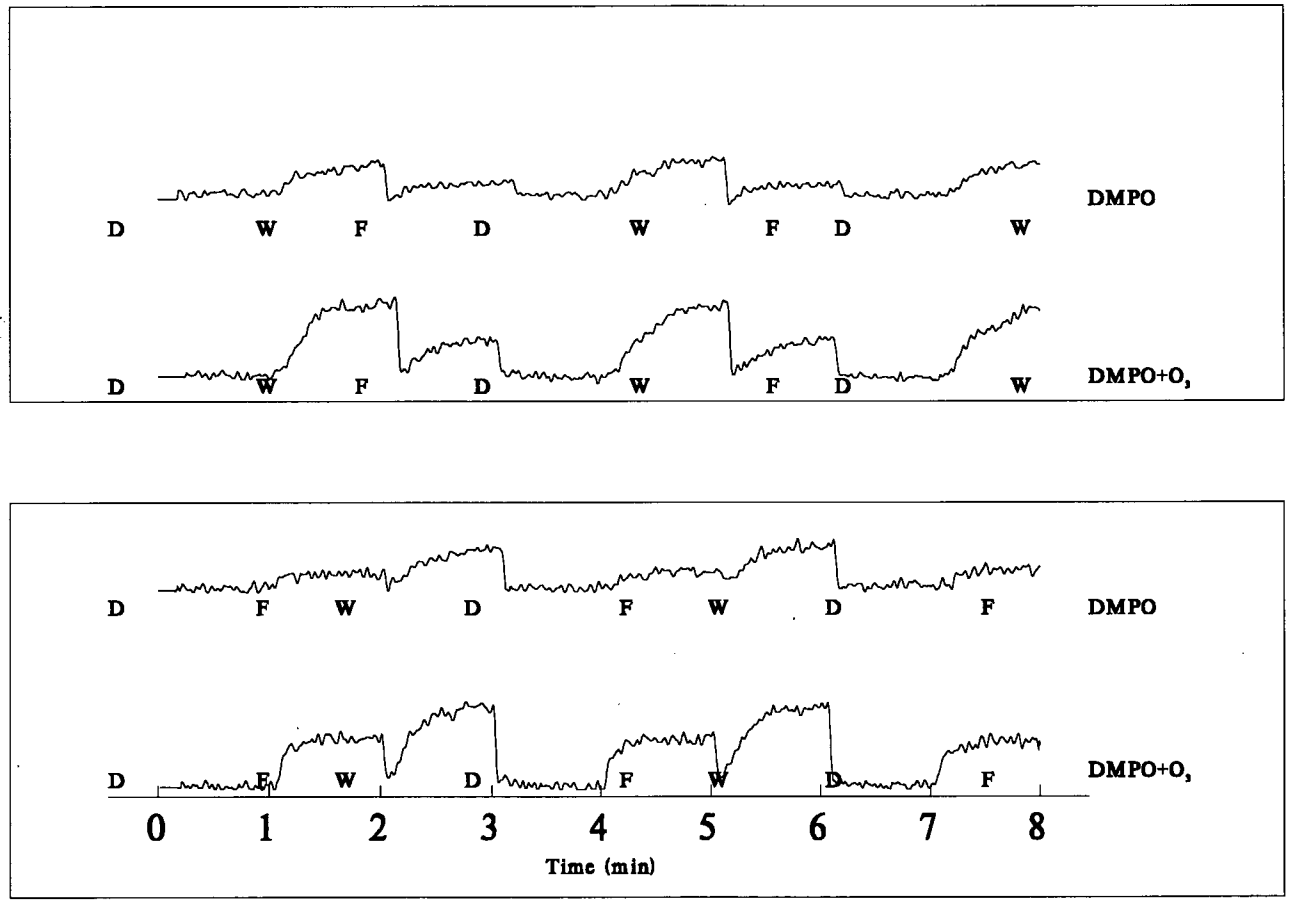


Fig. 4.17 Typical EPR kinetic Signal I traces for foxtail leaves (after infiltration with DMPO) in medical air or during exposure to 120 ppb O_3 with different sequences of illumination. D = dark, W = white light, F = 710nm light.

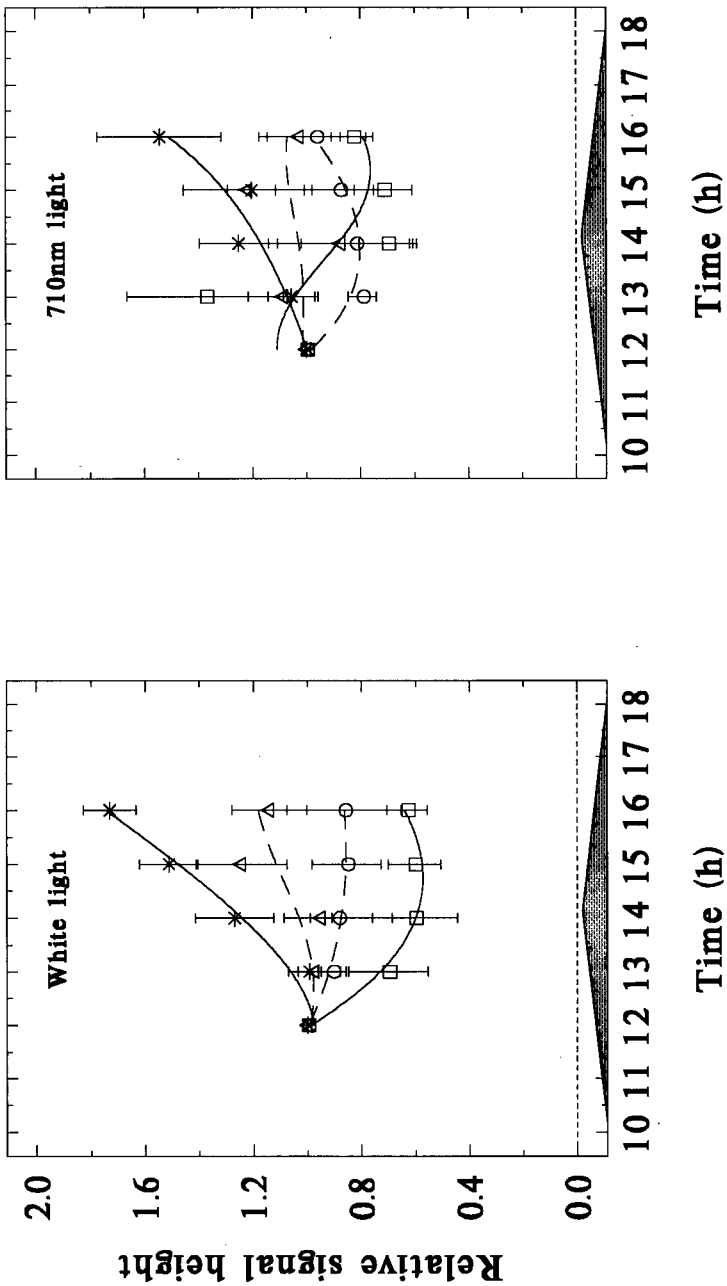


Fig. 4.18 Effects of O_3 on relative signal height in white and 710nm light for foxtail vacuum-infiltrated with distilled water or 4-POBN. (○). control; (△). O_3; (□). 4-POBN; (✱). 4-POBN + O_3. Bars are standard errors. n=4

situation occurred with the signal in 710 light (Fig. 4.16). The signals only differed significantly between DMPO+O_3 and the control from about 14:00 to 16:00hrs.

Table 4.3 Effects of 120 ppb O_3 on kinetics of Signal I in foxtail subjected to different sequences of illumination in foxtail.

Observation	Frequency
1. W > F signal in size in the control.	2/2
2. W>>F signal in size in O_3	2/2
3. Saturates faster in W after D than after F in control	2/2
4. Saturates slower after D in O_3 than with the control, in two phases	2/2

A summary of the EPR signal kinetics is shown in Table 4.4 while a typical trace of the signals from which the summary is derived is given in Fig. 4.17.

Table 4.4 Effects of 120ppb O_3 on kinetics of Signal I in foxtail infiltrated with DMPO, subjected to different sequences of illumination.

Observation	Frequency
1. W > F signal in DMPO control and DMPO + O_3.	2/2
2. Slow saturation in W unchanged after D or F in O_3+DMPO and DMPO	2/2
3. Saturation in F slower after W than after D in O_3 + DMPO	2/2

Infiltrating with 4-POBN resulted in an apparent decline in the relative signal height (Signal I and II) recorded in white light over time relative to the control (Fig. 4.18), although the difference was barely significant. Exposure to O_3 in the presence of 4-POBN, however, reversed this trend.

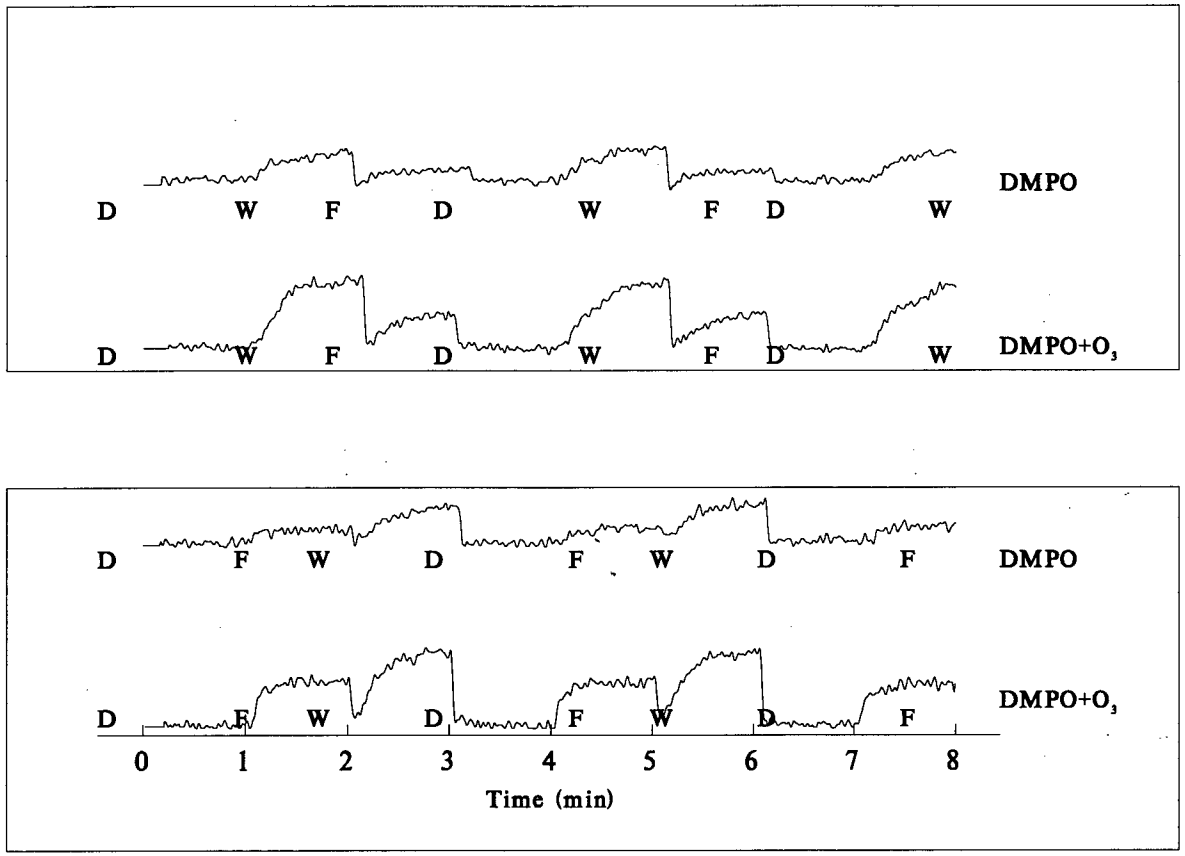


Fig. 4.17 Typical EPR kinetic Signal I traces for foxtail leaves (after infiltration with DMPO) in medical air or during exposure to 120 ppb O_3, with different sequences of illumination. D = dark, W = white light, F = 710nm light.

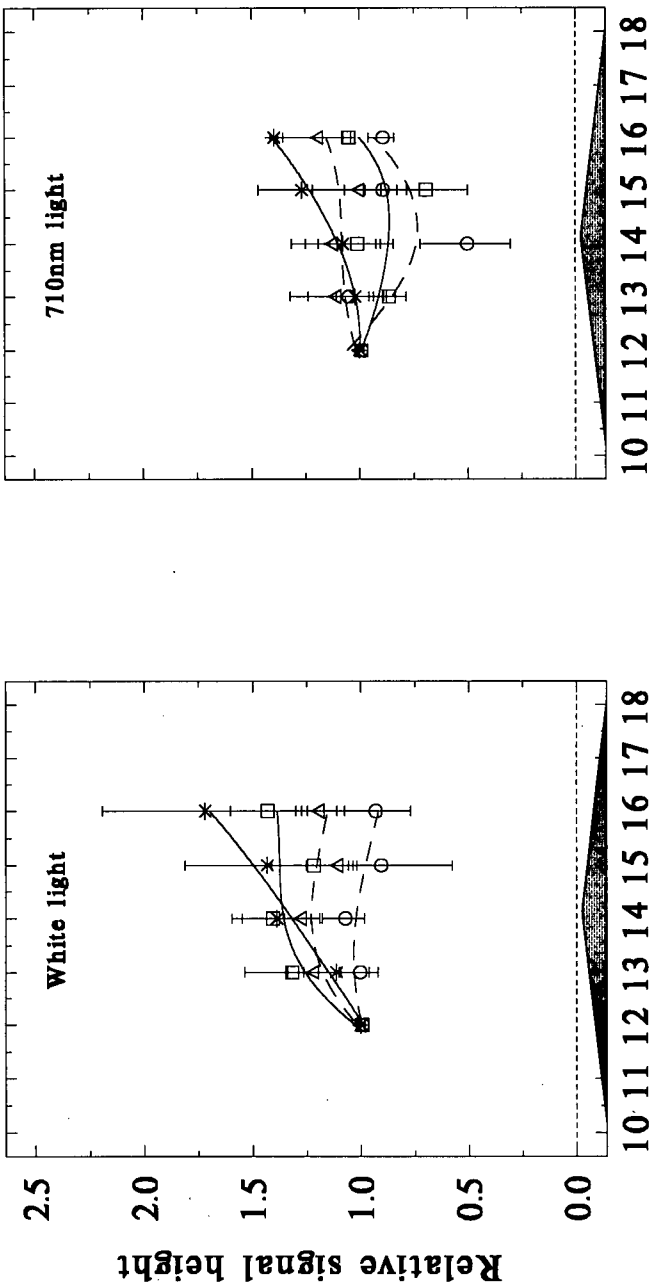


Fig. 4.16 Effects of O_2 on relative signal height in the dark or in white and 710nmlight for foxtail vacuum-infiltrated with distilled water or DMPO. (○), control: (◐), O_2; (□), DMPO; (✱), DMPO + O_2. Bars are standard erros. n=4.

The relative signal height in W also rose in O_3 alone relative to the control. Similar trends were seen for the signal in 710nm light. In this case, however, the control and spin trap treatments remain virtually unchanged over time, but O_3 exposure in the absence or presence of 4-POBN still caused an increase in relative signal height over time. These increases were somewhat smaller than those observed in white light.

With regard to the kinetics of Signal I, the spin trap 4-POBN had no effect on the relative sizes of signals W and F with or without O_3 (Fig. 4.19 and Table 4.5). However, both the signal heights were larger in the presence of O_3. The rise in Signal I in both W and F appeared to be damped by O_3 in the presence of 4-POBN, as was the case in leaves infiltrated with water (Fig.4.15) or DMPO (Fig.4.17).

Table 4.5 Effects of 120 ppb O_3 on kinetics of Signal I in foxtail infiltrated with 4-POBN, subjected to different sequences of illumination.

Observation made	Experimental results
1. W > F signal in size in 4-POBN control and 4-POBN + O_3.	4/4
2. Signal size greater in O_3 + 4-POBN than 4-POBN control	4/4
3. F saturates slower after W than after D in 4-POBN control and O_3 + 4-POBN	4/4

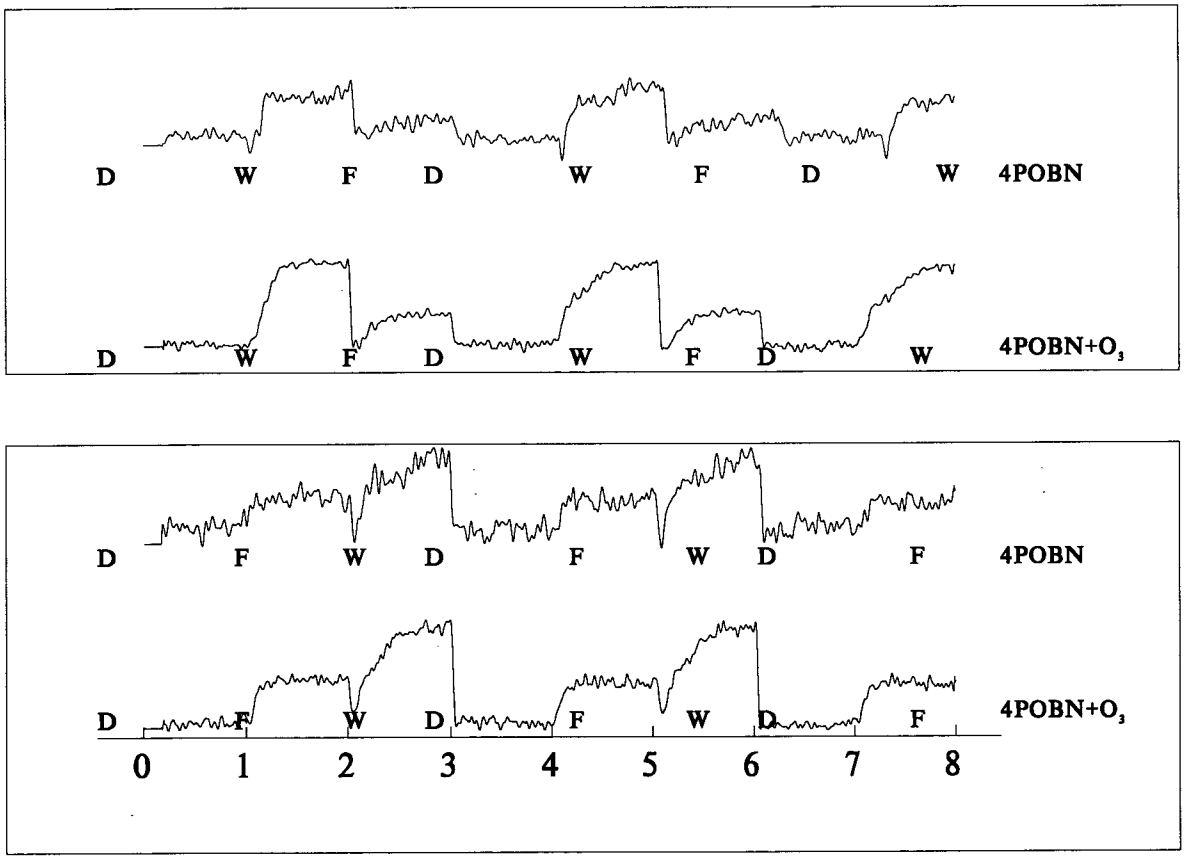


Fig. 4.19 Typical EPR kinetic Signal I traces for foxtail leaves (after infiltration with 4-POBN) in medical air or during exposure to 120 ppb O_3, with different sequences of illumination. D = dark. W = white light. F = 710nm light.

4.3.1.3 Effects of O_3 on chlorophyll fluorescence

Ozone had no effect on relative quantum yield (Y) (Fig. 4.20a), or photochemical quenching (qP) (Fig. 4.20b). However, O_3 increased both qN and NPQ significantly relative to the controls which decreased over time (Fig. 4.20c and d, respectively).

4.3.1.4 Effects of O_3 on chlorophyll fluorescence in the presence of spin traps

DMPO, O_3 and DMPO + O_3 had no effect on Y or qP relative to the control (Fig 4.21a, and b). Ozone exposure alone led to increases in qN and NPQ (Fig. 4.21c and d) but these increases were not seen with O_3 in the presence of DMPO. Similarly, there were no significant effects of any treatment with 4-POBN on Y or qP (Fig. 4.22 a and b respectively). Although the non-photochemical quenching parameters qN and NPQ declined over time in the control, the changes in both parameters were erratic in the treatments with O_3, 4-POBN and O_3 + 4-POBN all of which tended to remain constant (Fig. 4.22c and d) and hence reflected a degree of stimulation.

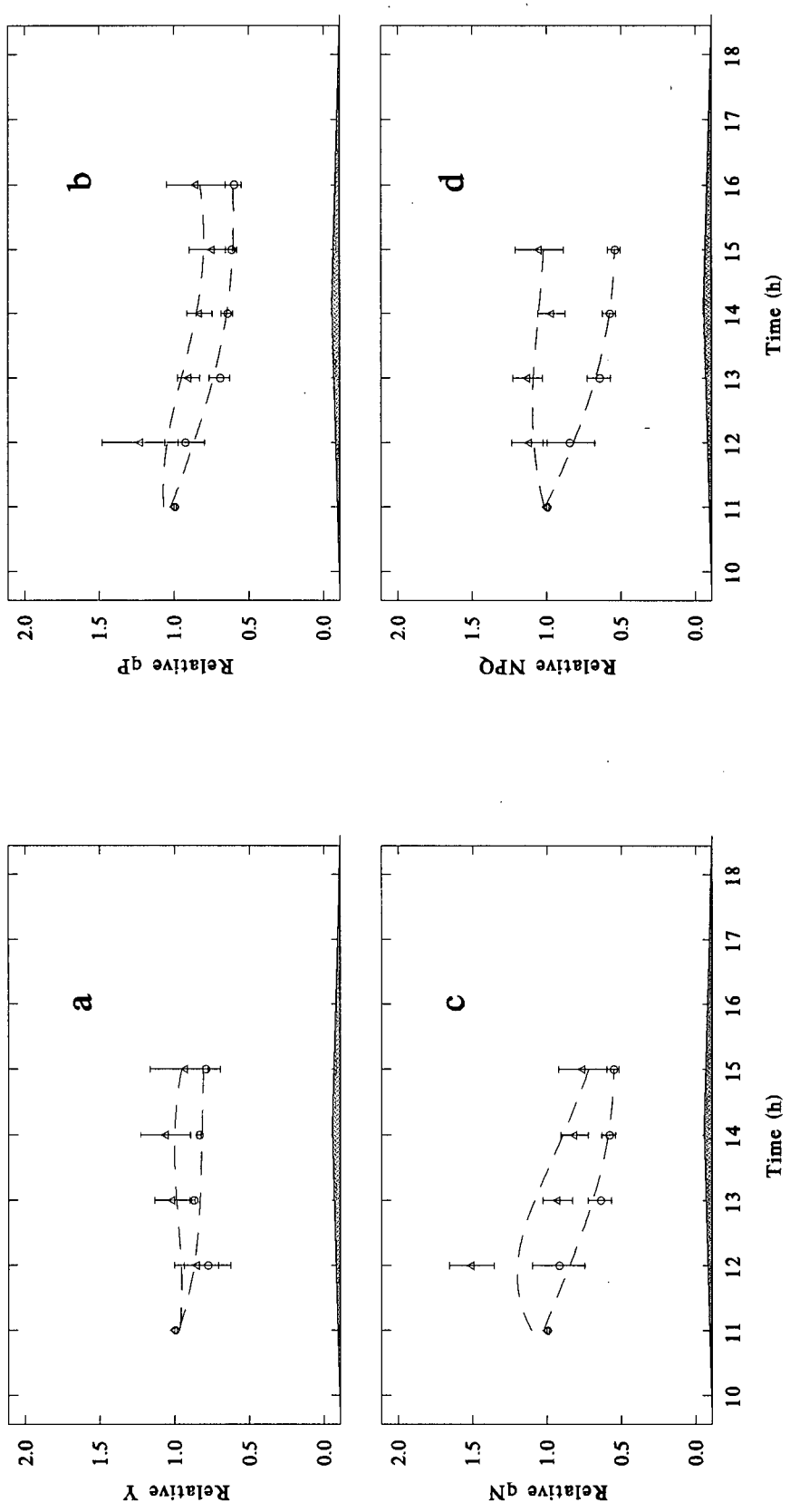


Fig. 4.20 Effects of O_3 on a. quantum yield (Y); b. photochemical quenching (qP); and non-photochemical quenching c.qN and d. NPQ in foxtail. (○), control; (△), O_3. Bars are standard errors. n=8.

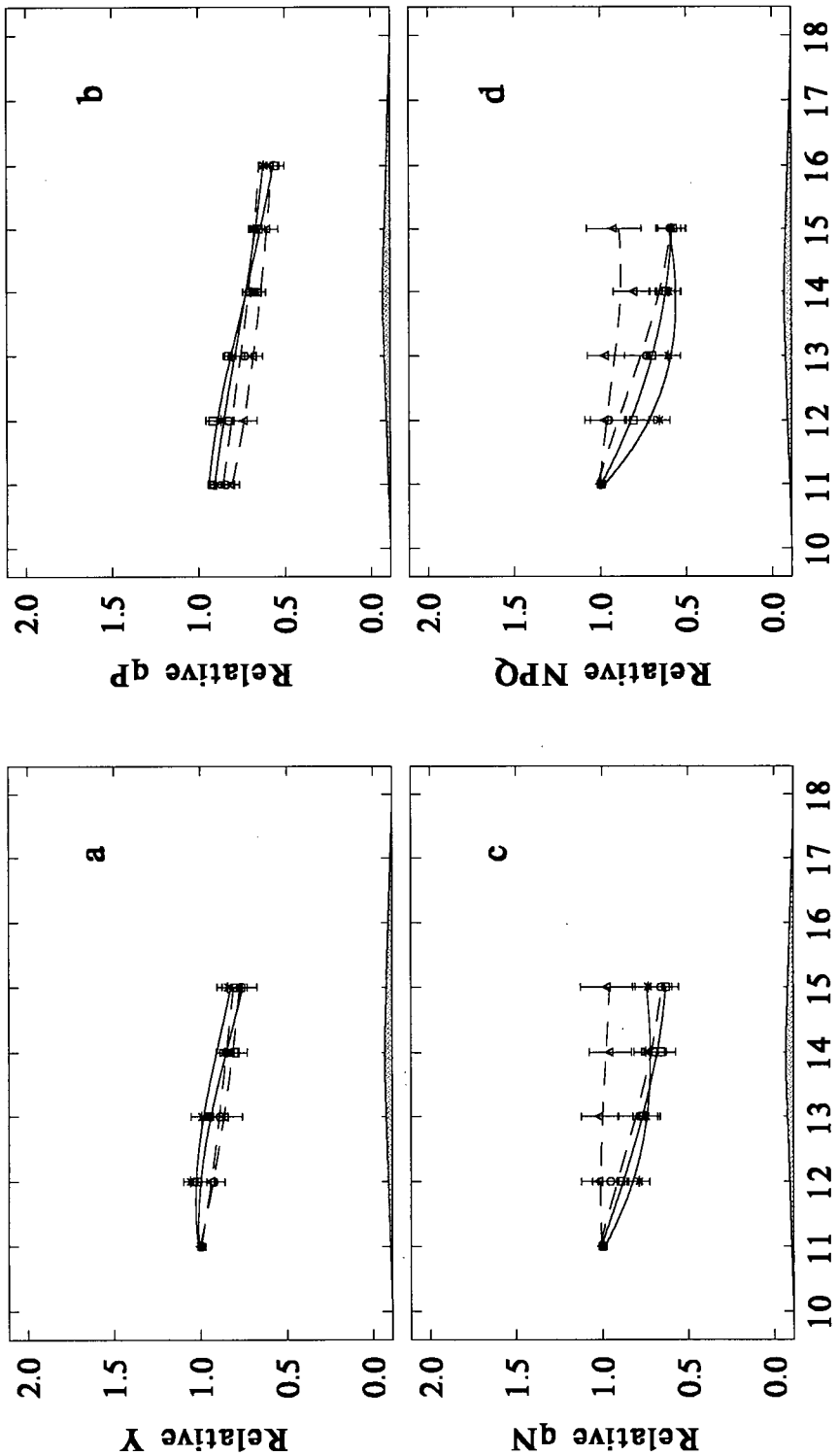


Fig. 4.21 Effects of DMPO on the time course of a. quantum yield (Y); b. photochemical quenching(qP) and non-photochemical quenching c. and d. in foxtail. (○). control; (△). O_2; (□). DMPO; (✱). DMPO+O_2. Bars are standard errors. n=4.

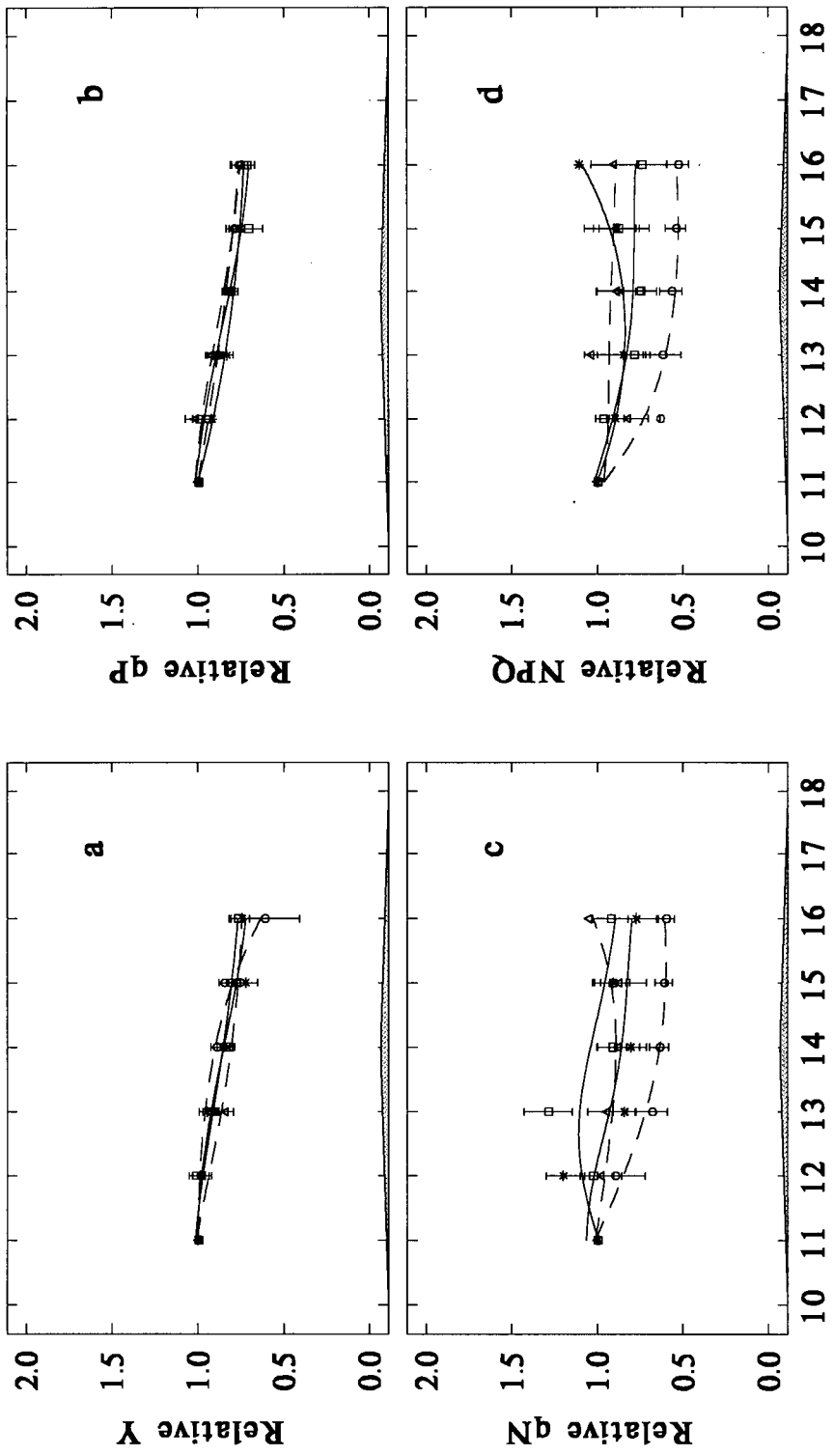


Fig. 4.22 Effects of 4-POBN on the time course of a. quantum yield (Y); b. photochemical quenching (qP); and non-photochemical quenching c. qN and d. NPQ in bluegrass. (○), control; (△), O_2; (□), 4POBN; (✱), 4POBN+O_2.Error bars are standard errors, n=4.

4.4 Discussion

The C-3 grass, *Poa pratensis*, and the C-4 grass, *Setaria viridis* exhibited many distinct differences in the magnitudes of the photosynthetic radicals observed using electron paramagnetic resonance (EPR) spectrometry and in the parameters obtained from chlorophyll fluorescence measurements, presumably related to differences in their leaf anatomy and the relative distributions of photosystem I and II in their chloroplasts.

In control Kentucky bluegrass leaves, irradiation with far-red light typically leads to the appearance of a larger radical signal I, attributed to the oxidized P700 chlorophyll in photosystem I, than that observed in a mixture of red and far-red light or in white light as shown in Fig. 4.7. This pattern of response is typical of radish (Runeckles and Vaartou, 1992) and many other C-3 species, including the blue-green alga, *Anacystis*, observed in an early study by Kok and Beinert (1962).

In contrast, the pattern observed in the present studies with the C-4 grass, foxtail, was reversed, with Signal I in white light consistently exceeding that observed in far-red light (Fig. 4.15). In addition, regardless of the sequence of illumination, the appearance of Signal I in foxtail was noticeably slower than in Kentucky bluegrass (cf. Fig. 4.7 and Fig. 4.15). The "overshoot" and the following decline observed in Kentucky bluegrass at the start of exposure to white light (most noticeable after darkness) was never observed in foxtail, while in many cases the foxtail signals appeared to fail to reach a maximum within the 1-minute of specific light exposure (Fig. 4.15). The overshoot and subsequent decline in Kentucky bluegrass are interpreted as being the result of the lag in the supply of electrons from photosystem II reaching the photosystem I centres permitting the amount of $P700^{+}$ radical to rise initially above the equilibrium level.

The signals observed in intact foxtail leaves, with approximately the same surface areas illuminated within the EPR cavity as in Kentucky bluegrass, showed much poorer signal: noise ratios. This could be accounted for in part by lower chlorophyll content, since the foxtail leaves used were approximately two weeks younger than those of Kentucky bluegrass (to permit them to fit within the dewar insert in the cavity) and were noticeably less green, and possibly by differences in the light gradients within the leaves, as described by Pfündel et al. (1996).

EPR signals observed in intact leaves are inherently poorer in quality than those observed with chloroplast suspensions or algal cells, because of the increased heterogeneity of the tissue. Although there appear to have been no reports of tissue orientation dependence of EPR spectra of the photosynthetic radicals in leaves, such dependence has been demonstrated for the manganous ion which is largely concentrated in the chloroplasts (McCain et al., 1984). However, since with both grass species used in the present study the leaves were placed in the cavity with their blades perpendicular to the illumination, any possible contribution of orientation to increased heterogeneity in foxtail must relate to the orientation of the chloroplasts within the mesophyll or bundle sheath cells or both.

The sluggish responses to different light exposures in foxtail suggest that the light intensities used were insufficient to cause rapid saturation of photosystem centers. However, the intensity of the far-red band used was only reduced about 15% relative to the 710nm component of the white light. Nevertheless, the greater signal I observed in white rather than 710nm in the foxtail leaf suggests greater light harvesting by PSI centers in white light. Although it was originally thought that PSII was absent from the frequently granaless bundle sheath chloroplasts in C-4 plants, evidence from studies with *Zea mais* indicated that a form of PSII is present in the bundle sheath thylakoids, and that its light-harvesting complex (LHCII) functions as an antenna for the PSI present (Bassi et al., 1995).

Recently, Pfündel et al. (1996) have shown that such involvement of light capture in the red region of the spectrum in the activation of PSI in bundle sheath chloroplasts also occurs in *Setaria viridis* and other C-4 species. This would account for the observations of greater Signal I levels in white than in far-red light in foxtail.

The slow maximization of Signal I on switching from white to far-red light (Fig. 4.15, upper trace) possibly results both from the low light intensity and the need for PSI centers to uncouple from LHCII and couple with LHCI antenna systems. Switching from 710nm to white light would require the reverse process, which seems to occur more rapidly as indicated by the faster increase to the maximum.

It should also be noted that, in contrast to Kentucky bluegrass, in which a distinct Signal II was observed in darkness (e.g. see Fig.4.6), the dark signal II in foxtail was rarely distinguishable above the noise level. This is surprising in view of the fact that between the hourly observations, the leaves were maintained in white light, and as a result would have been expected to demonstrate the slow Signal II component associated with the tyrosine residue (Y_D) on the D2 protein.

In Kentucky bluegrass, exposure to O_3 resulted in changes in both EPR signal characteristics and chlorophyll fluorescence parameters that indicate disturbance of the electron-flow between the photosystems. Thus O_3 suppressed the overshoot of Signal I observed at the start of illumination with white light in the control (Fig. 4.7) and the signal in white light continued to rise to the same level as that observed in 710nm light. This is typical of the situation observed in other systems in which the flow of electrons from PSII is blocked by the use of inhibitors such as 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) observed as early as 1962 by Kok and Beinert. Close inspection of the rise of Signal I in white light observed in many traces frequently revealed two components: a rapid rise to the

level observed in the controls followed by a somewhat slower rise to the maximum (Fig. 4.7). In white light in O_3 the fast rise probably reflects the immediate formation of $P700^+$, and the slower subsequent rise the establishment of a new equilibrium level determined by the reduced electron flow from PSII. The same mechanism could account for the slow maximizing of signal I in white light in foxtail in the presence of O_3 (Fig. 4.15).

In Kentucky bluegrass, any inhibiting effect of O_3 on the flow of electrons to PSI cannot account for the slow decay of signal I during O_3 exposure observed at the end of illumination, particularly with 710nm light (Fig. 4.7). Enlarged traces of these changes in air or O_3 are shown in Fig. 4.23. In darkness the decay in Signal I can only occur by transfers of electrons that are not light dependent, i.e. to NADP or to cytochrome b_6 (cyclic flow). Transfer to NADP is catalyzed by ferredoxin/NADP-oxidoreductase, which is light-induced (Pschorn et al., 1988). Inspection of the decay of Signal I on the transition from white to darkness in air shows an immediate drop to the baseline (Fig. 4.23b). From 710nm light there is an immediate drop to a low level (X in Fig. 4.23a) followed by a slow, reproducible decline to the baseline, suggesting that there was less oxidoreductase activity as a result of the lower overall light intensity (approximately one tenth of white light). As a result the final decay was rate-limited by the reduced oxidoreductase, while the rapid drop to X occurred by cyclic electron transfer to cytochrome b_6 and transfer to NADP although at a reduced rate. In the presence of O_3, the final decay from white light was also retarded (from Y in Fig. 4.23d) and a greater retardation was observed in the final decay from far-red light (from XY in Fig.4.23c). On the assumption that O_3 did not affect light-activation, the decline from white could be accounted for by an O_3 inhibition of the oxidoreductase activity (from Y to the baseline) which becomes more pronounced in the drop from 710nm light because of the reduced activation of the enzyme (from XY to the baseline).

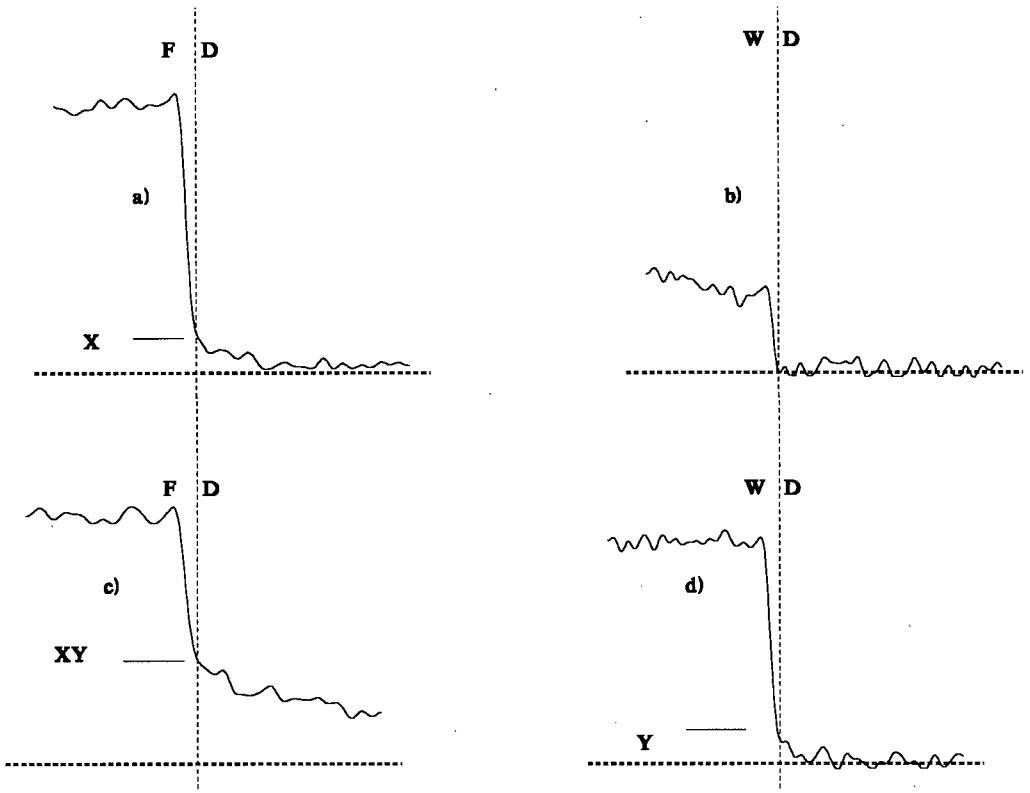


Fig. 4.23 Signal I decay kinetics; a. in 710nm light in the control; b. in white light in the control; c. in 710nm light during exposure to 120 ppb O_3; and d. in white light during exposure to 120 ppb O_3. D = dark. W = white light. F = 710nm light. X = limit imposed by reduced action of ferredoxin/NADP-reductase. and Y = limit imposed by inihibition of cyclic electron flow.

A summary of the changes in the different EPR signals and chlorophyll fluorescence parameters observed in both grasses at the end of 6h exposure to O_3 is presented in Table 4.6. The 6h exposure to the O_3 wave which peaked at 120 ppb after 4h showed increases in Signal II observed in darkness, and the composites of Signals I and Signal II observed in both white and 710nm light over the controls, in both species. It should be pointed out that the changes summarized in Table 4.6 refer to changes in relative signal heights after standardization based on the initial values at 11:00h.

Table 4.6. Summary of the changes in EPR signals and chlorophyll fluorescence parameters in Kentucky bluegrass and foxtail after 6h exposure to O_3 with 120ppb maximum at 4h. All changes are relative to controls in medical air.

Treatment	EPR signals in			Fluorescence			
	Dark	White light	Far-red light	Y	qP	qN	NPQ
Bluegrass:							
O_3	+	+	+	-	-	+	+
DMPO	0	(+)	0	-	-	(+)	+
O_3 + DMPO	+	+	+	-	-	**0**	+
4-POBN	na	0	(+)	-	-	+	+
O_3 + 4-POBN	na	+	+	**0**	**0**	**0**	**0**
Foxtail:							
O_3	na	+	(+)	0	0	+	+
DMPO	na	+	0	0	0	0	0
O_3 + DMPO	na	+	+	0	0	**0**	**0**
4-POBN	na	(-)	0	0	0	+	+
O_3 + 4-POBN	na	++	+	0	0	(+)	+

Parentheses indicate marginal changes. Sign indicates increase or decrease. Boldface/underline indicates changes involving interactions between O_3 and spin traps. na = not available.

The actual heights of the original dark signals in Kentucky bluegrass were much less than those observed in white or far-red light. The heights of the actual signals in 710nm light in the controls were approximately double those in white light as indicated by the heights in the kinetic traces shown in Fig. 4.7. Hence, Fig.4.6 indicates not only that the effects of O_3 on the white light signal were greater than those on the signal in 710nm light (as a result of reduced electron-flow from photosystem II discussed above) but that Signal I was also increased as a result of the prolonged exposure to O_3.

The increased dark signal seen in Kentucky bluegrass is due to Signal II_s, which arises from Y_D^+. Although Y_D does not appear to participate directly in oxygen evolution, it is thought to play a role in stabilizing and protecting the oxygen-evolving complex (Nugent et al., 1987), perhaps by providing a supply of electrons as a alternative to the supply from Y_Z^+, the normal donor to P680, in the event of perturbation of the water splitting complex. Since the spectra of Signal II_s and Signal II_f have identical characteristics, the combined Signal II (slow +fast) in white light in the in the absence of O_3, would lead to an increase in the peak-to-peak height of the white light composite signals I and II by increasing the magnitude of the high field minimum, although the low field maximum would be little affected. This increase would be independent of any change in Signal I. However, since O_3 causes an increase in the dark signal attributable to Y_D^+, this suggests that the normal flow of electrons from water-splitting through Y_Z^+ to P680 has been impaired, and that the increase in the white light signal is the result of increases in both Signal II_s and Signal I. Of these changes only the enhancement of Signal I would be revealed by the traces of its low field maximum.

During a single exposure to O_3 the fluorescence quantum yield and photochemical quenching decreased over time relative to the control (Fig. 4.11), which is in agreement with the fluorescence response observed in the presence of DCMU (Krause and Weis, 1991) or atrazine (Bolhar-Nordenkampf et al., 1989). These together with the O_3-induced increases in non-photochemical

quenching and the changes in EPR signals are compatible with the earlier suggestion that interference with water-splitting is a major consequence of exposure to O_3 (Schreiber et al., 1978; Shimazaki, 1987).

In foxtail, exposure to O_3 also resulted in increases in the EPR signals in white and 710nm light, (Fig. 4.14), although the increases were much less than those observed with Kentucky bluegrass. The saturation of Signal I in white light was slower in O_3 than in the control (Fig. 4.15) again suggesting that O_3 was interfering with electron-flow to photosystem I. However, the lack of clear dark signals in foxtail precluded drawing any conclusions as regards Y_Z^+ or Y_D^+. The minor increases in Signal I induced by O_3 were insufficient to lead to any significant changes in fluorescence yield or photochemical quenching. However, the increased non-photochemical quenching caused by O_3 may have been caused by effects on the water-splitting side of PSII as suggested for Kentucky bluegrass.

Although the objective of infiltration with spin traps was to detect the formation of oxyradicals such as hydroxyl and superoxide anion, in no spectra with or without exposure to O_3 were there any indications of the formation of spin-adducts attributable to such radicals, in spite of recent evidence for O_3-induced superoxide formation presented by Runeckles and Vaartnou (1997). The lack of adduct spectra could indicate the lack of penetration of the traps within the leaf tissue to the sites of radical formation, decomposition of the traps within the leaf tissue, or, more likely, the small amounts of adducts formed relative to the noise in the spectra.

The evidence of effects of the traps themselves and of significant interactions between the effects of O_3 and the traps on some of the EPR signals and chlorophyll fluorescence parameters argues for their penetration (or the penetration of reaction products) into the chloroplasts. Although it is not possible to exclude decomposition of the spin traps within the tissues, the efficacy of the traps to form adducts

with superoxide anion *in vitro* was tested prior to use, as shown in Appendix B. In the only other report of the use of spin traps with intact leaf tissues, Mehlhorn et al. (1990) applied the analogue of 4-POBN, N-*tert*-butyl-α-phenylnitrone (PBN) dissolved in toluene to the surface of bean and pea leaves, and detected a single broad signal after exposure to O_3. The species responsible for the adduct could not be identified, and showed no hyperfine splitting. Its *g*-value, 2.0037 to 2.0039 is between those of Signal I (2.0025) and Signal II (2.0046). However, it is surprising that no Signal II was apparently detected in either species, since the leaves were exposed to O_3 in light. Preliminary tests in the present studies with grass leaves using toluene as the solvent were found to result in severe tissue injury. As a result, DMPO and 4-POBN were used because of their solubilities in water, permitting their introduction into the leaves by vacuum-infiltration.

Both DMPO and 4-POBN caused minor changes in the EPR spectra relative to control leaves of Kentucky bluegrass or foxtail (Table 4.6) and the traps tended to lead to decreased quantum yield and qP and increased qN and NPQ in Kentucky bluegrass and to increased non-photochemical quenching in foxtail, suggesting that both were reacting in some way with components of photosystem II. Although the effects of the traps on EPR signals in Kentucky bluegrass were marginal, in foxtail DMPO resulted in a small but significant increase in the white light signal, while 4-POBN tended to suppress it. Since both hydroxyl and superoxide radicals produced by photooxidation of chlorophyll in the presence of oxygen can be trapped by DMPO (Harbour and Bolton, 1975, 1978) trapping of such oxyradicals at their sites of formation within the photosystems would reduce their reactivity in the destructive photooxidation of chlorophyll and hence might be expected to result in small increases in Signal I such as those shown Figs. 4.8 and 4.9 with bluegrass.

4-POBN caused only minor changes in the EPR signals in either species. However, it resulted in significant decreases in quantum yield and photochemical quenching and increased non-

photochemical quenching, indicating perturbation of photosystem II in Kentucky bluegrass. In contrast, no such effects were observed with DMPO in foxtail, although they were observed in Kentucky bluegrass (Table 4.6).

With exposure to O_3, the major effects of infiltration with either spin trap were to cancel the effect of O_3 on fluorescence parameters. Although DMPO did not influence the O_3-induced changes in Y, qP and NPQ, it negated the effect on qN, while 4-POBN reversed the changes in each parameter. In foxtail, DMPO removed the O_3-induced increases in non-photochemical quenching and 4-POBN reduced the increase in qN. Both traps may have been effective scavengers of oxyradicals resulting from O_3, even though the spectra of their adducts could not be detected. As a result the effects of such radicals on the integrity of PSII were reduced. However, the nature of these overall changes induced by the spin traps themselves, especially in the presence of O_3, are difficult to interpret in terms of specific mechanisms.

In summary, the effects of O_3 on both Kentucky bluegrass and foxtail revealed by the observed changes in Signals I and II are indicative of reduced electron flow through PSI, and through PSII. This suggests that the main effect of O_3 is on the electron acceptor side of PSII, and may involve impairment of the water-splitting process, as suggested by Schreiber et al. (1978) and others. The observed O_3-induced reduction in chlorophyll fluorescence quantum yield and photochemical quenching, and increase in both non-photochemical quenching parameters (qN and NPQ) are compatible with such an interpretation. Effects of O_3 on the kinetics of changes in Signal I on switching between different light exposures suggested that O_3 may also inhibit ferredoxin/NADP oxidoreductase. The spin traps DMPO and

4-POBN had little effect on the EPR signals and fluorescence parameters; nor did they result in the appearance of signals identifiable as the adducts of hydroxyl or superoxide radicals. Nevertheless, the

traps appeared to have interfered with the normal functioning of photosystem II in Kentucky bluegrass because they resulted in increased non-photochemical quenching of fluorescence, and gave rise to small increases in Signal II. These effects were less clear in foxtail. The effects of O_3 on both EPR signals and fluorescence parameters increased over time indicating that they were responses to the increasing O_3 concentration.

Finally, the differences observed between the two species in the kinetics of Signal I with changing illumination are in agreement with reports that in C-4 species such as foxtail, the light-harvesting complex of PSII contributes to light-capture by PSI in the bundle-sheath chloroplasts.

5. GENERAL DISCUSSION

The maximum concentrations of O_3 and NO_2 used in these studies with radish and of O_3 used with the two grass species were close to the thresholds at which adverse effects might be expected. Nevertheless, significant effects of exposure were observed on the functioning of the photosynthetic apparatus in all three species. Furthermore, the effects were observed using exposure profiles that simulated those occurring in ambient air, and avoided sudden exposures to the pollutant gas.

A major difference between the radish and the grass studies was that the observations on photosynthesis (by chlorophyll fluorescence measurements) in the former were made after a week of daily exposures, while those on the grasses (which involved both EPR and fluorescence measurements) were made during the first exposure period. This difference may account for the differences observed between the two with regard to photosynthetic functioning as a result of exposure to O_3. In radish, repeated daily exposures to O_3 led to apparent increases in quantum yield and photochemical quenching, whereas during a single exposure the grasses showed decreases in both parameters. Although no measurements of these parameters were made on radish during the first exposure period for comparison, the effects observed suggest acclimation to the daily exposure regime. Adaptation of radish to O_3 has been demonstrated in terms of leaf growth, although at higher exposures (constant 170ppb) than those used in the present study (Walmsley et al., 1980). The effects appeared within a few days of the start of exposure, and ultimately led to significant decreases in leaf and hypocotyl dry weight relative to filtered air controls. At the lower exposures used in the present study, no adverse effects of O_3 on growth were found, leading to the suggestion that the increased photosynthetic activity indicated by the fluorescence parameters was offset by increased respiration for maintenance and repair, a phenomenon reported in bean plants exposed to O_3 (Amthor, 1988; Amthor and Cumming, 1988).

The adverse effects of O_3 observed on grass photosynthesis were clearly responses of tissues that had not previously experienced O_3 levels greater than 10-15ppb. Furthermore, the effects tended to build up over time as the level of O_3 rose to its 120ppb maximum and then began to decline. The effects observed both on photosynthetic EPR signals and chlorophyll fluorescence parameters generally continued to rise after the maximum O_3 concentration had been reached. However, in radish the fluorescence parameters tended to remain relatively stable during the period of O_3 exposure, again suggesting acclimation of tissues resulting from the previous exposures. The combination of previous exposure to O_3 and the exposure profile used certainly modified stomatal response, as indicated by the diminished daily rhythm of stomatal closure.

The long-term O_3 exposures of radish failed to cause any significant decreases in dry matter accumulation, in spite of the reported sensitivity of the species and cultivar used. This lack of response may have been a result of the use of the sine-wave exposure profile in which, even with a peak concentration of 120ppb O_3, the total exposure would have been one half of that provided by a square-wave of 120ppb. The observations of Musselman et al. (1983; 1986) that adverse effects of O_3 on bean were dependent on the maximum concentration rather than the total exposure were obtained with concentrations reaching peak levels of 500ppb. Such levels resulted in significantly greater growth reductions than square-wave exposures to 200ppb with the same total exposure duration. They also resulted in significantly greater foliar necrotic injury, suggesting a relationship between injury and dry matter accumulation in contrast to numerous reports of a lack of such relationships in several species. Since the rating of radish as a sensitive species was based on square-wave exposures, the absence of injury and lack of adverse growth effects observed in the present study suggests that such ratings may be of questionable relevance to exposures experienced in the field. Nevertheless, if radish is indeed a sensitive species, the lack of growth effects observed here suggests that an air quality

objective or standard for O_3 based on the avoidance of levels exceeding 120ppb would be justifiable in minimizing the likelihood of adverse effects on vegetation.

However, the observation that exposures to sequences of O_3 and NO_2 tended to result in diminished growth relative to the controls illustrate the problems of attempting to quantify the effects of one pollutant in the absence of another likely to occur with it in ambient air. Viewed in this light, the O_3 level of 120ppb appears to be too high to ensure that adverse effects on vegetation will not occur.

The effects of treatment with O_3 observed as EPR signals indicate that these signals can be a useful complement to other non-invasive methods of assessing changes in the functioning of the photosynthetic apparatus in intact leaves, such as photometric chlorophyll estimation and the measurement of chlorophyll fluorescence. In this exploratory study, the focus was on overall changes in the EPR signals associated with PSI and PSII. However, no observations on pure Signal I (and thereby on photosystem I alone) were made since this would have involved a prohibitive number of signal subtractions of each dark signal (II) from the immediately preceding signals (I + II) obtained in white or far-red light. However, an indication of the specific effects of O_3 on photosystem I was obtained from the kinetic traces of Signal I height obtained with the spectrometer locked onto its low field maximum. These observations indicated that O_3 inhibited electron flow through PSI. The observations of concurrent changes to Signal II and chlorophyll fluorescence parameters lead to the suggestion that a major site of O_3 impact is on the acceptor side of PSII. This is in agreement with the original suggestion of Schreiber et al. (1978) based on chlorophyll fluorescence kinetics that O_3 inhibits water-splitting. It should be noted that in radish, dark-adapted fluorescence measurements made prior to the start of the O_3 exposure showed no residual effect of previous exposures that indicate impairment of PSII. The present studies of Signal I kinetics in the grasses suggest that an

additional effect of O_3 may be on ferredoxin/NADP oxidoreductase or some other electron transfer downstream of PSI.

The observation of marked differences between the heights of the low field maximum of Signal I in white and far-red light between C-3 Kentucky bluegrass and C-4 foxtail suggest that such differences may be used to demonstrate the contribution of PSII to light harvesting in the bundle sheath chloroplasts of C-4 species. In Kentucky bluegrass the ratio white/far-red is approximately 0.5 in air, while in foxtail the ratio is certainly greater than unity and may approach a value of 2.0. The interpretation that this reflects an involvement of PSII light-harvesting in electron flow through PSI in spite of the reported low photosystem II activity *per se* in C-4 bundle-sheath chloroplasts is supported by the recent findings of Pfündel et al. (1996) that PSII light absorption contributes significantly to PSI functioning in foxtail and other C-4 species. A white/far-red Signal I ratio of approximately 0.5 in Kentucky bluegrass (and other C-3 species) implies an approximate balance between PSI and PSII. If fully functional PSII is absent from the bundle-sheath chloroplasts of C-4 species but both systems are approximately equal in the mesophyll cell chloroplasts, a ratio less than 0.5 would be expected in contrast to the values >1 observed in foxtail. Hence observations of the white/far-red Signal I ratio may provide an alternative means of assessing the contribution of PSII light harvesting in bundle-sheath chloroplasts, although this has yet to be examined in other C-4 species.

In spite of EPR evidence for the formation of the superoxide anion radical during O_3 exposure (Runeckles and Vaartnou, 1997) the use of two infiltrated spin traps (DMPO and 4-POBN) failed to reveal any obvious indications of the formation of the adducts of either hydroxyl or superoxide anion radicals. Both traps resulted in effects on chloroplast functioning, as revealed by changes in EPR spectra and chlorophyll fluorescence. The absence of evidence of adduct formation may merely indicate that their formation could not be detected in the presence of the large signal noise levels

inevitable with the use of heterogeneous leaf tissue. Although the use of signal subtraction methods might have revealed the presence of such adducts, this would have required averaging over a prohibitively large number of replications.

6. SUMMARY AND CONCLUSIONS

1. A system was developed to provide sine-wave exposures of plants or leaves to O_3 and/or NO_2 in growth chambers or in the cavity of an electron paramagnetic resonance (EPR) spectrometer. The sine-wave exposures simulated the diurnal patterns of exposure to the pollutant gases observed in ambient air.

2. Daily exposures of radish plants to O_3 or NO_2 alone and in various sequences, in which the maximum concentration of either pollutant reached 120ppb, revealed that O_3 had no effects on dry matter accumulation after four weeks, while growth was stimulated by exposures to NO_2, particularly if they occurred early in the day.

3. Chlorophyll fluorescence measurements of quantum yield and photochemical quenching made after 6 days of exposure indicated stimulation of photosynthesis in leaves exposed to O_3 alone or to early NO_2 alone. Exposures to NO_2 late in the day resulted in no stimulation of fluorescence and exposures to early + late NO_2 resulted in stimulation to an intermediate level. These increases suggest increased photosynthetic CO_2-fixation. Although exposures to O_3 resulted in a decrease in non-photochemical quenching, exposures to NO_2 late in the day resulted in increases in non-photochemical quenching possibly indicating disturbance of the pH status of the thylakoid membranes resulting from the production of nitric and nitrous acids by dissolution of the absorbed NO_2.

4. With O_3, the possibly increased CO_2-assimilation in the absence of increased growth is interpreted as indicating that exposure increased respiratory demands for maintenance and repair. In contrast, increased CO_2-assimilation may account for the observed early NO_2-induced increase in growth as evidenced by increased quantum yield and photochemical quenching.

5. Sequential exposures to NO_2 and O_3 resulted in progressive decreases in dry matter production relative to either the control or O_3 treatment which became significant in the combination of O_3 with

NO_2 exposures both early and late in the day. However, fluorescence quantum yields and photochemical quenching remained higher if NO_2 preceded or followed the O_3 exposure and only dropped to the control level with exposure to both early and late NO_2 and O_3. These differences are interpreted as indicating that the stimulations induced by O_3 and NO_2 involve different mechanisms, and that, in the sequential exposures, the mechanisms are counteracting.

6. Repeated O_3-exposure suppressed the diurnal pattern of stomatal behaviour and caused stomata to remain open while NO_2 tended to enhance the diurnal pattern. The consequences of these stomatal responses were that, although exposure to NO_2 had little effect on the estimated flux of O_3 into the leaf, O_3 caused increased fluxes of NO_2 both early and late in the day.

7. No visible symptoms of necrotic foliar injury were observed in any treatment. While this was expected in the case of exposures to NO_2, the exposures to O_3 were close to the threshold at which small amounts of visible injury might have been expected.

8. The lack of significant effects of repeated exposures to either O_3 or NO_2 or their sequences on ethylene release is interpreted as indicating that the effect of O_3 in stimulating ethylene release reported by others may have resulted from the induction of foliar injury or incipient injury in the leaf tissues used rather than from any action on the normal processes involved in ethylene synthesis.

9. In both a C-3 grass, Kentucky bluegrass, and a C-4 grass, green foxtail, exposure to O_3 resulted in significant time-dependent increases in EPR Signal II (related to PSII activity) and Signal I (related to PSI activity), obtained by exposure of the leaves to white or far-red light. The effects are interpreted as indicating a major effect of O_3 on the acceptor side of PSII.

10. Examination of the behaviour of the kinetics of the increases and decreases of the low field maximum of Signal I observed on switching between darkness and illumination with white or far-red light suggests that O_3 may also interfere with the functioning of feredoxin/NADP oxidoreductase or some other electron transfer on the downstream side of PSI.

11. The observation that the ratio of the heights of the low field Signal I maxima in white to far-red light vary from about 0.5 in Kentucky bluegrass to >1 in foxtail suggests that the observation of such ratios can provide information on the contribution made by PS II light-harvesting to PSI functioning in the chloroplasts of bundle-sheath cells in C-4 plants.

12. The use of infiltrated spin traps (DMPO and 4-POBN) in attempting to find evidence for the increased formation of either the hydroxyl or superoxide anion radicals during exposure to O_3 was unsuccessful because of the inability of the system to resolve any adduct signals from the high noise level inherent in the use of intact leaf tissue. Nevertheless, changes from the controls in both EPR signals and chlorophyll fluorescence parameters, during exposures to O_3 of tissues infiltrated with either spin trap suggested that the traps had penetrated the chloroplasts and had reacted perhaps as scavengers of endogenous radicals which would otherwise have been detrimental to chloroplast functioning.

13. The lack of growth effects on radish due to repeated daily exposures to O_3 suggests that the peak concentration reached was insufficient to exceed the threshold for adverse effects in this species. If, as has been reported, radish is a species sensitive to O_3, this implies that air quality objectives or standards based on the avoidance of exceedances of 120ppb will provide protection to most vegetation.

14. However, the adverse effects of exposures to sequences of O_3 and NO_2 not exceeding 120ppb also indicate that air quality objectives or standards based on effects observed on the individual pollutants will be less adequate in providing protection in situations where both pollutants are present in the ambient air.

7. BIBLIOGRAPHY

Adaros, G., H.J. Weigel & H.J. Jager. Single and interactive effects of low levels of O_3, SO_2 and NO_2 on the growth and yield of spring rape. **Environ. Pollut. 72: 269-286 (1991).**

Adepipe, N.O., and D.P. Ormrod. Hormonal regulation of ozone phytotoxicity in *Raphanus sativus*. **Zeit. Pflanzenphysiol. 68: 453-8. (1972).**

Agrawal, M. & S.B. Agrawal. Effects of ozone exposure on enzymes and metabolites of nitrogen metabolism. **Scientia Hortic. 43: 169-177 (1990).**

Amthor, J.S. Growth and maintenance respiration in leaves of bean (*Phaseolus vulgaris* L.) exposed to ozone in open-top chambers in the field. **New Phytol. 110: 319-325 (1988).**

Amthor, J.S. & J.R. Cumming. Low levels of ozone increase bean leaf maintenance respiration. **Can. J. Bot. 66(4): 724-726 (1988).**

Anderson, J.M. & B. Anderson. The dynamic photosynthetic membrane and regulation of solar energy conversion. **Trends Biol. Sci. 13: 351-355 (1988).**

Anderson, W.C. & O.C. Taylor. Ozone induced carbon dioxide evolution in tobacco callus cultures. **Physiol. Plant. 28: 419-423 (1973).**

Asada, K. & M. Takahashi. Production and scavenging of active oxygen in photosynthesis. In **"Photoinhibition", D.J. Kyle, C.B. Osmond & C.J. Arntzen, eds. Elsevier Science, Amsterdam. Pp. 227-287 (1987).**

Atkinson, R. & W.P.L. Carter. Kinetics and mechanisms of the gas-phase reactions of ozone with organic compounds under atmospheric conditions. **Chem. Rev. 84: 437-470 (1984).**

Bae, G.Y., N. Nakajima, K.Ishizuka & N. Kondo. The role in ozone phytotoxicity of the evolution of ethylene upon induction of 1-aminocyclopropane-1-carboxylic acid synthase by ozone fumigation in tomato plants. **Plant Cell Physiol. 37(2): 129-134 (1996).**

Bahl, A. & G. Kahl. Air pollutant stress changes the steady-state transcript levels of three photosynthesis genes. **Environ. Pollut. 88: 57-65 (1995).**

Barnes, J.D. & T. Pfirrmann. The influence of CO_2 and O_3, singly and in combination, on gas exchange, growth and nutrient status of radish (*Raphanus sativus* L.). **New Phytol. 121: 403-412 (1992).**

Barnes, J.D., K. Reiling, A.W. Davison & C.J. Renner. Interactions between ozone and winter stress. **Environ. Pollut. 53: 235-254 (1988).**

Barry, B.A. & G.T. Babcock. Tyrosine radicals are involved in the photosynthetic oxygen-evolving system. **Proc. Natl. Acad. Sci. USA 84: 7099-7103 (1987).**

Bassi, R., & J. Marquardt, & J. Lavergne. Biochemical and functional properties of photosystem II in agranal membranes from maize mesophyll and bundle-sheath chloroplasts. **Eur. J. Biochem. 233: 7 9-719 (1995).**

Beckerson, D.W. & G. Hofstra. Stomatal responses of white bean to O_3 and SO_2 singly or in combination. **Atmos. Environ. 13: 533-535 (1979).**

Beckerson, D.W. & G. Hofstra. Effects of sulphur dioxide and ozone, singly or in combination, on membrane permeability. **Can. J. Bot. 58: 451-457 (1980).**

Beinert, H., B. Kok & G. Hoch. The light-induced electron paramagnetic resonance signal of photocatalyst P700*. **Biochim. Biophys. Res. Commun. 3: 209-213 (1962).**

Bender, J., H.J. Weigel & H.J. Jager. Response of nitrogen metabolism in beans (*Phaseolus vulgaris* L.) after exposure to ozone and nitrogen dioxide, alone and in sequence. **New Phytol. 119: 261-267 (1991).**

Benedict, H.M. & W.H. Breen. The use of weeds as a means of evaluating vegetation damage caused by air pollution. **Proc. 3rd Nat. Air Pollut. Sympos., Pasadena, 3: 177-190 (1955).**

Bennett, J.H. & A.C. Hill. Absorption of gaseous air pollutants by a standardized plant canopy. **J. Air Pollut. Contr. Assn. 23: 203-206 (1973).**

Bennett, J.H., A.C. Hill & D.M. Gates. A model for gaseous pollutant sorption by leaves. **J. Air Pollut. Contr. Assn. 23: 957-962 (1973).**

Bennett, J.P. & V.C. Runeckles. Effects of low levels of ozone on plant competition. **J. Appl. Ecol. 14: 877-880 (1977).**

Bennett, J.P., H.M. Resh & V.C. Runeckles. Apparent stimulation of plant growth by air pollutants. **Can. J. Bot. 52:35-41 (1973).**

Bennett, J.P., R.J. Oshima & L.F. Lippert. Effects of ozone on injury and dry matter partitioning in pepper plants. **Environ. Exper. Bot. 19: 33-39 (1979).**

Bicak, C.J.. "Plant Response to Variable Ozone Regimes of Constant Dosage". **M.Sc. Thesis, University of British Columbia, Vancouver B.C. (1978).**

Black, V.J., D.P. Ormrod & M.H. Unsworth. Effects of low concentration of ozone, singly and in combination with sulphur dioxide on net photosynthesis rates of *Vicia faba* L.. **J. Exper. Bot. 33: 1302-1311 (1982).**

Blumenfeld, L.A., M.G. Goldfield, A.I. Tzapin & S.V. Hangulov. Electron spin resonance signal I in leaves of higher plants: behaviour under simultaneous and separate illumination with red and far red light. **Photosynthetica 8(3): 168-175 (1974).**

Bolhar-Nordenkampf, H.R., S.P. Long, N.R. Baker, G. Oquist, U. Schreiber & E.G. Lechner. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. **Functional Ecol. 3: 497-514 (1989).**

Borg, D.C. Applications of electron spin resonance in biology. In **"Free radicals in biology", W.A. Pryor, ed. Vol. 1. Academic Press, New York. Pp. 69-147 (1976).**

Brennan, T. & L.E. Anderson. Inhibition by catalase of dark-mediated glucose-6-phosphate dehydrogenase activation in pea chloroplasts. **Plant Physiol. 66: 815-817 (1980).**

Bull, J.N., A.T. Mansfield. Photosynthesis in leaves exposed to SO_2 and NO_2. **Nature (London) 250: 443-444. (1974).**

Butler, L.K. & T.W. Tibbetts. Stomatal mechanisms determining genetic resistance to ozone in *Phaseolus vulgaris* L. **J. Amer. Soc. Hort. Sci. 104: 213-216 (1979).**

Capron, T.M. & T.A. Mansfield. Inhibition of net photosynthesis in tomato in air polluted with NO and NO_2. **J. Exper. Bot. 27: 1181-1186 (1976).**

Carlson, R.W.. Interaction between SO_2 and NO_2 and their effects on photosynthetic properties of soybean Glycine max. **Environ. Pollut. (Ser. A) 32: 11-38 (1983).**

Chameides, W.L. & J.P. Lodge. Tropospheric ozone: Formation and fate. In **"Surface Level Ozone Exposures and Their Effects on Vegetation", A.S. Lefohn, ed. Lewis Publishers, Chelsea, MI. Pp. 5-30 (1992).**

Chanway, C.P. & V.C. Runeckles. Effect of ethylene diurea (EDU) on ozone tolerance and superoxide dismutase activity in bush bean. **Environ. Pollut. (Ser. A) 35: 49-56 (1984).**

Chapman, S. A theory of upper atmospheric ozone. **Mem. Roy. Meteorol. Soc. 3:103-125 (1930).**

Chimiklis, P.E. & R.L. Heath. Ozone-induced loss of intracellular potassium ion from *Chlorella sorokiniana*. **Plant Physiol. 56: 723-727 (1975).**

Connor, H.D., V. Fischer & R.P. Mason. A search for oxygen-centered free radicals in the lipoxygenase/linoleic acid system. **Biochem. Biophys. Res. Commun. 141(2) : 614-621 (1986).**

Cooley, D.R. & W.J. Manning. The impact of ozone on assimilate partitioning in plants: a review. **Environ. Pollut. 47: 95-113 (1987).**

Coulson, C. & R.L. Heath. Inhibition of the photosynthetic capacity of isolated chloroplasts by ozone. **Plant Physiol. 53: 32-38 (1974).**

Craker, L.E.. Ethylene production from ozone injured plants. **Environ. Pollut. 1: 299-304 (1971).**

Czech, M., & W. Nothdurft. Investigations of the damage to field and horticultural crops by chlorine, nitrous and sulfur dioxide gases. **Landwirt. Forsch. 4:1-36 (1952).**

Dann, M.S. & E.J. Pell. Decline of activity and quantity of ribulose bisphosphate carboxylase/oxygenase and net photosynthesis in ozone-treated potato foliage. **Plant Physiol. 91: 427-432 (1989).**

Darrall, N.M. The effect of air pollutants on physiological processes in plants. **Plant Cell Environ. 12: 1-30 (1989).**

Debus, R.J., B.A. Barry, G.T. Babcock & L. McIntosh. Site-directed mutagenesis identifies a tyrosine radical involved in the photosynthetic oxygen-evolving system. **Proc. Natl. Acad. Sci. USA 85: 427-430 (1988a).**

Debus, R.J., B.A. Barry, I. Sithole, G.T. Babcock & L. McIntosh. Directed mutagenesis indicates that the donor to P680+ in photosystem II is tyrosine-161 of the D1 polypeptide. **Biochemistry 27: 9071-9074 (1988b).**

Decleire, M., W. de Cat, L. de Temmerman & H. Baeten. Changes of peroxidase, catalase, and superoxide dismutase activities in ozone-fumigated spinach leaves. **J. Plant Physiol. 116: 147-152 (1984).**

Dekker, J.P., J.J. Plijter, L. Ouwehand & H.J. Van Gorkom. Kinetics of manganese redox transitions in the oxygen-evolving apparatus of photosynthesis. **Biochim. Biophys. Acta 767: 176-179 (1984).**

Dijak, M. & D.P. Ormrod. Some physiological and anatomical characteristics associated with differential ozone sensitivity among pea cultivars. **Environ. Exper. Bot. 22: 395-402 (1982).**

Dugger, W.M., Jr. & R.L. Palmer. Carbohydrate metabolism in leaves of rough lemon as influenced by ozone. **Proc. 1st Int. Citrus Sympos. 2: 711-715 (1969).**

Dugger, W.M., Jr. & I.P. Ting. Physiological and biochemical effects of air pollution oxidants on plants. **Rec. Adv. Phytochemistry 3: 31-58 (1970a).**

Dugger, W.M., Jr. & I.P. Ting. Air pollution oxidants - their effects on metabolic processes in plants. **Ann. Rev. Plant Physiol. 21: 215-234 (1970b).**

Engle, R.L. & W.H. Gabelman. Inheritance and mechanism for reistance to ozone damage in onion, *Allium cepa* L. **Proc. Amer. Soc. Hort. Sci. 89: 423-430 (1966).**

Evans, L.S., & I.P. Ting. Ozone-induced membrane permeability changes. **Amer. J. Bot. 60: 155-162 (1973).**

Evans, L.S., & I. P. Ting. Effects of ozone ^{86}Rb-labelled potassium transport in leaves of *Phaseoulus vulgaris* L. **Atmos. Environ. 8: 855-861 (1974a).**

Evans, L.S. & I.P. Ting. Ozone sensitivity of leaves; relationship to leaf water content, gas transfer resistance and anatomical characteristics. **Amer. J. Bot. 61(6): 592-597 (1974b).**

Farage, P.K., S.P. Long, E.G. Lechner & N.R. Baker. The sequence of change within the photosynthetic apparatus of wheat following short-term exposure to ozone. **Plant Physiol. 95: 529-535 (1990).**

Feder, W.A.. Reduction in tobacco pollen germination and tube elongation, induced by low levels of ozone. **Science 160: 1122 (1968).**

Feder, W.A. Plant response to chronic exposure of low levels of oxidant type air pollution. **Environ. Pollut. 1: 73-79 (1970).**

Flagler, R.B., R.P. Patterson, A.S. Heagle & W.W. Heck. Ozone and soil moisture deficit effect on nitrogen metabolism of soybean. **Crop Sci. 27: 1177-1184 (1987).**

Fuentes, J.D. & T.J. Gillespie. A gas exchange system to study the effects of leaf surface wetness on the deposition of ozone. **Atmos. Environ. A26: 1165-1173 (1992).**

Garsed, S.G. Uptake and distribution of pollutants in plant and residence time of active species. In **"Gaseous air pollutants and plant metabolism". M.J. Koziol and F.R. Whatley eds. Butterworths, London. Pp. 83-103 (1982).**

Genty, B., J.M. Briantais & N. Baker. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. **Biochim. Biophys. Acta 990: 87-92 (1989).**

Godde, D. & J. Buchhold. Effect of long term fumigation with ozone on the turnover of the D-1 reaction center polypeptide of photosystem II in spruce (*Picea abies*). **Physiol. Plant. 86: 568-574 (1992).**

Godzik, S., M.R. Ashmore & J.N.B. Bell. Responses of radish cultivars to long-term and short-term exposures to sulphur dioxide, nitrogen dioxide, and their mixture. **New Phytol. 100: 191-197 (1985).**

Goodyear, S.N. & D.P. Ormrod. Tomato response to concurrent and sequential NO_2 and O_3 exposures. **Environ. Pollut. 51: 315-326 (1988).**

Greitner, C.S. & W.E. Winner. Increases in $d^{13}C$ values of radish and soybean plants caused by ozone. **New Phytol. 108(4): 489-494 (1988).**

Grimes, H.D., K.K. Perkins & W.F. Boss. Ozone degrades into hydroxyl radical under physiological conditions. A spin trapping study. **Plant Physiol. 72: 1016-1020 (1983).**

Grimm, A.G. & J. Fuhrer. The response of spring wheat (*Triticum aestivum* L.) to ozone at higher elevations. III. Responses of leaf and canopy gas exchange and chlorophyll fluoroescence to ozone flux. **New Phytol. 122: 321-328 (1992).**

Guderian, R., D.T. Tingey & R. Rabe. Effects of photochemical oxidants on plants. In **"Air Pollution by Photochemical Oxidants", R. Guderian, ed. Springer-Verlag, Berlin. Pp. 127-333. (1985).**

Gupta, A.S., R.G. Alscher, & D. McCune. Response of photosynthesis and cellular antioxidants ozone in *Populus* leaves. **Plant Physiol. 96: 650-655 (1991).**

Haagen-Smit, A.J., Darley, E.F., Zaitin, M., Hull,T., and Noble, W. Investigation on injury to plants from air pollution in the Los Angles area. **Plant Physiol. 27: 18-34 (1952).**

Haber, F. & J. Weiss. The catalytic decomposition of hydrogen peroxide by iron salts. **Proc. Roy. Soc., London, Ser. A, 147: 332-351 (1934).**

Harrison, B.H. & W.A. Feder. Ultrastructural changes in pollen exposed to ozone. **Phytopathology 64: 257-258 (1974).**

Hassan, I.A., M.R. Ashmore & J.N.B. Bell. Effects of O3 on the stomatal behaviour of Egyptian varieties of radish (*Raphanus sativus* L. cv. Baladey) and turnip (*Brassica rapa* L. cv. Sultani). **New Phytol. 128: 243-249 (1994).**

Harbour, J.r. & J.r. Bolton. Superoxide Formation in Spinach Chloroplasts: Electron Spin Resonancedetection by Spin Trapping. **Biochem. Biophys. Res. Comm. 64(3): 803-807 (1975).**

Harbour, J.R. & J.R. Bolton. The involvement of the hydroxyl radical in the destructive photoxidation of chlorophylls *in vivo* and *in vitro*. **Phochem. Photobiol. 28: 231-234 (1978).**

Heagle, A.S.. Ozone and crop yield. **Ann. Rev. Phytopathol. 27: 397-423 (1989).**

Heagle, A.S. & M.B. Letchworth. Relationships among injury, growth and yield responses of soybean cultivars exposed to ozone at different light intensities. **J. Environ. Qual. 11: 690-694 (1982).**

Heagle, A.S., D.F. Body & E.K. Pounds. Effect of ozone on yield of sweet corn. **Phytopathology 62: 683-687 (1972).**

Heagle, A.S., R.B. Philbeck & W.M. Knott. Thresholds for injury, growth and yield loss caused by ozone on field corn hybrids. **Phytopathology 69: 21-26 (1979b).**

Heagle, A.S., R.B. Philbeck, H.H. Rogers & M.B. Letchworth. Dispensing and monitoring ozone in open-top field chambers for plant-effects studies. **Phytopathology 69: 15-20 (1979c).**

Heagle, A.S., S. Spencer & M.B. Letchworth. Yield response of winter wheat to chronic doses of ozone. **Can. J. Bot. 57: 1999-2005 (1979a).**

Heagle, A.S., L.W. Kress, P.J. Temple, R.J. Kohut, J.E. Miller & H.E. Heggestad. Factors influencing ozone dose-yield response relationships in open-top field chamber studies. In **"Assessment of Crop Loss from Air Pollutants", W.W. Heck, O.C. Taylor & D.T. Tingey, eds. Elsevier Applied Science, London. Pp. 141-179 (1988).**

Heagle, A.S., R.B. Philbeck, & M.B. Letchworth. Injury and yield response of spinach cultivars to chronic doses of ozone in open-top field chambers. **J. Environ. Qual. 8(3): 368-373 (1979d).**

Heath R.L. Responses of plants to air pollution. In **"Responses of Plants to Air Pollution". J.B. Mudd & M. Kozlowski, eds. Academic Press, New York Pp. 23-55 (1975)**

Heath, R.L. Initial events in injury to plants by air pollutants. **Ann. Rev. Plant Physiol. 31: 395-431 (1980).**

Heath, R.L. Air pollutant effects on biochemicals derived from metabolism: organic, fatty and amino acids. In **"Gaseous Air Pollutants and Plant Metabolism", M.J. Koziol & F.R. Whatley, eds. Butterworths, London. Pp. 275-290 (1984).**

Heath, R.L. The biochemistry of ozone attack on the plasma membrane of plant cells. **Rec. Adv. Phytochem. 21: 29-54 (1987a).**

Heath, R.L. Oxidant air pollutants and plant injury. In **"Models in Plant Physiology and Biochemistry", D.W. Newman & K.G. Wilson, eds. CRC Press, Boca Raton, FL. Vol. III. Pp. 63-66 (1987b).**

Heath, R.L. Biochemical mechanisms of pollutant stress. In **"Assessment of Crop Loss from Air Pollutants", W.W. Heck, O.C. Taylor & D.T. Tingey, eds. Elsevier Applied Science, London. Pp. 259-286 (1988).**

Heath, R.L. Possible mechanisms for the inhibition of photosynthesis by ozone. **Photosyn. Res. 39:439-451 (1994).**

Heath, R.L, & F.J. Castillo. Membrane disturbances in response to air pollutants. In **"Air Pollution and Plant Metabolism," S. Schulte-Holstede, N.M Darrall, L.W. Blank, & A.R. Wellburn eds. Elsevier, London. Pp 55-75 (1988).**

Heck, W.W. Assessment of crop losses from air pollutants in the United States. In **" Air Pollution's Toll on Forests and Crops", J.J. MacKenzie & M.T. El-Ashry eds. Yale University Press, Pp. 235-315 (1989).**

Hill, A.C. & J.H. Bennett. Inhibition of apparent photosynthesis by nitrogen oxides. **Atmos. Environ. 4: 341-348 (1970).**

Hill, A.C. & N. Littlefield. Ozone. Effect on apparent photosynthesis, rate of transpiration and stomatal closure in plants. **Environ. Sci. Technol. 3(1): 52-56 (1969).**

Hill, R. & F. Bendall. Function of the two cytochrome components in chloroplasts: a working hypothesis. **Nature (London). 186: 136-1137 (1960).**

Hoganson, C.W. & Babcock, G.T. Electron-transfer events near the reaction center in O_2-evolving photosystem II preparations. **Biochemistry 27: 5848-5855 (1988).**

Hogsett, W.E., S,R. Holman, M.L. Gumpertz & D.T. Tingey. Growth response in radish to sequential and simultaneous exposures of NO_2 and SO_2. **Environ. Pollut. (Ser. A) 33: 303-325 (1984).**

Hoigne, J. & H. Bader. Ozonation of water: role of hydroxyl radicals as oxidizing intermediates. **Science, 190: 782-784 (1975).**

Holmes, J.J., H.G. Weger & D.H. Turpin. Chlorophyll a fluorescence predicts total photosynthetic electron flow to CO_2 or NO_3-/NO_2- under transient conditions. **Plant Physiol. 91: 331-337 (1989).**

Horsman, D.C. & A.R. Wellburn. Synergistic effect of SO_2 and NO_2 polluted air upon enzyme activity in pea seedlings. **Environ. Pollut. 8: 124-133 (1975).**

Hove, L.W.A. van, M.E. Bossen, M.G.J. Mensink & O. van Kooten. Physiological effects of a long term exposure to low concentrations of NH_3, NO_2 and SO_2 on Douglas fir (*Pseudotsuga menziesii*). **Physiol. Plant. 86: 559-567 (1992).**

Howell, R.K. Influence of air pollution on quantities of caffeic acid isolated from leaves of *Phaseoulus vulgaris*. **Phytpathol. 60: 1626-1629 (1970).**

Ito, O., F. Mitsumori & T. Totsuka. Effects of NO_2 and O_3 alone or in combination on kidney bean plants (Phaseolus vulgaris L.): products of $^{13}CO_2$ assimilation detected by ^{13}C nuclear magnetic resonance. **J. Exper. Bot. 36: 281-289 (1985).**

Jacks, T.J. & O. Hinojosa. Superoxide radicals in intact tissues and in dimethyl sulphoxide-based extracts. Phytochemistry 33(3): 563-568 (1993).

Kaji, M., T. Yoneyama, T. Totsuka & H. Iwaki. Absorption of atmospheric NO_2 by plants and soils VI. Transformation of NO_2 absorbed in the leaves and transfer of the nitrogen through the plants. **Res. Rep. Natl. Inst. Environ. Stud., Japan 11: 51-58 (1980).**

Kanofsky, J.R. & P. Sima. Singlet oxygen production from the reactions of ozone with biological molecules. **J. Biol. Chem. 266: 9039-9042 (1991).**

Kaur H. & B. Halliwell. Aromatic hydroxylation of phenylalanine as an assay for hydroxyl radicals. Measurement of hydroxyl radical formation from ozone and in blood from premature babies using improved HPLC methodology. **Analytical Biochemistry. 220(1):11-5 (1994)**

Keen, N.T. & O.C. Taylor. Ozone injury in soybeans: isoflavone accumulation is related to necrosis. **Plant Physiol. 55: 731-733 (1975).**

Klein, H., H.-J. Jäger, W. Domes, & C.H. Wong. Mechanisms contributing to differential sensitivity of plants to SO_2. **Oecology 33: 203-208 (1978).**

Knowles, P.F., D. Marsh & H.W.E. Rattle. Magnetic Resonance of Biomolecules. **John Wiley & Sons, London. 343 p (1976).**

Kok, B. & H. Beinert. The light induced EPR signal of photocatalyst P700. II. Two light effects. **Biochem. Biophys. Res. Comm. 9(4): 349-354 (1962).**

Kostka-Rick, R. & W.J. Manning. Partitioning of biomass and carbohydrates in field-grown radish under ambient concentrations of ozone, and treated with the anti-ozonant ethylene-diurea (EDU). **New Phytol. 121: 187-200 (1992).**

Koziol, M.J., F.R. Whatley & J.D. Shelvey. An integrated view of the effects of gaseous air pollutants on plant carbohydrate metabolism. In **"Air Pollution and Plant Metabolism", S. Schulte-Hostede, N.M. Darrall, L.W. Blank & A.R. Wellburn, eds. Elsevier, London. Pp. 148-168 (1988).**

Krause G.H., & E. Weis. Chlorophyll fluorescence and photosynthesis: The basics. **Ann. Rev. Plant. Physiol. Plant. Mol. Biol. 42: 313-349 (1991).**

Kress, L.W. & J.M. Skelly. Response of several eastern forest tree species to chronic doses of ozone and nitrogen dioxide. **Plant Dis. 66: 1149-1152 (1982).**

Kuchino, Y., F. Mori, H. Kasai, S. Iwai, K. Miura, E. Ohtsuka & S. Nishimura. Misreading of DNA templates containing 8-hydroxyguanosine at the modified base and at adjacent residues. **Nature (London) 327: 77-79 (1987).**

Laisk, A., O. Kull & H. Moldau. Ozone concentration in leaf intercellular air spaces is close to zero. **Plant Physiol. 90: 1163-1167 (1989).**

Langebartels, C., K. Kerner, S. Leonardi, M.Schaudner, M.Trost, W.Kerner & H. Sandermann, Jr. Biochemical plant responses to ozone 1. Differential induction of polyamine and ethylene biosynthesis in tobacco. **Plant Physiol. 95: 882-889 (1991).**

Lee, E.H. & J.H. Bennett. Superoxide dismutase. A possible protective enzyme against ozone injury in snap beans (*Phaseolus vulgaris* L.). **Plant Physiol. 69: 1444-1449 (1982).**

Leffler, H.R. & J.H. Cherry. Destruction of enzymatic activities of corn and soybean leaves exposed to ozone. **Can. J. Bot. 52: 1233-1238 (1974).**

Lefohn, A.S. & D.T. Tingey. The co-occurrence of potentially phytotoxic concentrations of various gaseous air pollutants. **Atmos. Environ. 18: 2521-2526 (1984).**

Macdowall, F.D.H.. Importance of soil in the absorption of ozone by a crop. **Can. J. Soil Sci. 54: 239-240 (1974).**

MacLean, D.C., D.C. McCune, L.H. Weinstein, R.H. Mandl, & G.N. Woodruff. Effects of acute hydrogen fluoride and nitrogen dioxide exposures on citrus and ornamental plants of central Florida. **Environ. Sci. Technol. 2: 444-449 (1968).**

Malhotra, S.S. & A.A. Khan. Biochemical and physiological impact of major pollutants. In **"Air Pollution and Plant Life", M. Treshow, ed. Wiley, Chichester, England. Pp. 113-157 (1984).**

Manderscheid, R., J. Bender, H.-J. Weigel & H.-J. Jager. Low doses of ozone affect nitrogen metabolism in bean (*Phaseolus vulgaris* L.) leaves. **Biochem. Physiol. Pflanzen 187: 283-291 (1991).**

Marie, B.A. & D.P. Ormrod. Effects of air pollutants on dry matter partitioning in developing rutabaga and cabbage plants. **Environ. Pollut. 53(1-4): 448-449 (1988).**

Matsushima, J. Influence of SO_2 and NO_2 on assimilation of amino acids, organic acids and saccharoid in *Citrus natsudaidai* seedlings. **Bull. Fac. Agr., Mie Univ. 44: 131-139 (1972)**

Matyssek, R., M.S.G. Gunthardt, T. Keller, & C. Scheidegger. Impairment of gas exchange and structure in birch leaves (*Betula pendula*) caused by low ozone concentrations. **Trees: Structure and Function. 5(1): 5-13 (1991).**

McCain, D.C., T.C. Selig, Govindjee & J.L. Markley. Some plant leaves have orientation-dependent EPR and NMR spectra. **Proc. Natl. Acad. Sci. USA 81: 748-752 (1984).**

McKersie, B.D., P. Hucl & W.D. Beversdorf. Solute leakage from susceptible and tolerant cultivars of Phaseolus vulgaris following ozone exposure. **Can. J. Bot. 60: 73-78 (1982).**

Mehlhorn, H. & A.R. Wellburn. Stress ethylene formation determines plant sensitivity to ozone. **Nature (London) 327: 417-418 (1987).**

Mehlhorn, H., B.J Tabner & A.R. Wellburn. Electron spin resonance evidence for the formation of free radicals in plant exposed to ozone. **Physiol. Plant. 79: 377-383 (1990).**

Mehlhorn, H., J.M. O'Shea & A.R. Wellburn. Atmospheric ozone interacts with stress ethylene formation by plants to cause visible plant injury. **J. Exper. Bot. 42: 17-24 (1991).**

Middleton, J.T., E.F Darley, & R.F. Brewer. Damage to vegetation from polluted atmospheres. **J. Air Pollut. Contr. Assn. 8: 9-15 (1958).**

Miller, J.E.. Effects of ozone and sulfur dioxide stress on growth and carbon allocation in plants. **Rec. Adv. Phytochemistry 21: 55-100 (1987).**

Miller, A.-F. & G.W. Brudvig. A guide to electron paramagnetic resonance spectroscopy of photosystem II membranes. **Bioch. Biophys. Acta 1056: 1-18 (1991).**

Moldau, M., J. Sober & A. Sober. Differential sensitivity of stomata and mesophyll to sudden exposure of bean shoots to ozone. **Photosynthetica 24: 446-458 (1990).**

Molina, M.J. & F.S. Rowland. Stratospheric sink for chlorofluoromethanes: chlorine-atom catalyzed destruction of ozone. **Nature (London) 249: 810-812 (1974).**

Mottley, C., H.D. Connor & R.P. Mason. [^{17}O]oxygen hyperfine structure for the hydroxyl and superoxide radical adducts of the spin traps DMPO, PBN and 4-POBN. **Biochem. Biophys. Res. Commun. 141(2): 522-628 (1986).**

Mudd, J.B.. Effects of oxidants on metabolic function. In **"Effects of Gaseous Air Pollution on Agriculture and Horticulture", M.H.Unsworth & D.P.Ormrod, eds. Butterworth Scientific, London. Pp. 189-203 (1982).**

Murabayashi, M., M. Awaya, H. Tsuji & T. Matsuno. Effects of ozone on photosynthetic electron transport in spinach (II), **Bulletin of the Institute of Environmental Science and Technology, Yokohama National University 7: 43-48 (1981).**

Murray, A.J.S. & A.R. Wellburn. Differences in nitrogen metabolism between cultivars of tomato and pepper during exposure to glasshouse atmospheres containing oxides of nitrogen. **Environ. Pollut. (Ser. A) 39: 303-316 (1985).**

Musselman, R.C., R.J. Oshima & R.E. Gallavan. Significance of pollutant concentration distribution in the response of 'Red Kidney' beans to ozone. **J. Amer. Soc. Hort. Sci. 108: 347-351 (1983).**

Musselman, R.C., A.J. Huerta, P.M. McCool & R.J. Oshima. Response of beans to simulated ambient and uniform ozone distributions with equal peak concentration. **J. Amer. Soc. Hort. Sci. 111(3): 470-473 (1986).**

Nash, T.H. Sensitivity of lichens to nitrogen dioxide fumigation. **Bryologist. 79: 103-106 (1976).**

Natori, N. & T. Totsuka. Effects of mixed gas on transpiration rate of several woody plants 2. Synergistic effects of mixed gas on transpiration rate of *Euonymus japonica*. **Res. Rep. Natl. Inst. Environ. Stud., Japan No. 65, pp. 45-52 (1984).**

Neubert, A., D.Kley & J. Wildt. Uptake of NO, NO_2 and O_3 by sunflower (*Helianthus annuus* L.) and tobacco plants (*Nicotiana tabacum* L.): dependence on stomatal conductivity. **Atmos. Environ. 27A(14): 2137-2145 (1993).**

Nugent, J.H.A, C. Demetriou, & C.J. Lockett. Electron donation in photosystem II. **Biochim. Biophys. Acta 894: 534-542 (1987).**

Nugent, J. H. A., D. J. MacLachlan, S. E. J Rigby, & M. C. W Evans,. Spectroscopic studies of the managanese complex of Photosystem II. **Photosynthesis Res. 38(3): 341-350 (1993).**

Okano, K., T. Totsuka, T. Fukuzawa & T. Tazaki. Growth reponses of plants to various concentrations of nitrogen dioxide. **Environ. Pollut. (Ser. A) 38: 361-373 (1985).**

Olszyk, D.M. & D.T. Tingey. Joint action of O_3 and SO_2 in modifying plant gas exchange. **Plant Physiol. 82: 401-405 (1986).**

Ormrod, D.P., D.T. Tingey, M.L. Gumpertz & D.M. Olszyk. Utilization of a response-surface technique in the study of plant responses to ozone and sulfur dioxide mixtures. **Plant Physiol. 75: 43-48 (1984).**

Oshima, R.J., O.C. Taylor, P.K. Braegelmann & D.W. Baldwin. Effect of ozone on the yield and plant biomass of a commercial variety of tomato. **J. Environ. Qual. 4(4): 463-464 (1975).**

Oshima, R.J., P.K. Braegelmann, D.W. Baldwin, V.Van Way & O.C. Taylor. Responses of five cultivars of fresh market tomato to ozone: a contrast of cultivar screening with foliar injury and yield. **J. Amer. Soc. Hort. Sci. 102(3): 286-289 (1977).**

Oshima, R.J., P.K. Braegelmann, R.B. Flagler & R.R. Teso. The effects of ozone on the growth, yield and partitioning of dry matter in cotton. **J. Environ. Qual. 8(4): 474-479 (1979).**

Pell, E.J. & E. Brennan. Changes in respiration, photosynthesis, adenosine 5'-triphosphate, and total adenylate content of ozonated pinto bean foliage as they relate to symptom expression. **Plant Physiol. 51: 378-381 (1973).**

Pell, E.J. & N.S. Pearson. Ozone-induced reduction in quantity of ribulose-15-bisphosphate carboxylase in alfalfa foliage. **Plant Physiol. 73: 185-187 (1983).**

Pell, E.J., N. Eckardt & A.J. Enyedi. Timing of ozone stress and resulting status of ribulose bisphosphate carboxylase/oxygenase and associated net photosynthesis. **New Phytol. 120: 397-405 (1992).**

Perchorowicz, J.T. & I.P. Tingey. Ozone effects on plant cell permeability. **Amer. J. Bot. 61:7 87-793 (1974).**

Pfündel, E., E. Nagel & A. Meister. Analyzing the light energy distribution in the photosynthetic apparatus of C-4 plants using highly purified mesophyll and bundle-sheath thylakoids. **Plant Physiol. 112: 1055-1070 (1996).**

Pschorn, R., W. Rukle & A. Wild. Structure and function of $NADP^+$-oxidoreductase. **Photosynthesis Res. 17: 217-229 (1988).**

Purvis, A.C.. Differential effects of ozone on in vivo nitrate reduction in soybean cultivars. I. Response to exogenous sugars. **Can. J. Bot. 56: 1540-1544 (1978).**

Reich, P.B., A.W. Schoettle & R.G. Amundson. Effects of low concentrations of O_3, leaf age and water stress on leaf diffusive conductance and water use efficiency in soybean. **Physiol. Plant. 63: 58-64 (1985).**

Reinert, R.A. & W.W. Heck. Effects of nitrogen dioxide in combination with sulfur dioxide and ozone on selected crops. In **"Air Pollution by Nitrogen Oxides", T. Schneider & L. Grant, eds. Elsevier, Amsterdam (1982).**

Reinert, R.A. & T.N. Gray. The response of radish to nitrogen dioxide, sulfur dioxide, and ozone, alone and in combination. **J. Environ. Qual. 10: 240-243 (1981).**

Reinert, R.A., D.T. Tingey & H.B. Carter. Ozone induced foliar injury in lettuce and radish cultivars. **J. Amer. Soc. Hort. Sci. 97(6): 711-714 (1972).**

Reinert, R.A. & J.S. Sanders. Growth of radish and Marigold following repeated exposure to nitrogen dioxide, sulfur dioxide and ozone. **Plant Dis. 66: 122-124 (1982).**

Reinert, R.A., D.S. Shriner & J.O. Rawlings. Responses of radish to all combinations of three concentrations of nitrogen dioxide, sulfur dioxide and ozone. **J. Environ. Qual. 11: 52-57 (1982).**

Robinson, E. & R.C. Robbins. Gaseous nitrogen compound pollutants from urban and natural sources. **J. Air. Pollut. Contr. Assn. 20: 303-306 (1970).**

Robinson, D.C. & A.R. Wellburn. Light-induced changes in the quenching of 9-aminoacridine fluorescence by photosynthetic membranes due to atmosphereic pollutants and their products. **Environ. Pollut. (Ser. A) 32: 109-120 (1983).**

Rowland-Bamford, A.J., S. Coghlan & P.J. Lea. Ozone-induced changes in CO_2 assimilation, O_2 evolution and chlorophyll a fluorescence transients in barley. **Environ. Pollut. 59: 129-140 (1989).**

Rowlands, J.R., E.M. Gause, C.F. Rodriguez & H.C. McKee. "Electron spin resonance studies of vegetation damage". **Final Report, SwRI Project 05-2622-01, Southwest Research Institute, San Antonio TX. 67p. (1970).**

Runeckles, V.C.. Uptake of ozone by vegetation. In **"Surface Level Ozone Exposures and Their Effects on Vegetation", A.S. Lefohn, ed. Lewis Publishers, Chelsea, MI. Pp. 153-184 (1992).**

Runeckles, V.C. & B.I. Chevone. Crop responses to ozone. In **"Surface Level Ozone Exposures and Their Effects on Vegetation", A.S. Lefohn, ed. Lewis Publishers, Chelsea, MI. Pp. 185-266 (1992).**

Runeckles, V.C. & K. Palmer. Pretreatment with nitrogen dioxide modifies plant response to ozone. **Atmos. Environ. 21: 717-719 (1987).**

Runeckles, V.C. & P.M. Rosen. Effects of ambient ozone pretreatment on transpiration and susceptibility to ozone injury. **Can. J. Bot. 55: 193-197 (1976).**

Runeckles, V.C. & M. Vaartnou. Observations on the in situ detection of free radicals in leaves using electron paramagnetic resonance spectrometry. **Can. J. Bot. 70: 192-199 (1992).**

Runeckles,V.C., & Vaartnou, M. EPR evidence for superoxide formation in leaves during exposure to low levels of ozone. **Plant Cell Environ. 20: 306-314 (1997).**

Sabaratnam, S., G. Gupta & C. Mulchi. Effects of nitrogen dioxide on leaf chlorophyll and nitrogen content of soybean. **Environ. Pollut. 51: 113-120 (1988a).**

Sabaratnam, S., G. Gupta & C. Mulchi. Nitrogen dioxide effects on photosynthesis in soybean. **J. Environ. Qual. 17(1): 143-146 (1988b).**

Sakaki, T., N. Kondo & K. Sugahara. Breakdown of photosynthetic pigments and lipids in spinach leaves with ozone fumigation: role of active oxygens. **Physiol. Plant. 59: 28-34 (1983).**

Salam, M.A., & G. Soja. Bush bean (*Phaseolus vulgaris* L.) leaf injury, photosynthesis and stomatal function under elevated ozone levels. **Water, Air Soil Pollut. 85: 1533-1538, (1995).**

Sanders, J.S. & R.A. Reinert. Screening Azalea cultivars for sensitivity to nitrogen dioxide, sulfur dioxide and ozone alone and in mixtures. **J. Amer. Soc. Hort. Sci. 107(1): 87-90 (1982).**

Sandhu, R. & G. Gupta. Effects of nitrogen dioxide on growth and yield of black turtle bean (*Phaseolus vulgaris* L.) cv. 'Domino'. **Environ. Pollut. 59: 337-344 (1989).**

Saran, M., C. Michel & W. Bors. Reactivities of free radicals. In **"Air Pollution and Plant Metabolism", S. Schulte-Hostede, N.M. Darrall, L.W. Blank & A.R. Wellburn, eds. Elsevier, London. Pp. 76-93 (1988).**

Saxe, H.. Effects of NO, NO_2 and CO_2 on net photosynthesis, dark respiration and transpiration of pot plants. **New Phytol. 103: 185-197 (1986).**

Schmidt, W., C. Neubauer, J. Kolbowski, U. Schreiber & W. Urbach. Comparison of effects of air pollutants (SO_2, O_3, NO_2) on intact leaves by measurments of chlorophyll fluorescence and P700 absorbance changes. **Photosyn. Res. 25: 241-248 (1990).**

Schreiber, U., W. Vidaver, V.C. Runeckles & P. Rosen. Chlorophyll fluorescence assay for ozone injury in intact plants. **Plant Physiol. 61: 80-84 (1978).**

Schreiber, U., W. Bilger & C. Neubauer. Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of in vivo photosynthesis. In **"Ecophysiology of photosynthesis", E.S. Schultze & M.M. Caldwell eds. Springer-Verlag, Berlin. Pp. 49-70 (1994).**

Sheng, S. & B.I. Chevone. Gas exchange response of soybean cultivars to short term exposure of sulfur dioxide and ozone. **Phytopathology 78: 1513 (1988).**

Shimazaki, K.. Thylakoid membrane reactions to air pollutants. In **"Air Pollution and Plant Metabolism", S. Schulte-Hostede, N.M. Darrall, L.W. Blank & A.R. Wellburn, eds. Elsevier, London. Pp. 116-133 (1988).**

Shriner, D.S., W.W. Heck, S.B. McLaughlin, D.W. Johnson, P.M. Irving, J.D. Joslin & C.E. Peterson. Response of vegetation to atmospheric deposition and air pollution. In **"Acidic Deposition: State of Science and Technology", Volume III, National Acid Precipitation Assessment Program, Washington, DC (1990).**

Smith, W.H.. Pollutant uptake by plants. In **"Air Pollution and Plant Life", M. Treshow, ed. Wiley, Chichester, England (1984).**

Srivastava, H.S. & D.P. Ormrod. Effects of nitrogen dioxide and nitrate nutrition on growth and nitrate assimilation in bean leaves. **Plant Physiol. 76: 418-423 (1984).**

Srivastava, H.S., P.A. Jolliffe & V.C. Runeckles. Inhibition of gas exchange in bean leaves by NO_2. **Can. J. Bot. 53: 477-474 (1975).**

Staehelin, J. & J. Hoigne. Decomposition of ozone in water. Rate of initiation by hydroxide ion and hydrogen peroxide. **Environ. Sci. Technol. 16: 676-681 (1982).**

Staehelin, J. & J. Hoigne. Decomposition of ozone in water in the presence of organic solutes acting as promoters and inhibitors of radical chain reactions. **Environ. Sci. Technol. 19: 1206-1213 (1985).**

Strand, M.. Photosynthetic activity of Scots pine (Pinus sylvestris L.) needles during winter is affected by exposure to SO_2 and NO_2 during summer. **New Phytol. 123: 133-141 (1993).**

Sugahara, K. Effects of air pollutants on light reactions in chloroplasts. In **"Gaseous air pollutants and plant metabolism", Koziol M.J & F.R. Whatley eds. Butterworths, London. Pp 169-180 (1982).**

Sugahara, K., K. Ogura, M. Takimoto & N. Kondo. Effects of air pollutant mixtures on photosynthetic electron transport systems. **Res. Rept. Natl. Inst. Environ. Stud., Japan 65(1): 155-164 (1984).**

Sutton, R. & I.P. Ting. Evidence for repair of ozone induced membrane injury: alteration in sugar uptake. **Atmos. Environ. 11: 273-275 (1977).**

Suzuki, S., M. Murabayashi, & T. Matsuno. Effects of ozone on photosynthetic electron transport in spinach (III), **Bulletin of the Institute of Environmental Science and Technology, Yokohama National University 8: 81-87 (1982).**

Taylor, G.E., Jr., P.J. Hanson & D.D. Baldocchi. Pollutant deposition to individual leaves and plant canopies: sites of regulation and relationship to injury. In **"Assessment of Crop Loss from Air Pollutants", W.W. Heck, O.C. Taylor & D.T. Tingey, eds. Elsevier Applied Science, London. Pp. 227-257 (1988).**

Taylor, O.C. Effects of oxidant air pollutants. **J. Occup. Med. 10:485-499 (1968).**

Taylor, O.C. & F.M. Eaton. Suppression of plant growth by nitrogen dioxide. **Plant Physiol. 41: 132-135 (1966).**

Taylor, O.C., C.R. Thompson, D.T. Tingey, & R.A. Reinert. Oxides of nitrogen. In **"Responses of plants to air pollution", Mudd, J.B. & T.T.Kozlowski, eds. Academic Press, New York. Pp. 122-139 (1975).**

Temple, P.J. & L.F. Benoit. Effects of ozone and water stress on canopy temperature, water use, and water use efficiency of alfalfa. **Agron. J. 80(3): 439-447 (1988).**

Thompson, C.R., E.G. Hensel, G. Kats & O.C. Taylor. Effects of continuous exposure of naval oranges to NO_2. **Atmos. Environ. 4: 349-355 (1970).**

Thompson, C.R., G. Kats, & E.G. Hensel. Effects of ambient levels of NO_2 on naval oranges. **Atmos. Environ. 4: 349-355 (1971).**

Thompson, J.E., R.L. Legge & R.F. Barber. The role of free radicals in senescence and wounding. **New Phytol. 105: 317-344 (1987).**

Tingey, D.T. & R.A. Reinert. The effects of ozone and sulphur dioxide singly and in combination on plant growth. **Environ. Pollut. 9: 117-125 (1975).**

Tingey, D.T., R.C. Fites & C. Wickliff. Foliar sensitivity of soybeans to ozone as related to several leaf parameters. **Environ. Pollut. 4: 183-192 (1973a).**

Tingey, D.T., R.C. Fites & C. Wickliff. Ozone alteration of nitrate reduction in soybean. **Physiol. Plant. 29: 33-38 (1973b).**

Tingey, D.T., R.G. Wilhour & C. Standley. The effect of chronic ozone exposures on the metabolite content of ponderosa pine seedlings. **For. Sci. 22: 234-241 (1976).**

Tomlinson, H. & S. Rich. Relating lipid content and fatty acid synthesis to ozone injury of tobacco leaves. **Phytopathology 59(9): 1284-1286 (1969).**

Troiano, J.J. & .A. Leone. Changes in growth rate and nitrogen content of tomato plants after exposure to NO_2. **Phytopathology 67: 1130-1133 (1977).**

Turner, N.C., P.E. Waggoner & S. Rich. Removal of ozone from the atmosphere by soil and vegetation. **Nature (London) 250: 486-489 (1974).**

U.S. Environmental Protection Agency. "Air Quality Criteria for Ozone and Other Photochemical Oxidants". **Report EPA/600/8-84/020cF, Vol. III. Environmental Criteria and Assessment Office, U.S. Environmental Protection Agency, Research Triangle Park, NC. (1986).**

Unsworth, M.H., P.V. Biscore, & L. Black. Analysis of gas exchange between plants and pollutant atmospheres. In **"Effects of air pollutants on plants", T.A. Mansfield, ed. Cambridge University Press, London Pp. 5-16 (1976).**

Vaartnou, M. "EPR Investigation of Free Radicals in Excised and Attached Leaves Subjected to Ozone and Sulphur Dioxide Air Pollution". **Ph.D. Thesis, University of British Columbia, Vancouver, B.C. (1988).**

Vermaas, W.F.J., H.B. Pakrasi & C.J. Arntzen. Photosystem II and inhibition by herbicides. In **"Models in Plant Physiology and Biochemistry". D.W. Newman & K.G. Wilson, eds. Vol.1. CRC Press, Boca Raton FL. Pp. 9-12 (1987).**

Vermaas, W.F.J., A.W. Rutherford, & Ö. Hansson. Site-directed mutagenesis in photosystem II of the cyanobacterium *Synechocystis* sp. PCC 6803: donor D is a tyrosyl residue in the D2 protein. **Proc. Natl. Acad. Sci. USA 85: 8477-81 (1988).**

Walmsley, L., M.R. Ashmore & J.N.B. Bell. Adaptation of radish Raphanus sativus L. in response to continuous exposure to ozone. **Environ. Pollut. (Ser. A) 23: 165-177 (1980).**

Weaver, E.C. & G.A. Corker. Electron paramagnetic resonance spectroscopy. In **"Encyclopaedia of Plant Physiology", Vol. V, "Photosynthesis", Springer -Verlag, Berlin Pp. 168-178 (1977).**

Weis, E. Opening lecture. In **"Air Pollution and Plants", C. Troyanowsky ed. VCH Publishers, Pp. 10-17. (1985).**

Wellburn, A.R.. Why are atmospheric oxides of nitrogen usually phytotoxic and not alternative fertilizers. **New Phytol. 115: 395-429 (1990).**

Wellburn, A.R.. The influence of atmospheric pollutants and their cellular products upon photophosphorylation and related events. In **"Gaseous Air Pollutants and Plant Metabolism", M.J. Koziol & F.R. Whatley, eds. Butterworths, London. Pp. 203-221 (1984).**

Wellburn F.A.M., & A.R. Wellburn. Variable patterns of antioxidant protection but similar ethene emission difference in several ozone-sensitive and ozone-tolerant plant species. **Plant Cell Environ. 19: 754-760 (1996).**

Wellburn, A.R., O. Majernik & F.A.M. Wellburn. Effects of SO2 and NO2 polluted air upon the ultrastructure of chloroplasts. **Environ. Pollut. 3: 37-49 (1972).**

Wellburn, A.R., C. Higginson, D. Robinson & C. Walmsley. Biochemical explanations of more than additive inhibitory effects of low atmospheric levels of sulphur dioxide plus nitrogen dioxide upon plants. **New Phytol. 88: 223-237 (1981).**

Williams, W.P. & J.F. Allen. State 1/State 2 changes in higher plants and algae. **Photosynth. Res. 10: 22-43 (1987).**

Wingsle, G., T. Nasholm, T. Lundmark & A. Ericsson. Induction of nitrate reductase in needles of Scots pine seedlings by NOx and NO_3-. **Physiol. Plant. 70: 399-403 (1987).**

Winner, W.E., C. Gillespie, W.-S. Shen & H.A. Mooney. Stomatal responses to SO_2 and O_3. In **"Air Pollution and Plant Metabolism", S. Schulte-Hostede, N.M. Darrall, L.W. Blank & A.R. Wellburn, eds. Elsevier, London. Pp. 255-271 (1988).**

Yocum, C.F.. Oxygen evolution by photosystem II: polypeptide structure and inorganic constituents. In **"Models in Plant Physiology and Biochemistry", D.W. Newman & K.G. Wilson, eds. CRC Press, Boca Raton FL. Vol.1, Pp. 3-5 (1987).**

Yoneyama, T. & H. Sasakawa. Transformation of atmospheric NO_2 absorbed in spinach leaves.
Plant Cell Physiol. 20(1): 263-266(1979).

Yoneyama, T., H. Sasakawa, & S. Ishizuka. Absorption of atmospheric NO_2 by plants and soils, II: Nitrite accumulation, nitrite reductase activity, and diurnal change, of NO_2 absorption in leaves. **Soil Sci. Plant Nutr. 25: 265-275 (1979).**

Zeevaart, A.J. Some effects of fumigating plants for short periods with NO_2. **Environ. Pollut. 11: 97 108 (1976).**

APPENDIX A.
Campbell 21x Program For Supplying Double No_2 & Single O_3 Sine-waves Daily to Two Growth Chambers With Continuous Time-shared Monitoring of O_3, NO_2 And NO

Internal memory locations:

1 1 = manual; 0 = auto
2 ON time for manual
3 20s register (sine-waves created by fraction of 20s that ports are opened by LOC7 value)
4 hour of day
5 12min register (0 to 11)
6 12min-period counter (new values for sine-wave computed every 12min)
7 10 * [1 + sin(LOC6*LOC15 - LOC29)] (sine-wave values in 1s units)
8 flipflop #1 (= 0 for 0-10min [LOC5 < 11]; = 1 for 11min [LOC5 = 11])
9 battery voltage check
10 multipliers for sine-waves (18 for NO_2; 9 for O_3)
11 flipflop #2 (for Subroutine #5; = 0 for NO_2; = 1 for O_3)
12 1s counter
13 O_3 storage
14 NO_2 storage
15 NO storage
16 thermistor (chamber #1) storage
17 thermistor (chamber #2) storage
18 thermistor (chamber #3) storage
19 O_3 accumulator
20 NO_2 accumulator
21 NO accumulator
22 thermistor (chamber #1) accumulator
23 thermistor (chamber #2) accumulator
24 thermistor (chamber #3) accumulator
25 photocell (chamber #1 lights ON/OFF)
26 photocell (chamber #2 lights ON/OFF)
27 photocell (chamber #3 lights ON/OFF)
28 output time-share label
29 value = 90
30 3min register

Ports: (the arrangement of ports is shown in Fig. A1)

1 NO_2 supply ON/OFF
2 O_3 generator ON/OFF
3 NO_2 sine waves (two waves)
4 O_3 sine wave
5 monitor time-share and sample line switching
6 monitor time-share and sample line switching

Excitation channels:

1 all thermistors
2 all photocells

Input channels:

1 output from O_3 monitor
2 NO_2 output from NO_x monitor
3 NO output from NO_x monitor
4 thermistor (chamber #1)
5 thermistor (chamber #2)
6 thermistor (chamber #3)
7 photocell (chamber #1)
8 photocell (chamber #2)
9 photocell (chamber #3)

Main program (LOC2 set to 1s)

[1st column: Boldface = line number;
[indent = parameter number.

[2nd column: Italics = Campbell instruction;
[parameter value (data, location (LOC) #, etc.).
[3rd column: description.
[To assist in reading the program, dotted [lines bracket conditional instruction sets.

```
01: P89 if .....
 01 1  LOC1
 02 1  =
 03 1  1
 04 30 then
02: P86 do
 01 1  sub #1
03: P86 do
 01 2  sub #2
04: P86 do
 01 0  sub #0
05: P95 endif.....
06: P86 do
 01 1  sub #1 (increment 1s timer or reset to 0)
07: P18 time
 01 2  hours into year
 02 24 MOD 24
 03 4  to LOC4 (hour of day)
08: P18 time
 01 1  minutes into day
 02 12 MOD 12
 03 5  to LOC5 (minutes into 12min period)
09: P86 do
 01 4  sub #4 (monitor time-share)
10: P89 if .....
 01 30 LOC30
 02 4  <
 03 2  2min
 04 30 then
11: P30 store
 01 0  0
 02 12 to LOC 12 (reset 1s counter)
12: P30 store
 01 0  0
 02 19 to LOC19
13: P30 store
 01 0  0
 02 20 to LOC20
14: P30 store
 01 0  0
 02 21 to LOC21
15: P30 store
 01 0  0
 02 22 to LOC22
16: P30 store
 01 0  0
 02 23 to LOC23
17: P30 store
 01 0  0
 02 24 to LOC24
18: P86 set
 01 21 flag #1 OFF
19: P95 end .....
20: P1  voltage (O3, NO2 and NO output)
 01 3  reps
 02 5  5v full scale
 03 1  = 1st input channel
 04 13 = 1st storage LOC
 05 1  multiplier
 06 0  offset
21: P11 thermistor 107 probe
 01 3  reps
 02 4  = 1st input channel
 03 01 excite all from channel 1
 04 16 = 1st storage LOC
 05 1  multiplier
 06 0  offset
22: P11 photocell (using thermistor 107
        command)
 01 3  reps
 02 7  = 1st input channel
 03 2  excite all from channel 2
 04 25 = 1st storage location
 05 1  multiplier
 06 0  offset
23: P18 time
 01 1  minutes into day
 02 3  MOD 3
 03 30 to LOC 30 (minutes in 3min period
24: P89 if
 01 30 LOC 30
 02 3  >=
 03 2  2
 04 11 set Flag #1 ON
25: P91 if flag .....
 01 11 flag #1 ON
 02 30 then
26: P32 increment
 01 12 LOC12
27: P33 accumulate .
 07 19 LOC19
 02 13 + LOC13
 03 19 in LOC19
28: P33 accumulate .
 01 20 LOC20
 02 14 + LOC14
 03 20 in LOC20
29: P33 accumulate .
 01 21 LOC21
 02 15 + LOC15
 03 21 in LOC21
30: P33 accumulate .
 01 22 LOC22
 02 16 + LOC16
 03 22 in LOC22
31: P33 accumulate .
 01 23 LOC23
 02 17 + LOC17
 03 23 in LOC23
32: P33 accumulate .
 01 24 LOC24
 02 18 + LOC18
 03 24 in LOC24
33: P95 endif.....
34: P89 if .....
 01 12 LOC12
 02 1  =
 03 60 60s
 04 30 then
35: P37 multiply
 01 19 LOC19
 02 0.0167 (*0.0167 = 1/60)
 03 19 in LOC19
36: P37 multiply
 01 20 LOC20
 02 0.0167 (*0.0167 = 1/60)
 03 20 in LOC20
37: P37 multiply
 01 21 LOC21
 02 0.0167 (*0.0167 = 1/60)
 03 21 in LOC21
38: P37 multiply
 01 22 LOC22
 02 0.0167 (*0.0167 = 1/60)
 03 22 in LOC22
```

39: *P37* multiply
01 23 LOC23
02 0.0167 (*0.0167 = 1/60)
03 23 in LOC23
40: *P37* multiply
01 24 LOC24
02 0.0167 (*0.0167 = 1/60)
03 24 in LOC24
41: *P86* do
01 10 set output flag
42: *P77* time stamp .
01 111 day; hour-min; secs
43: *P70* store output
01 10 reps (O_3, NO_2, NO, 3 chamber temperatures, 3 chamber lights, output time-share label)
02 19 = 1st storage LOC
44: *P95* endif .....
45: *P89* if .....
01 5 LOC5
02 1 =
03 11 11 (i.e. 12th min.)
04 30 then
46: *P30* store
01 1 1
02 8 in LOC8 (set flipflop #1 to 1)
47: *P95* endif .....
48: *P30* store
01 90 90
02 29 in LOC29
49: *P10* battery voltage
01 9 to LOC9
50: *P89* if .....
01 4 LOC4
02 1 =
03 22 22 (END of 2nd NO_2 wave)
04 30 then
51: *P30* store
01 0 0
02 11 in LOC11 (set flipflop #2 to 0)
52: *P30* store
01 0 0
02 6 in LOC6 (reset 12min. count)
53: *P30* store
01 0 0
02 8 in LOC8 (set flipflop #1 to 0)
54: *P20* set port
01 0 OFF
02 1 port #1 - NO_2 supply OFF
55: *P20* set port
01 0 OFF
02 2 port #2 - O_3 supply OFF
56: *P20* set port
01 0 OFF
02 3 port #3 - NO_2 sine-wave OFF
57: *P20* set port
01 0 OFF
02 4 port #4 - O_3 sine-wave OFF
58: *P95* endif .....
59: *P89* if .....
01 4 LOC4
02 3 >=
03 6 6 (1st NO_2 wave running)
04 30 then
60: *P89* if .....
01 4 LOC4
02 4 <
03 10 10 (NOT END of 1st NO_2 wave).
04 30 then
61: *P20* set port
01 1 ON
02 1 port #1 - NO_2 supply ON.
62: *P30* store
01 18 18 = NO_2 multiplier.
02 10 in LOC10
63: *P89* if .....
01 5 LOC5
02 1 =
03 0 0
04 30 then
64: *P86* do
01 3 sub #3
65: *P95* endif .....
66: *P86* do
01 5 sub #5
67: *P95* endif .....
68: *P95* endif .....
69: *P89* if .....
01 4 LOC4
02 1 =
03 10 10 (START of O_3 wave)
04 30 then
70: *P89* if .....
01 6 LOC6 (12min period count)
02 1 =
03 20 20≡240min (4hours; end of NO_2 wave)
04 30 then
71: *P30* store
01 0 0
02 6 in LOC6 (reset 12min period count)
72: *P95* endif .....
73: *P95* endif .....
74: *P89* if .....
01 4 LOC4
02 3 >=
03 10 10 (O_3 wave running)
04 30 then
75: *P89* if .....
01 4 LOC4
02 4 <
03 18 18 (NOT END of O_3 wave)
04 30 then
76: *P20* set port
01 1 ON
02 2 port #2 - O_3 supply ON
77: *P30* store
01 9 9 = O_3 multilpier
02 10 in LOC10
78: *P30* store
01 1 1
02 11 in LOC11 (flipflop #2)
79: *P20* set port
01 0 OFF
02 1 port #1 - NO_2 supply OFF
80: *P20* set port
01 0 OFF
02 3 port #3 - NO_2 sine-wave OFF
81: *P89* if .....
01 5 LOC5
02 1 =
03 0 0
04 30 then
82: *P86* do
01 3 sub #3
83: *P95* endif .....
84: *P86* do
01 5 sub#5

85: *P95* endif .....
86: *P95* endif .....
87: *P89* if .....
01 4 LOC4
02 3 >=
03 18 18 (2nd NO_2 wave running)
04 30 then
88: *P30* store
01 0 0
02 11 in LOC11 (flipflop #2)
89: *P20* set port
01 0 OFF
02 2 port #2 - O_3 supply OFF
90: *P20* set port
01 0 OFF
02 4 port #4 - O_3 sine-wave OFF
91: *P89* if .....
01 6 LOC6
02 1 =
03 40 40≡480min (8hours; end of O_3 wave)
04 30 then
92: *P30* store
01 0 0
02 6 in LOC6 (reset 12min period counter)
93: *P95* endif .....
94: *P89* if .....
01 4 LOC4
02 4 <
03 22 22 (NOT END of 2nd NO_2 wave)
04 30 then
95: *P20* set port
01 1 ON
02 1 port #1 - NO_2 supply ON
96: *P30* store
01 18 18 = NO_2 multiplier
02 10 in LOC10
97: *P89* if .....
01 5 LOC5
02 1 =
03 0 0
04 30 then
98: *P86* do
01 3 sub #3
99: *P95* endif .....
100: *P86* do
01 5 sub #5
101: *P95* endif .....
102: *P95* endif .....

Sub-routines:

[#1:;1s counter]

01: *P85* label sub
01 1 #1
02: *P32* increment
01 3 LOC3=LOC3+1
03: *P89* if .....
01 3 LOC3
02 3 >=
03 20 20s limit
04 30 then
04: *P30* store
01 0 0
02 3 in LOC3
05: *P95* endif .....
06: *P95* endsub

[#2; sine-waves ON/OFF]

07: *P85* label sub
01 2 #2
08: *P88* if .....
01 3 LOC3
02 3 >=
03 2 LOC2
04 30 then
09: *P20* set port
01 1 ON
02 3 port #3 - NO_2 sine-wave ON.
10: *P20* set port
01 1 ON
02 4 port #4 - O_3 sine-wave ON
11: *P94* else .....
12: *P20* set port
01 0 OFF
02 3 port #3 - NO_2 sine-wave OFF
13: *P20* set port
01 0 OFF
02 4 port #4 - O_3 sine-wave OFF
14: *P95* endif .....
15: *P95* endsub

[#3; 12min period counter]

16: *P85* label sub
01 3 #3
17: *P89* if .....
01 8 LOC8 (flipflop #1)
02 1 =
03 1 1
04 30 then
18: *P32* increment (12min period count)
01 6 LOC6=LOC6+1
19: *P30* store
01 0 0
02 8 in LOC8 (set flipflop #1 to 0)
20: *P95* endif .....
21: *P95* endsub

[#4; monitor time-share]

22: *P85* label sub (time-share routine)
01 4 4
23: *P89* if .....
01 5 LOC5
02 3 >=
03 0 0
04 30 then
24: *P89* if .....
01 5 LOC5
02 4 <
03 3 3
04 30 then
25: *P20* set port
01 1 ON
02 5 port #5 ON
26: *P20* set port
01 0 OFF
02 6 port #6 OFF
27: *P30* store
01 1 1
02 28 in LOC28
28: *P95* endif .....

29: *P95* endif .....
30: *P89* if .....
01 5 LOC5 .
02 3 >= .
03 3 3 .
04 30 then .
31: *P89* if ..... .
01 5 LOC5 . .
02 4 < . .
03 6 6 . .
04 30 then . .
32: *P20* set port . .
01 1 ON . .
02 6 port #6 (and port #5) ON
33: *P30* store . .
01 2 2 . .
02 28 in LOC28 . .
34: *P95* endif ..... .
35: *P95* endif .....
36: *P89* if .....
01 5 LOC5 .
02 3 >= .
03 6 6 .
04 30 then .
37: *P89* if ..... .
01 5 LOC5 . .
02 4 < . .
03 9 9 . .
04 30 then . .
38: *P20* set port . .
01 0 OFF . .
02 5 port #5 OFF. .
39: *P20* set port . .
01 0 OFF . .
02 6 port #6 OFF .
40: *P30* store . .
01 3 3 . .
02 28 in LOC28 . .
41: *P95* endif ..... .
42: *P95* endif .....
43: *P89* if .....
01 5 LOC5 .
02 3 >= .
03 9 9 .
04 30 then .
44: *P20* set port .
01 1 ON .
02 6 port #6 ON; port #5 OFF
45: *P30* store .
01 4 4 .
02 28 in LOC28 .
46: *P95* endif .....
47: *P95* endsub

[#5; create sine-wave and switch sample lines]

48: *P85* label sub
01 5 #5
49: *P36* multiply
01 6 LOC6
02 10 *LOC10
03 7 to LOC7
50: *P35* subtract
01 7 LOC7
02 33 -LOC29 (i.e., -90, to start wave at minimum)
03 7 to LOC7
51: *P48* sine
01 7 LOC7
02 7 to LOC7
52: *P34* add
01 7 LOC7
02 1 +1
03 7 to LOC7 (i.e., to raise minimum to zero)
53: *P37* multiply
01 7 LOC7
02 10 *10
03 7 to LOC7
54: *P88* if .....
01 3 LOC3 .
02 4 < .
03 7 LOC7 .
04 30 then .
55: *P89* if ..... .
01 11 LOC11 . .
02 1 = . .
03 1 1 . .
04 30 then . .
56: *P20* set port . .
01 1 ON . .
02 4 port #4 - O_3 sine-wave ON
57: *P94* else ..... .
58: *P20* set port . .
01 1 ON . .
02 3 port #3 - NO_2 sine-wave ON
59: *P95* endif ..... .
60: *P94* else .....
61: *P89* if ..... .
01 11 LOC11 . .
02 1 = . .
03 1 1 . .
04 30 then . .
62: *P20* set port . .
01 0 OFF . .
02 4 port #4 - O_3 sine-wave OFF
63: *P94* else ..... .
64: *P20* set port . .
01 0 OFF . .
02 3 port #3 - NO_2 sine-wave OFF
65: *P95* endif ..... .
66: *P95* endif .....
67: *P95* endsub

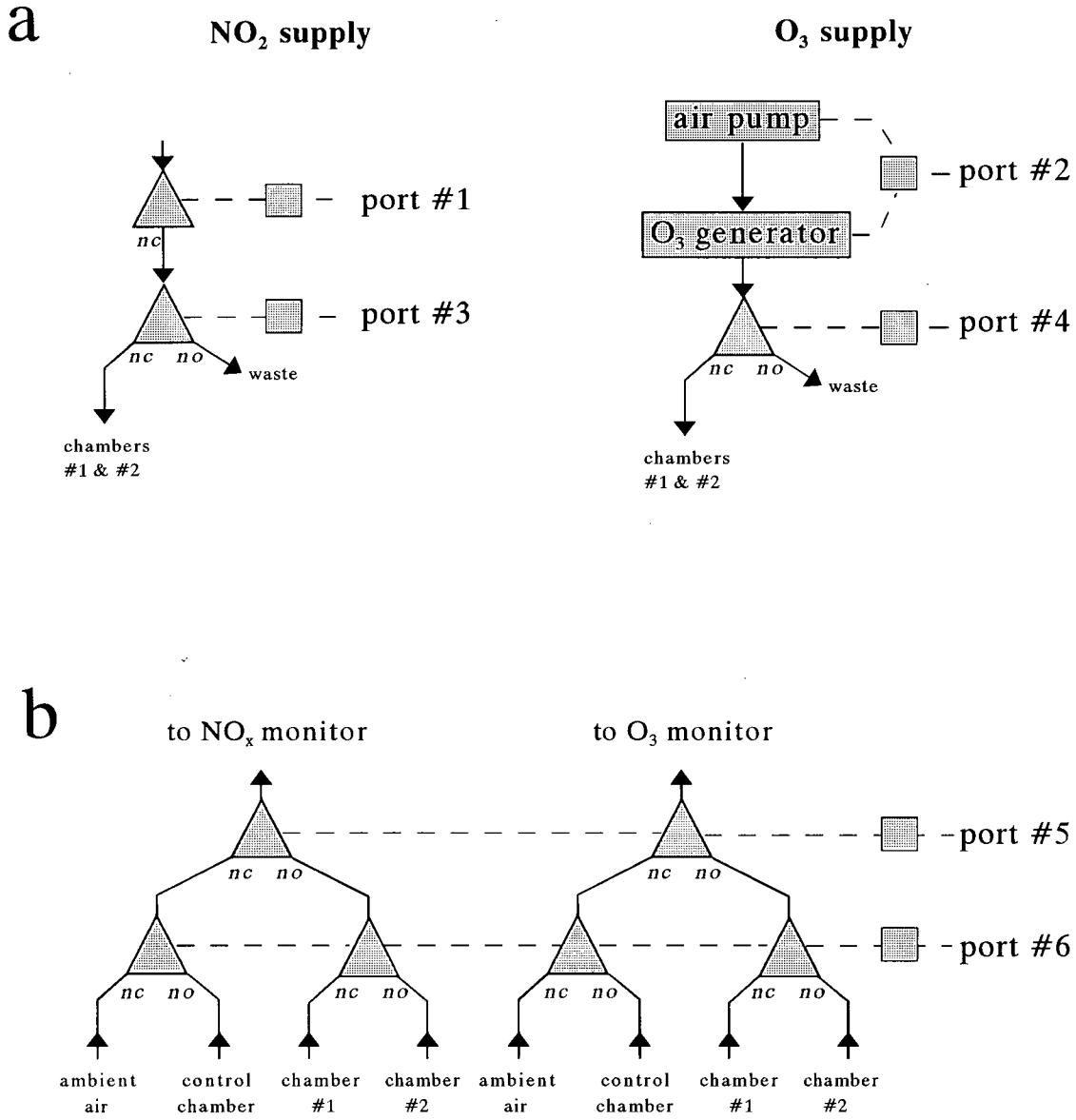


Fig. A1. Port assignments of solid-state relays, □, operating solenoid valves, △, controlling a) NO_2 or O_3 supply and sine-wave generation, and b) 3min time-share of sample lines to NO_x and O_3 monitors. *nc* = normally closed; *no* = normally open.

APPENDIX B

Electron Paramagnetic Resonance Spectroscopy

Spectroscopy based on electron paramagnetic resonance, EPR (= electron spin resonance, ESR), involves the absorption of microwave energy by an unpaired electron when it is exposed to a strong magnetic field. The basis of the absorption is the microwave-induced transition between magnetic energy levels of electrons with unpaired spins. Unpaired electrons occur in free radicals and many transitional metal ions. The simplest radical is the single electron. Although the energy transitions of interest could be obtained by varying the microwave frequency in a fixed magnetic field, practical considerations have led to the use of fixed microwave frequencies combined with varying the magnetic field strength.

Microwave energy is provided by a klystron oscillator - the most frequently used frequency is 9.5 GHz (X-band; $\lambda \approx 3$cm) with the range of magnetic field strengths centered on 3400G(auss) = 340mT(esla). The microwave energy is withdrawn through a waveguide to the sample cavity tuned to establish a standing wave.

The detection of the range of magnetic field strengths over which absorption by a sample within the cavity perturbs the standing wave leads to the EPR spectrum. Since the range of field strengths over which the electrons in free radicals absorb is very narrow and has to be detected against a background of noise from various sources, EPR spectra are usually presented as first derivatives in order to improve resolution.

Simple EPR spectra are characterized by their g-values (splitting factors) and linewidths:

$$g = h\nu/\beta H_0$$

where h=Planck's constant, ν = microwave frequency, β= the Bohr magneton, and H_0 = the magnetic field strength at which the maximum absorption occurs. The g-value is thus defined by the field strength at the absorption maximum, i.e. the point at which the first derivative signal crosses the abscissa. Linewidth is the difference between the field strengths at which the maximum and minimum of the first derivative spectrum occur, corresponding to the points of inflection of the absorption curve. With radicals in free solution or radicals with random orientation, isotropic signals are an average of the different resonances resulting from the degree of alignment of the molecular axis with the magnetic field and usually provide symmetrical first derivative signals reflecting the gaussian form of the absorbance spectrum.

The g-value of the free electron is 2.0023, and free radicals have g-values close to this value. However, since the free electron in a radical tends to be delocalized over the whole molecule or at least a large part of it, it comes into interactive contact with many nuclei which, if they have nuclear magnetic moments, affect the energy level of the electron and hence affect resonance. In small molecules, this hyperfine coupling interaction may lead to a number of discrete spectral lines (centred on H_0 and determined by the number of spin states of the

interacting nucleus), whose size is a function of the number of nuclei in the molecule involved in interactions. In large molecules, many nuclear interactions may be possible, leading to overlap of the numerous hyperfine splittings resulting in the appearance of a broad spectrum, with a clearly defined single low field maximum and single high field minimum in the first derivative signal.

If measurement of microwave frequencies is made during the course of determining a signal, *g*-values are computed directly, but if a set frequency is assumed, slight variations in frequency will affect *g*-values and may be corrected for by proportionality to the *g*-value of a standard such as diphenylpicrylhydrazyl (2.0036) and measurement of H_o of the standard.

Typical *g*-values for the two symmetrical signals observed in illuminated green leaves, algal cells, isolated chloroplasts or thylakoid fractions are:

2.0025, with a linewidth of 7.5-9.0G for Signal I, the signal produced by $P700^+$ in photosystem I (Beinert et al., 1962), and

2.0046, with a linewidth of 19G for Signal II, the signal of the tyrosine radicals Y_Z^+ (on the D1 protein = Signal II_{fast}) and Y_D^+ (on the D2 protein = Signal II_{slow}) of photosystem II (Miller and Brudvig, 1991).

The superoxide anion radical signal is markedly asymmetrical with a linewidth of 10G; its *g*-value of 2.001 is based on the high field minimum (Knowles et al., 1969).

Although the absolute error in *g*-value determination is usually ±0.0002, g-values differing by as little as 0.0001 can be determined by replication (Harbour et al., 1974).

The practical lower limit for the reliable detection of EPR signals is about 0.1μM using X-band frequencies because of the spectrometer noise level. With K-band frequencies (35GHz) sensitivity is increased 20-fold but the reduced size of the cavity size limits applications to small sample sizes.

APPENDIX C

In vitro Spin-trapping of Superoxide Anion Radical

The efficacy of the spin traps 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and α-(4-pyridyl-1-oxide)-N-*tert*butylnitrone (4-POBN) in forming spin adducts with superoxide anions in aqueous media *in vitro* was tested using a procedure developed by Jacks and Hinojosa (1993).

A solution of 10mM tetrabutylammonium perchlorate (TBAP; Sigma) in dimethyl sulphoxide (DMSO; Sigma) was diluted to 10% with distilled water for use as the solvent. DMSO is inert to superoxide anion radicals. The quaternary ammonium counter-ions of dissociated TBAP stabilize superoxide anions. Superoxide anions were obtained by dissolving potassium superoxide (Sigma) in the DMSO/TBAP solvent.

EPR spectra were obtained using the conditions described in Section 4.2, with the sample in a quartz cuvette instead of the quartz Dewar insert.

The figure below shows spectra obtained with a) the cuvette alone; b) DMSO/TBAP solvent; c) DMSO/TBAP containing 0.66mM KO_2; and d) DMSO/TBAP containing 0.66mM KO_2 and 80 mM 4-POBN.

The spectrum of the 4-POBN-OOH adduct shows the typical triplet of doublets which is true for such adducts (Connor et al., 1986; Mottley et al., 1986).

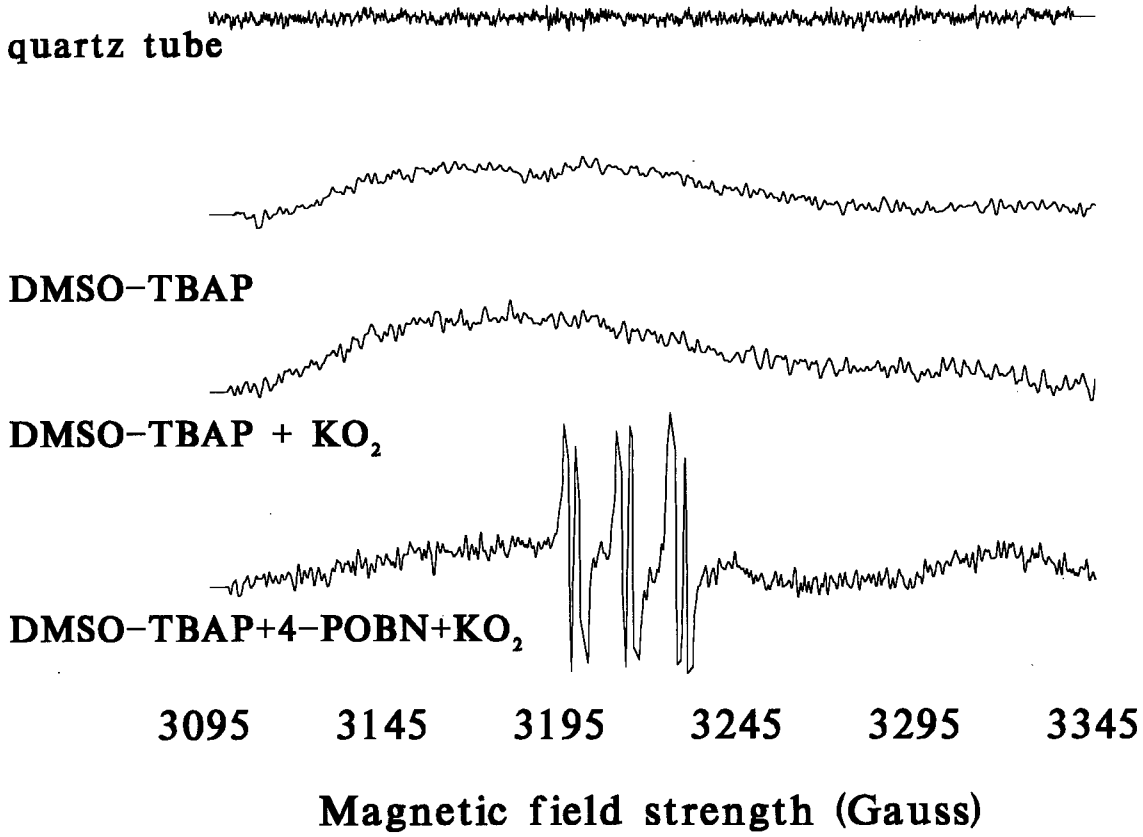


Fig. 4.24 Spectra of the 4–POBN–superoxide adduct in DMSO–TBAP.