# The Effects of Ozone and Nitrogen Oxides on Cereal/Aphid Interactions.

By

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## Abstract

The response of the rose grain aphid (*Metapolophium dirhodum*) on cereals polluted for different short durations, with either ozone or nitrogen oxides, was investigated using a small chamber fumigation system. The parameter used to measure aphid performance was The Mean Relative Growth Rate (MRGR) and was calculated following fumigation of the plant only. The Intrinsic Rate of Natural Increase ($r_m$) was calculated where a significant difference in MRGR was found. No general response was found for any pollutant following different fumigation durations, although significant increases in performance following certain fumigation regimes were observed.

The direct effect of the pollutant gases on the aphid was investigated using artificial diets and was found to be unimportant.

An explanation for the increases in aphid performance was sought in the plant biochemistry and changes known to benefit aphid performance were found to occur following ozone fumigation. Increases in soluble nitrogen levels, in overall levels of free amino acids, and in glutathione levels were found. Particular increases of the sulphur containing amino acids were observed. No such changes were found following nitrogen dioxide or nitric oxide fumigation, at the fumigation durations found to produce an increase in aphid performance.

The increases in sulphur containing compounds was investigated as a possible causal mechanism responsible for the increases in aphid performance. They were, however, found to be unimportant, at least in isolation from other biochemical changes found following fumigation.

The increases in sulphur containing compounds were further investigated using the tracer $^{35}S$, and possible alterations in aphid feeding patterns were observed. The amount of honeydew produced by aphids on ozone fumigated plants was found to decrease, and the contained concentration of amino acids was found to increase.

Finally, the effect of a long term low level ozone fumigation on the subsequent response of the aphid and plant to an acute fumigation was investigated, and was found to be minimal.

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# Chapter 1
# General Introduction

## 1.1 The Significance of Nitrogen Oxides and Ozone in the Atmosphere

### 1.1.1 Nitrogen Oxides

Nitric oxide (NO) and nitrogen dioxide ($NO_2$), known collectively as NOx, are emitted into the atmosphere as a result of the combustion of almost all fuels. The gases are formed by oxidation of nitrogen in the combustion air of a flame. In addition some fuels, particularly coal, contain nitrogenous compounds which when burnt contribute additional nitrogen oxide emissions. In the United Kingdom motor vehicles and coal fired power stations account for the majority of the nitric oxide and nitrogen dioxide emissions (PORG, 1990). The U.K. emits just over 2 million tonnes of nitrogen oxides per year.

Nitrogen oxides are also exhaled from soils, as a result of microbial activity. The extent of this is uncertain, but may account for 10% of that estimated from combustion sources over the U.K. as a whole. Biological processes within plant canopies may be a source of nitrogen dioxide but their magnitude is not yet established. Oxides of nitrogen are also produced by $NH_3$ oxidation, lightning and biomass burning.

Interest in the oxides of nitrogen has been particularly intense within the last five years because of their involvement in processes giving rise to acidic deposition and photochemical ozone formation. They have an environmental impact not only on the terrestrial ecosystem, but also on human health, global tropospheric chemistry, and effects on climate. The principal concern in this context of this study is the effects on the terrestrial ecosystem.

### Chemistry of Nitrogen Oxide Formation

High temperature combustion processes initiate the reaction between molecular oxygen and nitrogen to form predominantly nitric oxide:

$$N_2 + O_2 \Rightarrow 2NO$$

Since NO is fairly reactive a small fraction is immediately oxidised to nitrogen dioxide, but in most cases this is later formed in the atmosphere. At high concentrations close to emission sources, nitric oxide can be oxidised by molecular oxygen:

$NO + NO + O_2 \Rightarrow NO_2 + NO_2$

The reaction with ozone ($O_3$) is, however, generally the most important leading to nitrogen dioxide formation. An equilibrium occurs between products and reactants since $NO_2$ absorbs blue and UV light and is therefore decomposed to yield NO and $O_3$:

$NO + O_3 \Leftrightarrow NO_2 + O_2$

The position of the equilibrium depends on the ozone concentration and the light intensity, but generally the $NO_2$ concentration exceeds that of NO.

## Direct Effects of Nitrogen Oxides on Plants

A large body of literature concerning the direct effects of nitrogen oxides on plants has accumulated in recent years. Nitrogen oxide pollution differs from most other atmospheric pollutants since the nitrogen supply is often a limiting environmental factor. At low concentrations (of less than 100 ppb - where 1 ppb represents a volume mixing ratio of 1 volume of pollutant gas in $10^9$ volumes of air) for a few days, both stimulations and reductions in growth are reported. This variable response may reflect the nitrogen status of the plant. For example, Anderson and Mansfield (1979) found that when the amount of nitrogen available in the soil is low, tomato plants (*Lycopersicon esculentum*) can tolerate and even benefit from, nitric oxide in the atmosphere. However, even at low levels of soil nitrogen, if the concentration of NO rises above a certain critical level (in their studies, 40-80 ppb) deleterious effects are seen. Deleterious effects at low exposure levels were also seen by Taylor and Eaton (1966), who found that 15-26 ppb $NO_2$ for 10-22 days caused reductions in tomato (*L. esculentum*) leaf area and dry weight. The metabolic activity of the plant can also influence the impact of nitrogen dioxide, for example at night or at low temperatures, the plant is less able to detoxify the damaging products of nitrogen dioxide (Yoneyama *et al*, 1979). Knowledge of the effects of nitric oxide is much less than that of nitrogen dioxide, but it is thought that the mechanisms whereby damage is caused to plants are similar for the two pollutants (Anderson and Mansfield, 1979). The importance of a mixture of pollutants in the atmosphere has been realised over the last ten years. For example, Ashenden and Williams (1980) exposed *Lolium multiflorum* and *Phleum pratense* to rural levels of $NO_2$ and $SO_2$, both singly and in combination, for 20 weeks over the winter, and found greater growth reductions when the gases were present together, than the sum of the damage found for each gas individually.

## Concentrations of Atmospheric Nitrogen Oxides

Over the 17 years of reliable monitoring, at U.K. sites, in both rural and urban areas, the annual mean nitric oxide concentration averages 9 ppb, and the annual mean nitrogen dioxide concentration averages 15 ppb. (Data is taken from PORG, 1990). The concentrations are highest in urban areas and in the proximity of major roads, and lowest in rural and remote areas. Annual mean urban concentrations of NO and $NO_2$ are 5-10 times, and 2-4 times those at rural sites, respectively. Certain urban sites exhibit highest hourly mean concentrations in the range of 100-400 ppb, and the highest hourly mean concentrations recorded anywhere in the U.K. for nitric oxide and nitrogen dioxide from 1972 - 1988 were 2000 ppb and 1817 ppb, respectively. Both these extremes were observed at a roadside site, 0.5m from the kerb, in central London. The number of hours that nitric oxide and nitrogen dioxide hourly mean concentrations have exceeded 100 ppb has been increasing at certain sites. From 1978 - 1985 over all the monitored sites only one hourly mean nitrogen dioxide measurement exceeded 100 ppb. Between 1986 - 1987, 32 hours exceeded 100 ppb. Ten hourly mean nitric oxide concentrations exceeded 100 ppb at a particular Midlands site in 1978, whereas 190 hourly means exceeded 100 ppb in 1987. At some rural sites the annual mean concentrations of nitric oxide and nitrogen dioxide have almost doubled between 1979 and 1987. Some rural areas have levels at the lower limits of detection of the samplers, with levels increasing to, but not exceeding 16 ppb. For various climatic and anthropogenic reasons winter mean concentrations of nitrogen dioxide are significantly higher than summer means (Campbell, 1988), a fact that is also true for NO.

### 1.1.2 Ozone

Ozone is a so called 'secondary pollutant' since it is not emitted directly into the atmosphere, and is only formed as a result of reactions between other pollutants and atmospheric gases. It is further termed a 'photochemical pollutant' which indicates that the driving force behind the atmospheric reactions is sunlight. The presence of oxidising gases in the atmosphere has been known since the discovery of ozone in 1840 by the Swiss chemist Schonbein. However, it was not until the 1940's that the environmental implications of this gas began to be realised. The first signs of problems were seen in the Los Angeles basin, but they were considered exceptional and research was not initiated in Europe until the late 1960's. In the last ten years interest in photochemical oxidants (also including peroxyacetyl nitrate, and hydrogen peroxide) has increased with the realisation that they are involved in processes giving rise to acidic deposition, and in effects on trees, crops, non-biological materials and human health.

## Chemistry of Ozone Formation

The main chemical reaction producing ozone in the atmosphere is the recombination of atomic and molecular oxygen:

$O + O_2 + M \Rightarrow O_3 + M$

where M can be any molecule such as nitrogen or oxygen which dissipates the energy released in the reaction, and so prevents the ozone decomposing. At altitudes of less than 20km, photodissociation of nitrogen dioxide is the main oxygen atom production reaction:

$NO_2$ + radiation ($280 < \lambda < 430$) $\Rightarrow NO + O$

The nitric oxide produced in this reaction reacts rapidly with the ozone formed as above, to produce $NO_2$ again:

$NO + O_3 \Rightarrow NO_2 + O_2$

When any nitric oxide is oxidised to nitrogen dioxide by other processes, net formation of ozone occurs. This can be by reaction with peroxy radicals $RO_2$, where R is a hydrogen atom or organic radical. The most important peroxy radicals are those derived from the oxidation of hydrocarbons initiated by photochemically generated hydroxyl (OH) radicals. Hydrocarbons are emitted from motor vehicles, industrial sources and solvents. Ozone formation can therefore only occur in the sunlit (day time) atmosphere and the amount formed will depend on the concentrations of nitrogen oxides and hydrocarbons present in the atmosphere.

This dependence on sunlight accounts for the general occurrence of ozone episodes during the summer. Other factors may help promote the development of high ozone levels, including high temperatures (i.e. greater than 20°C) which promote certain reactions, low wind speeds (i.e. 0-5 m/s) and a restricted boundary layer. This inhibits atmospheric dispersion and allows the build up of pollutant concentrations. Once formed in stable weather, ozone persists for several days and may be transported long distances. Meteorological factors are therefore also important in the build up of ozone episodes.

## Direct Effects of Ozone on Plants

At high concentrations the characteristic symptoms of ozone damage are brown or white flecking on the leaves. At lower concentrations effects on the growth rate of the plant and its physiological activities can occur, in the absence of any visible damage. The precise mechanism by which ozone damages plant is still under investigation, but it is thought that the initial site of attack is the cell membrane. Plants vary greatly in their sensitivity to ozone, and this sensitivity is further modified by environmental conditions. For example, some plants tend to be less sensitive when light intensities are low, when the humidity is low, or when the water supply is restricted (see Huttunen, 1984). The presence of other pollutants is also an important factor. The most sensitive known plant is the tobacco cultivar Bel-W3, which can be injured by concentrations of approximately 40 ppb. Visible injury to other sensitive species, however, may only be produced when the concentrations reach 100 ppb for a few hours (PORG, 1987). The most comprehensive series of experiments on the relationship between crop yield and ozone concentration, was conducted by NCLAN (National Crop Loss Assessment Network study) in the U.S.A. This demonstrated that yields of sensitive crops were reduced by 10-15% with a mean ozone concentration of 50 ppb and an exposure regime of 7 hours per day, over the course of a growing season. Ozone is also implicated in the decline in forest health in Germany and in Southern California. Long term effects of ozone on natural ecosystems may also alter the competitive balance of species and produce changes in habitat structure.

## Concentrations of Atmospheric Ozone

In unpolluted air over the United Kingdom and the European continent the ozone concentration varies greatly, depending on the time of year and the weather conditions. The average U.K. annual mean and summertime mean concentrations are 19 ppb and 24 ppb, respectively, but there are considerable year to year variations. (Data is taken from PORG, 1987). Ozone is an 'episodic' pollutant as a result of the conditions required for its formation and accumulation and peaks in levels are therefore observed as isolated events superimposed on a variable background distribution.

There is some evidence that the doubling in background ozone concentration which has been observed over the last century has been associated with increasing emissions of the oxides of nitrogen. Hourly mean ozone concentrations during the summer at rural or suburban locations typically exceed 60 ppb for 100-200 hours, 80 ppb for up to 100 hours, 100 ppb for 10-50 hours and 120 ppb for 10 hours. The highest hourly mean concentration was recorded at Harwell, at 258 ppb, during the 1976 summer.

Ground level ozone concentrations in urban areas near busy roads or combustion plant tend to be lower than in the suburban and rural areas immediately adjacent to them. Ozone is detectable on almost every day of the year at every monitoring site around the U.K. but is lowest during the winter time. It is not possible to discern if there is a long term trend in the 14 years of reliable monitoring between 1972 and 1985, because of year to year variations.

## 1.2 Objectives of the Study

The increase in levels of ozone and nitrogen oxides during this century has only relatively recently been considered as a potential modifier of environmental relationships. Initial studies reported only correlations between incidences of high levels of air pollution and visible damage to plants. Experimental investigations were then conducted, either excluding pollution from a natural system (filtration studies), or adding pollutants to a non-polluted system (fumigation studies). Subsequently the importance of a mixture of pollutants has been realised, and more recently the interaction with other environmental variables in mitigating the plant response has been appreciated. Included amongst these variables are cold stress, water stress, mineral deficiency and the interaction with insects. It is this secondary interaction with insects, particularly aphids, which is the subject of this study.

In recent years a large body of literature has accumulated relating phytophagus insect performance to levels of air pollution. Sulphur dioxide is the most frequently studied pollutant, in this respect, and increases in performance are often seen in moderately polluted air. Rather less work has been conducted with ozone and nitrogen oxides, and this is reviewed in chapter 2.

The initial aim of this study was to characterise the response of the rose grain aphid, *Metopolophium dirhodum*, to cereals polluted for different short durations with either ozone or nitrogen oxides. The level of pollution chosen for this was 100 ppb, which although high, was not unrealistic. It was chosen to elicit a response, rather than to mimic frequently seen pollution episodes. The fumigations were conducted in small chambers in the greenhouse, using plants in pots. The aim was to find a trend in the response, or to isolate pollution regimes which produced a significant difference in subsequent aphid performance. Since the fumigation of the plant was separated from the subsequent assay of aphid performance, the possibility that under natural conditions (where aphid and plant would be exposed together) the aphid would itself be affected by the pollution was considered. This was done by fumigating the aphids whilst feeding

on artificial diets, and also by fumigating both aphid and plant together. The remainder of the thesis, with the exception of chapter 7, is devoted to the attempt to explain the mechanism behind the observed differences in aphid performance.

An explanation of the difference in aphid performance on polluted and control plants was sought in the plant biochemistry. A particular biochemical difference was then simulated using both artificial diets and nutrient enhanced hydroponically growing plants. Aphids were fed on these substrates in an attempt to demonstrate a causal relationship responsible for the change in growth rate observed on the polluted plants. A further investigation into the possible origin of the biochemical differences following fumigation was undertaken using radio-labelled sulphate. This work allowed uptake of the tracer by the aphid and its deposition in honeydew to be considered, and stimulated an investigation into the quantity and amino acid quality of honeydew produced by aphids on polluted and control plants.

Finally, the effect of a long term low level ozone fumigation of wheat, on subsequent aphid performance was considered. Various plant parameters were also investigated and the possible modifying effect of this chronic treatment, to the response to a final acute short term fumigation was studied.

The importance of aphids as a serious pest of cereals places this work in context. Pesticide spraying is the normal solution to control increases in aphid numbers, which further threatens the environment. Although varieties of cereals can be chosen which are more resistant to air pollution, the only long term answer is a reduction in levels of air pollution.

# Chapter 2

# Indirect Effects of Ozone and Nitrogen Oxides on *Metopolophium dirhodum*, Mediated via the Plant.

## 2.1 Introduction.

This chapter initially reviews the literature concerning the interaction between phytophagus insects and air pollution. It then moves on to study the performance of the rose grain aphid (*Metopolophium dirhodum*), on wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*), following fumigation of the cereal, for short durations with one of three air pollutants:

1) Ozone,
2) Nitrogen Dioxide and
3) Nitric Oxide (Wheat only).

## 2.2 Literature Review

The first recorded incident of numerical changes in insect fauna due to air pollution was noticed by Beling in 1831 (in Riemer and Whittaker, 1989). He noticed a seven fold higher density of larvae of the lepidopteran *Epinotia tedella* on Norway spruce (*Picea abies*) around an iron foundry, compared with levels at unpolluted sites. Following this, relationships were generally noticed around point sources of pollution where increases in forest pests often occurred. Such correlative observations have frequently appeared in the literature, but it is only since approximately 1980 that manipulative experiments have been used to study the relationship between air pollution and insect performance. These studies have been extensively reviewed by Riemer and Whittaker (1989) and Hughes (1988) amongst others.

Controlled experiments have been conducted both in the field and in the laboratory. One of the first laboratory tests was conducted by Hughes *et al.* (1981), in which they demonstrated that female Mexican bean beetles (*Epilachna varivestis* Mulsant) preferred to feed on leaves of Pinto beans (*Phaseolus vulgaris* L.) which had been prefumigated for seven days with sulphur dioxide, at 150 ppb. They went on to show that beetles which had been fed leaves fumigated with $SO_2$, at 200 ppb, for seven days, had a significantly shorter development time, reached a greater weight and laid more eggs, than those on control plants (Hughes *et al.* 1982). They then conducted the first controlled field exposures and demonstrated similar effects when $SO_2$ was added to ambient air (Hughes *et al.* 1983). Dohmen *et al.* (1984) conducted the first plant/aphid

interaction studies and demonstrated stimulatory effects on the growth of the black bean aphid *Aphis fabae* with both ambient London air, and with $SO_2$ and $NO_2$ fumigation.

### 2.2.1 Ozone/Insect Interactions

These early experiments were conducted primarily with sulphur dioxide, but as the importance of ozone as an atmospheric pollutant was realised, controlled experiments investigating its interaction with phytophagous insects were undertaken. Jeffords and Endress (1984) assessed the feeding preference of gypsy moth larvae (*Lymantria dispar* L.) for white oak (*Quercus alba* L.) foliage, which had been exposed for eleven seven hour periods to three concentrations of ozone. They found that insects preferred to feed on foliage which had been exposed to the highest ozone concentration (150 ppb) but that the median concentration of 90 ppb was less preferred than control (ambient) air. Endress and Post (1985) then conducted paired feeding tests with Mexican bean beetle on soybean (*Glycine max* L.), and found that they preferentially fed on foliage exposed to ozone, compared with control plants. During a 37 day period the foliage was exposed on 16 occasions, for seven hours, to a total of 112 hours. Four different ozone concentrations were used, 25, 50, 78 and 150 ppb. The insects frequently chose foliage which had received the greatest ozone stress, and the effects lasted up to 23 days after the last exposure.

Trumble *et al.* (1987) also showed that ozone prefumigation of tomato plants (*Lycopersicon esculentum* M.) produced faster development times and better survival of the tomato pin worm (*Keiferia lycopersicella* W.). The survival of the pinworm was reduced if the plants were fumigated whilst it was feeding upon them, which may indicate a direct toxicity of the gas, but this was not further investigated. The ozone exposure regime was 280 ppb ozone for three hours. Chappelka *et al.* (1988) used open top chambers and 0, 30 and 60 ppb ozone for the duration of the growing season, to investigate the performance of the Mexican bean beetle on soybean. The insects were introduced to the plants post fumigation and a significant linear positive correlation was found between the levels of ozone exposure and the degree of defoliation of the plants. They also found that development time decreased, and larval weight increased when fed on ozone fumigated foliage in the laboratory. Whittaker *et al.* (1989) found that the sizes of egg batches of the chrysomelid beetle *Gastrophsa viridula* were larger,

the survival and productivity of the larvae was higher, and the consumption lower on *Rumex obtusifolius* L. fumigated with 70 ppb ozone, for 15 days, for 7 hours a day, compared with filtered air controls.

Studies with chewing insects such as these therefore clearly indicate that ozone exposure of the host plant leads to an increase in insect performance.

### 2.2.2 Ozone/Aphid Interactions

Not all the work conducted with ozone has produced such a clear pattern of insect response. Work with aphids has produced contradictory results with different plant/aphid interactions. Braun and Fluckiger (1989) found that ambient air, containing on average 40 ppb ozone (and very low concentrations of other pollutants), inhibited population growth of *Aphis fabae* on *Phaseolus vulgaris*, but stimulated *Phyllaphis fagi* on *Fagus sylvatica*. Dohmen (1988) found that ozone fumigations of *Vicia faba* with 85 ppb for 2 or 3 days, caused decreased aphid growth, but at higher ozone concentrations, or in the presence of NOx, an increase was observed. Whittaker *et al.* (1989) found no clear trend in growth rate for pea and dock aphids (*Acyrthosiphon pisum* and *Aphis rumicis* respectively) fumigated with ozone concentrations ranging from 21 to 206 ppb. However, Warrington (1989) found that spring barley (*Hordeum vulgare*) grown in greenhouse air augmented with ozone at 70, 120 and 170 ppb, for 7 hours a day, for approximately 2, 3 and 4 months, produced significant increases in growth rate of *Rhopalosiphum padi*. The increases were larger on the younger plants and at the higher ozone levels.

Ashmore *et al.* (1989) also found that the size of an introduced population of *Aphis fabae* was subsequently significantly larger on *Vicia faba* growing in open top chambers receiving unfiltered air, compared with chambers receiving filtered air. The average ozone concentration was 21 ppb, with average concentrations of less than 2 ppb for NO and $SO_2$ and varying between 1 and 4 ppb for $NO_2$. The relative growth rate of the aphids was also increased on the plants in the ambient air chambers. These results were not, however, endorsed in a subsequent experiment (Brown *et al.* 1990), when significant decreases or no significant differences were observed. This was probably attributable to different pollution and climatic conditions, but further experiments are required to test this. Brown *et al.* (1991) also conducted short continuous fumigations of *Vicia faba* with ozone at 100 ppb, and found that the subsequent mean relative growth rates of *Aphis fabae* were altered according to the fumigation duration. Fumigation durations of less than 24 hours produced an increase in growth rate, whilst durations

of greater than 24 hours produced decreases in growth rate. When these experiments were repeated using episodic ozone doses of 8 hours per day at 100 ppb, significant increases in growth rates, compared with controls, were found for all durations up to 72 hours.

Work with trees has produced similarly inconsistent results. Ozone fumigation of *Pinus sitchensis* both continuously and episodically (8 hours in 24 hours), at 100 ppb for durations ranging from 4 to 96 hours, proved to have no significant effect on the subsequent growth rate of *Cinara pilicornis* (on old shoots), *C. pini* and *Elatobium abietinum* (McNeill and Whittaker, 1990). The effects on *C. pilicornis* on new shoots were strongly temperature dependent. Reduced growth rate of *Schizolachnus pineti* (on the same host) was observed following continuous ozone exposure, with no effect apparent when the fumigation was episodic.

It therefore appears that ozone exerts a variable effect, which depends upon the particular host plant/aphid interaction, the ozone fumigation regime, and probably certain environmental variables such as temperature (see also Brown *et al*, 1990).

### 2.2.3 Nitrogen Dioxide/Aphid Interactions

There appear to be no published investigations of the interaction of nitrogen dioxide with herbaceous species and insects, other than with aphids. Dohmen *et al.* (1984) demonstrated a significant increase in the growth rate of *Aphis fabae* on *Vicia faba* fumigated with $NO_2$ at 200 ppb for seven days, compared with charcoal filtered air. Similarly following fumigation for 7 hours with 100 ppb $NO_2$, Houlden *et al.* (1990) found consistent increases in the growth rate of aphids on a wide variety of crops. Fumigations for three and seven days, with $NO_2$ at 100 ppb, also produced significant increases in the growth rate of *Sitobion avenae* on *Triticum aestivum*, and *Aphis fabae* on *Vicia faba*. The only significant decrease in growth rate in this study was observed for *Acyrthosiphon pisum* on *Vicia faba* following fumigation for 7 hours with 100 ppb $NO_2$.

Fumigation of tree seedlings has shown significant increases in relative growth rate for *E. abietinum* on *P. sitchensis*, *C. pilicornis* on *P. sitchensis* and *S. pineti* on *Pinus sylvestris*, compared with controls. The exposure regime was a continuous fumigation with $NO_2$ at 100 ppb for durations ranging from 4 to 96 hours (McNeill and Whittaker, 1990).

### 2.2.4 Ambient Air/Aphid Interactions

Fluckiger has studied the effects of filtration of ambient air at a motorway verge on various plant/aphid interactions. The studies involved a cocktail of air pollutants produced by vehicle emissions, of which nitrogen oxides are a major component. Bolsinger and Fluckiger (1984) showed that a population of *Aphis fabae* was much larger on *Viburnum opulus* plants in ambient air chambers next to a motorway compared with populations in filtered air chambers. The mean concentration of $NO_2$ in this experiment was 208 ppb. This effect was also observed for *Aphis pomi* on *Crataegus monogyna* using the same experimental design (Braun and Fluckiger, 1985). The mean $NO_2$ concentration in this experiment varied between 125 and 306 ppb. Dohmen (1985) conducted similar studies in ambient Munich air and demonstrated that the growth rate of *Macrosiphon rosae* on roses (*Rosa* sp.) was, on average, 20% higher than in charcoal filtered air. Pollution monitoring at this site demonstrated that the dominant pollutant was nitrogen dioxide with a mean of 40 ppb and a range in concentration from 15 to 280 ppb. Ozone and $SO_2$ were also present, each with means of 25 ppb. The levels of nitric oxide were not recorded. Indeed no records of NO concentrations in any of the ambient studies could be found. There are also no studies which directly investigate plant/insect interactions with NO.

Such results provide good evidence that air pollutants alter the sensitive interaction between herbivores and their host plants. In a natural situation many factors, both biotic and abiotic, interact and elucidating changes due to air pollution alone can be difficult. It is for this reason that carefully controlled experimental manipulations of natural interactions are useful. When an interaction has been understood at this level, further interacting factors can be included until finally a situation more closely resembling realistic conditions can be considered.

The experiments in this chapter investigate further aphid/host plant/air pollution interactions, and the critical exposure durations required for the pollution effects to be apparent are varied. The effects of nitric oxide on insect/host plant relationships have not been considered previously, and clarification of the effects of ozone and nitrogen dioxide is required before mechanisms investigating the alteration in growth rates can be considered. All the fumigations are conducted under controlled conditions, in small

fumigation chambers situated within a greenhouse. The fumigations range in duration from 4 to 96 hours and the level of exposure for all pollutants was held as close as practically possible to 100 ppb.

## 2.3 Experimental Investigation - Materials and Methods

### 2.3.1 The Cereal Species Used, and Plant Growth Conditions

Two varieties of spring cereals were used in these experiments; wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*). The barley cultivar Atem was used because of its natural resistance to powdery mildew (which was proving a problem with the initial variety chosen, Maris Huntsman), and the wheat cultivar Ralle was chosen as it is the variety used in Denmark in similar open top chamber studies of cereal/aphid interactions.

The soil was made up according to the U.C. Soil Mix D (Matkin and Chandler, 1957) - which is 75% peat moss and 25% sand. The following nutrients have been found to produce healthy plants, and were added to 400 litres of soil;

574g Osmocote
380g $CaCO_3$
476g $MgCO_3$
56g Iron Sulphate
56g Frit
286g Super Phosphate

Osmocote is a commercial fertilizer produced by Monroe Horticultural Services designed to give a continual slow release of N, P and K in the ratio 18:11:10, respectively. Pots (11cm in diameter and 9.5cm deep) were filled with soil and three seeds planted in each. They were placed in trays and well watered. The trays were monitored throughout the experiment to ensure they contained water at all times. They were situated in a greenhouse, with a 16 hour photophase and temperatures of 20°C during the day (when possible - see below), and 15°C at night. After approximately 10 days the seedlings were thinned to one plant per pot, and exactly five weeks after planting, the plants were randomised between and within the trays, and any obviously small plants discarded. At this point the plants had reached stage 5 (end of tillering stage) on the Feekes scale (Large, 1954). The trays were then transferred to the fumigation chambers.

### 2.3.2 The Aphid Species Used, and its Culturing.

The rose grain aphid, *Metopolophium dirhodum*, was used for all experiments. It is a moderately sized (1.9 - 2.8 mm long), light green aphid with a dark green stripe running centrally along its back. It spends the winter as an egg on roses, and migrates to grasses and cereals in spring. It is usually found on the lower leaves of cereals, which turn yellow and senesce prematurely, but individuals are also found on the top leaves in the later growth stages of the plant. Watt and Wratten (1984) established field caged populations of this aphid on winter wheat (*Triticum aestivum*), at either an early or a late growth stage, and found significant reductions in grain weight due to the aphid, only when infestation occurred at the early growth stage. In addition to causing feeding injury to the plant by sucking phloem sap from the tissues, *M. dirhodum* can also transmit mild strains of Barley Yellow Dwarf Virus.

The aphids were cultured in a controlled temperature (C.T.) room at 20°C during the day and 15°C during the night, with a 16 hour photophase, and 60% relative humidity. The plants used for the culture were as near to five weeks old as possible. New plants were added to the culture at weekly intervals as older damaged plants were removed. Light levels were 140μE $m^{-2}$ $s^{-1}$.

### 2.3.3 Fumigation Conditions

#### 2.3.3.1 Ozone Fumigation

Ozone was generated from oxygen, using an electric discharge generator (type BA 0203012, Wallace and Tiernan, Tonbridge, England). This overcame the problems of contamination by nitrogen oxides which are encountered when air is used. Ambient air was pumped through a filter containing 20% 'purafil', positioned before activated charcoal. Purafil is a commercially available absorbant, consisting of aluminium hydroxide pellets impregnated with potassium permanganate. It has the property of converting NO to $NO_2$, which in this system is then largely removed by the charcoal. The filter removed at least 90% of the ambient ozone, and most of the already very low rural levels of $SO_2$, $NO_2$, and NO, although the level of these pollutants was not measured. Filtered air was pumped into two chambers, with ozone added just before the air inlet to one. The air entered at the side of the chamber, exited at the opposite side, and left the greenhouse in an exhaust pipe. The chamber dimensions were 60cm x 60cm x 60cm and there were an average of two air changes a minute.

Since the chambers were situated in a greenhouse they received natural light but this was supplemented with metal halide lamps from 6am to 10pm which ensured that even on dull days the light levels did not fall below 152μE $m^{-2}$ $s^{-1}$, at plant height, and that the photophase was never shorter than 16 hours. The greenhouse was heated when necessary to ensure the temperature did not fall below 20°C during the day, and 15°C at night. The flow of ozone to the chamber was controlled using a flow meter, and the treatment and control chambers could be reversed. The initial experiments were performed at least twice with the ozone added to the opposite chamber (see tables of results). Since no significant differences in the results were found when this was done, this was not continued for the later experiments. This was as expected since all the physical parameters of the chambers (as detailed above) were the same. The levels of ozone were continuously monitored using a UV photometer (Dasibi type AH-1003), and checked at least once every 2 hours to maintain 100 ppb. A chart recording was continually made which showed that the variation in levels was rarely greater than 10%. More variation was found on intermittently cloudy days, in which case levels were controlled more frequently. During the night, fumigation was continued and the chart recording showed that levels also varied little. Fumigations ranging in duration from 4 to 96 hours were undertaken. In an attempt to ensure that all plants were in the same physiological state, in terms of circadian rhythms, all experiments began at 10am.

### 2.3.3.2 Nitrogen Dioxide Fumigation

Nitrogen dioxide (1% in Nitrogen) was obtained from BOC Special Gas Division. Charcoal and purafil filtered air (as above) was pumped into a pair of perspex chambers at a rate of 3.03 air changes a minute. The chamber dimensions were 91cm x 91cm x 91cm, and air entered at one side and exited at the opposite side. $NO_2$ was added just before the air inlet to one of the chambers, and the flow was regulated using a flow meter. The levels of $NO_2$ were monitored at least once every two hours, using an Ambient NOx monitor (W H Monox MKII - supplied by British Gas). The levels generally varied little from 100 ppb, once the correct concentration had been established. No monitoring occurred between 11pm and 9am, although fumigation was continuous. The chambers were again situated in a greenhouse and therefore received natural light, supplemented from 6am to 10pm with metal halide lamps. These maintained a light level, at plant height, of not less than 149μE $m^{-2}$ $s^{-1}$. The greenhouse was heated when necessary to ensure the temperatures did not fall below 20°C during the day and 15°C at night. There was no facilty to exchange the treatment and control chambers, but a

bioassay was performed in which a standard experimental procedure was followed, but $NO_2$ was not added (as in Houlden *et al*, 1990). This showed no significant differences between chambers, with respect to host plant or aphid performance.

### 2.3.3.3 Nitric Oxide Fumigation.

The same chambers were used as for nitrogen dioxide. Nitric oxide (1% in Nitrogen) was obtained from BOC special gas division. The fumigation conditions were the same as for nitrogen dioxide. The levels were also monitored using the Monox and were maintained at 100 ppb as for the $NO_2$ fumigations.

## 2.3.4 Temperature Problems.

Although an attempt was made to conduct the experiments during the winter months when the temperatures in the chambers were more controllable, this was not always possible, and some experiments were conducted in the summer when the temperatures in the greenhouse frequently exceeded 20°C. Temperature data from The Meteorological Office, Bracknell, (measurements made at Heathrow, 19 kilometers east of Silwood Park) was therefore obtained and used in the analysis of results. Since the temperature in the fumigation chambers was normally higher than the measured outdoor ambient temperature a calibration curve of ambient temperature versus chamber temperature was constructed using a thermocouple (V. Brown pers. com.). The relationship between ambient temperature and temperature in the chambers was found to be:

$$Chamber\ \ Temp\ \ =\ \ (1.22\ \ x\ \ Ambient\ \ Temp)\ \ +\ \ 2.6$$

The ambient values were transformed to give chamber values, and any values found to be less than 20°C were altered to this value, since the greenhouse heating maintained this as a minimum daily temperature.

## 2.3.5 Calculation of Relative Growth Rate of the Aphid.

The Relative Growth Rate (RGR) technique (van Emden, 1969) was used as a measure of aphid growth since it allows the performance of aphids to be compared over a range in initial size. It also suggests something of the quality of the plant material on which the aphid is feeding:-

'If significant differences in relative growth rates of aphids are found between treatments, it seems reasonable to suppose that the plants giving lower aphid relative growth rates are poorer substrates for the aphid growth.' (van Emden, 1969)

Relative growth rates are also much quicker to calculate than parameters such as fecundity or reproductive rates, which is important since during the time following fumigation the physiology of both the aphid and the plant can vary considerably.

Standardised two day old aphid nymphs were produced in the culture to coincide with the end of the fumigation. This was done by placing adults in small cages on a leaf for 24 hours, then removing the adults and leaving the nymphs in the cages for a further 24 hours. The experimental plants were removed from the chambers and the nymphs separately weighed (using a Perkin-Elmer AD-2Z microbalance), and caged onto the second youngest leaf of each plant. Two aphids were weighed onto each plant. The first cage, plus aphid, was positioned approximately 1cm from the main axis of the plant, and the second a further 3cm along the leaf. The aphids and plants were then transfered to the controlled temperature room used for aphid culture, and after three days (72 hours), the nymphs were reweighed, and the RGR of each aphid calculated according to the formula:

$$RGR = \frac{\ln\ (Final\ \ Weight) - \ln\ (Initial\ \ Weight)}{Number\ \ of\ \ Days}$$

This produces results in units of $\mu g\ \mu g^{-1} day^{-1}$.

## 2.3.6 Treatment of MRGR Results.

A mean of the two RGRs from each plant was calculated. Occasionally a negative RGR for one of the aphids occurred, in which case the value was discarded as the aphid was assumed to have been damaged. If only one aphid survived its relative growth rate was recorded, and in the analysis of the results a mean value of two RGRs from one plant was weighted twice as strongly as a single value. The total number of plants per fumigation duration (i.e. the total number of plants for both treatments, and for all experiments) are shown in the results tables. Each fumigation was analysed as a one way ANOVA in GLIM (Version 3.77 update 0, of The Royal Statistical Society, London).

Five different pollutant/cereal combinations were produced:

1) Ozone and barley
2) Ozone and wheat
3) Nitrogen dioxide and barley
4) Nitrogen dioxide and wheat
5) Nitric oxide and wheat.

The fumigation of a particular cereal with a particular pollutant for a particular duration was sometimes repeated. This depended on the level of replication in the initial experiment, and the level of significance obtained. The experiment was repeated if the replication level was low, or if an almost significant difference in growth rate was initially obtained. For the fumigations with ozone, some experiments were repeated to allow the treatment and control chambers to be exchanged. The results from the repeated experiments were merged and analysed by two way analysis of variance using replicate experiment and air quality as factors. These results are presented graphically.

Since the chamber temperature was not controllable, the same experiment (i.e the same cereal, pollutant and fumigation duration) was sometimes conducted at different temperatures. The different fumigation durations for the same pollutant/cereal interaction were also unintentionally conducted at different temperatures. All the fumigations for the same pollutant/cereal combination were therefore considered together. Two temperature variables were included in the analysis; the maximum temperature, and the mean temperature (recorded between the hours of 0900 and 2100), experienced in the chambers. The data was analysed in two ways;

(i) As unbalanced weighted ANCOVAs of MRGR. Three ANCOVAs were conducted for each pollutant/cereal interaction, using the covariates maximum temperature, mean temperature and experimental duration (the duration of the plant exposure in the fumigation chambers), in turn. Air quality (i.e filtered air or polluted air) was used as a factor in each case.

(ii) As three regressions, for each pollutant/cereal interaction. MRGR was regressed against maximum temperature, mean temperature and experimental duration.

The means for each experiment, the standard errors, and the temperature data, are tabulated in the results.

### 2.3.7 Calculation of the Intrinsic Rate of Natural Increase of the Aphid.

In certain cases, when a significant difference in MRGR was found between the polluted and filtered air, the Intrinsic Rate of Natural Increase ($r_m$) was calculated. This was done using the estimation technique of Wyatt and White (1977).

Plants were grown and the fumigation repeated. Immediately following fumigation, adult aphids were placed onto the second youngest leaf of each plant for 24 hours and then removed to leave nymphs. All but two of the nymphs were also removed and these were caged separately on the leaf and monitored until they were adult and produced nymphs. The nymphs generally took about 10 days before they themselves reproduced, and the nymphs they produced were counted and removed daily. The $r_m$ value for each aphid was then calculated according to the formula:

$$r_m = 0.74 \quad ((lnM_d) \quad / \quad d)$$

Where d is the prereproductive time. (From birth to first reproduction)
$M_d$ is the number of progeny produced in the ensuing period of length d.

If both aphids on each plant survived to produce an $r_m$ value a mean was taken which was weighted twice as strongly as a single value from each plant. The results were analysed as one way ANOVAs in GLIM.

### 2.3.8 Relationship between RGR and $r_m$

It would be expected that these two parameters are closely related in which case a significant increase or decrease in MRGR would also produce a significant increase or decrease in the $r_m$. The relationship between the two parameters was tested by placing one adult on each of 18 five week old wheat plants for 24 hours then removing it together with all but 2 of the nymphs produced. After a further 24 hours (2 days old) these nymphs were weighed, returned to the plants for three days, reweighed, returned to the plants again, and the RGR calculated. They were then monitored until they produced nymphs, and the number produced in the time taken for them to first reproduce recorded and the $r_m$ value calculated.

## 2.4 Results.

(The standard error bars are omitted from all the graphs for clarity of the results, they are presented in the tables.)

### 2.4.1 Ozone Fumigation

#### 2.4.1.1 Barley

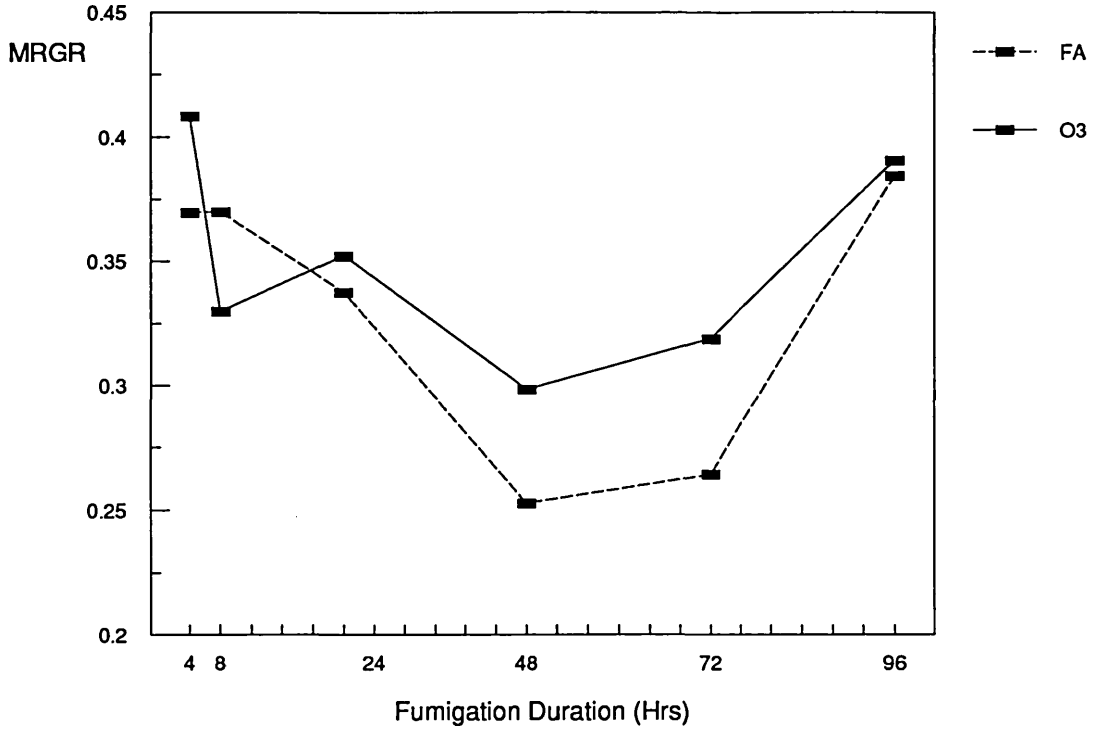


**Figure 2.1** **MRGR of aphids on Barley Following Fumigation with Ozone for the Durations Shown.**

Increases in aphid MRGR were seen in ozone for each fumigation duration, except 8 hours, but none of the differences were significant.

**Analysis of Combined Data Set**

**(i) Covariate Analysis**

It can be seen from Table 2.1 that the individual fumigations were conducted at different temperatures. When the twelve fumigations were considered together and the covariates maximum temperature, mean temperature and fumigation duration considered in the analysis there was no significant difference in MRGR due to air quality. These covariate tables are presented in the appendix, as an example of the analysis.

### (ii) Regression Analysis

The regressions of MRGR versus maximum temperature and mean temperature revealed a significant decrease in MRGR as the temperature increased ($p < 0.018$ and $p < 0.035$, respectively). However, the r squared values were low (0.228 and 0.189, respectively) which indicated either that the majority of the variation in MRGR cannot be explained by temperature differences, and must be attributed to unknown factors, or that the relationship is highly non-linear. The regression of duration of the plants in the chambers against the aphid MRGR produced no significant relationship. Consideration of interaction effects between temperature, duration, and air quality was not justified because of the small sample size.

**Table 2.1 Results of the Ozone/Barley Fumigation Series.**

| DUR | NO. OF EXPS | NO. OF PLANTS | MRGR FA | MRGR $O_3$ | S.E. FA | S.E. $O_3$ | % +/- in $O_3$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **4** | 2 | 28 | 0.369 | 0.408 | 0.018 | 0.025 | + 9.5 | 2.31 | 0.14 | 23.5<br>26.4 | 21.1<br>22.7 |
| **8** | 3 | 36 | 0.369 | 0.3298 | 0.018 | 0.018 | - 10.8 | 2.66 | 0.11 | 26.3<br>27.6<br>20.0 | 23.2<br>24.6<br>20.0 |
| **24** | 2 | 25 | 0.337 | 0.352 | 0.031 | 0.031 | + 4.2 | 0.13 | 0.72 | 26.4<br>27.8 | 22.5<br>24.2 |
| **48** | 3 | 31 | 0.253 | 0.298 | 0.020 | 0.018 | + 15.3 | 3.40 | 0.08 | 30.2<br>28.5<br>20.0 | 25.2<br>24.3<br>20.0 |
| **72** | 1 | 30 | 0.264 | 0.319 | 0.035 | 0.021 | + 17.1 | 1.63 | 0.21 | 24.1 | 20.0 |
| **96** | 1 | 21 | 0.384 | 0.391 | 0.026 | 0.020 | + 1.58 | 0.03 | 0.95 | 20.0 | 20.0 |

**KEY to tables 2.1 to 2.5**

| | |
|---|---|
| DUR | Duration of Fumigation (Hours). |
| NO. OF EXPS | Number of times fumigation was repeated. |
| NO. OF PLANTS | Number of plants, for both treatments, pooled for all experiments |
| MRGR FA | Mean Relative Growth Rate of aphids on plants from Filtered Air. |
| MRGR $O_3$ | Mean Relative Growth Rate of aphids on plants from FA + Ozone. |
| S.E. FA | Standard Error of MRGR of aphids on plants from Filtered Air. |
| S.E. $O_3$ | Standard Error of MRGR of aphids on plants from FA + Ozone. |
| % +/- in $O_3$ | Percentage increase or decrease in MRGR of aphids on Ozonated plants. |
| F | F ratio. |
| p | Probability. |
| MAX TEMP | Maximum Temperature experienced in the chambers (0900 to 2100). |
| MEAN TEMP | Mean Temperature in the chambers (0900 to 2100). |

### 2.4.1.2 Wheat

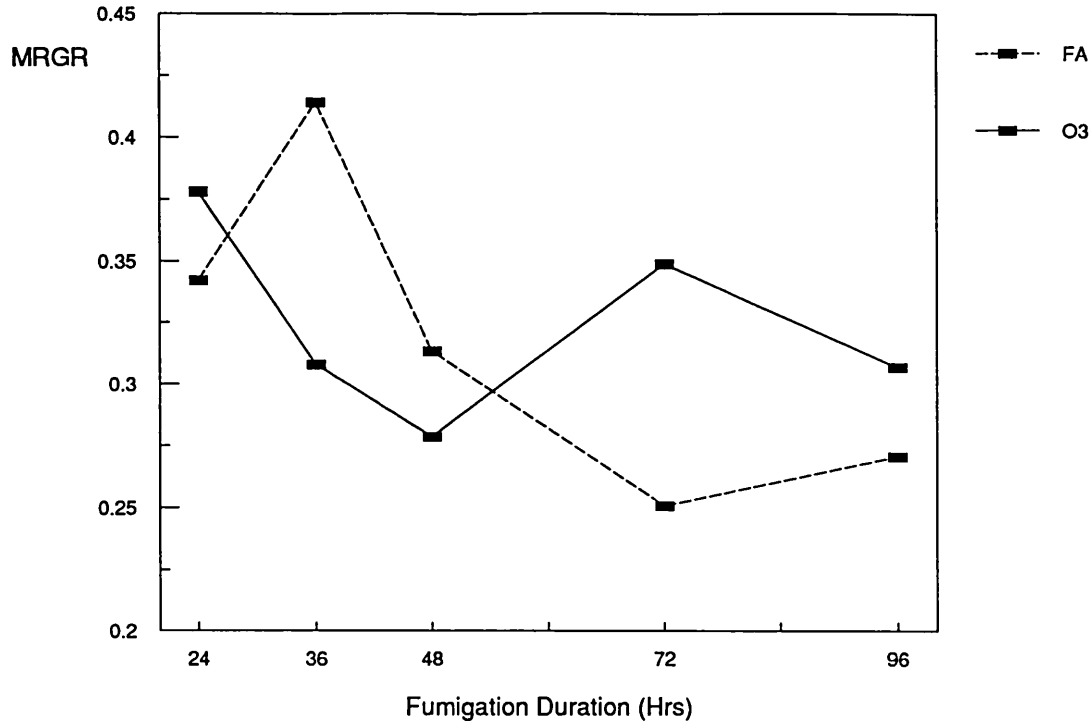


**Figure 2.2** **MRGR of aphids on Wheat Following Fumigation with Ozone for the Durations Shown.**

The results show both increases and decreases in MRGR for the different durations, although only one significant difference (an increase) in growth rate following 72 hours of ozone fumigation was seen ($p < 0.013$). This fumigation duration was investigated further by conducting an intrinsic rate of natural increase study.

#### Analysis of Combined Data Set

#### (i) Covariate Analysis

These fumigations were conducted in the winter months and as Table 2.2 shows the average temperature remained constant at 20°C, but the maximum temperature exceeded 20C for the 36, 72 and 96 hour fumigations. Consideration of the effects of experimental duration and maximum temperature in the analyses of covariance produced non-significant differences in MRGR due to air quality.

### (ii) Regression Analysis

The regressions of the six fumigations showed that there was no effect of maximum temperature in the chambers on the subsequent growth rate of the aphids. However, the duration of the experiment significantly reduced the MRGR of the aphid ($p < 0.011$).

## Intrinsic Rate of Natural Increase Study

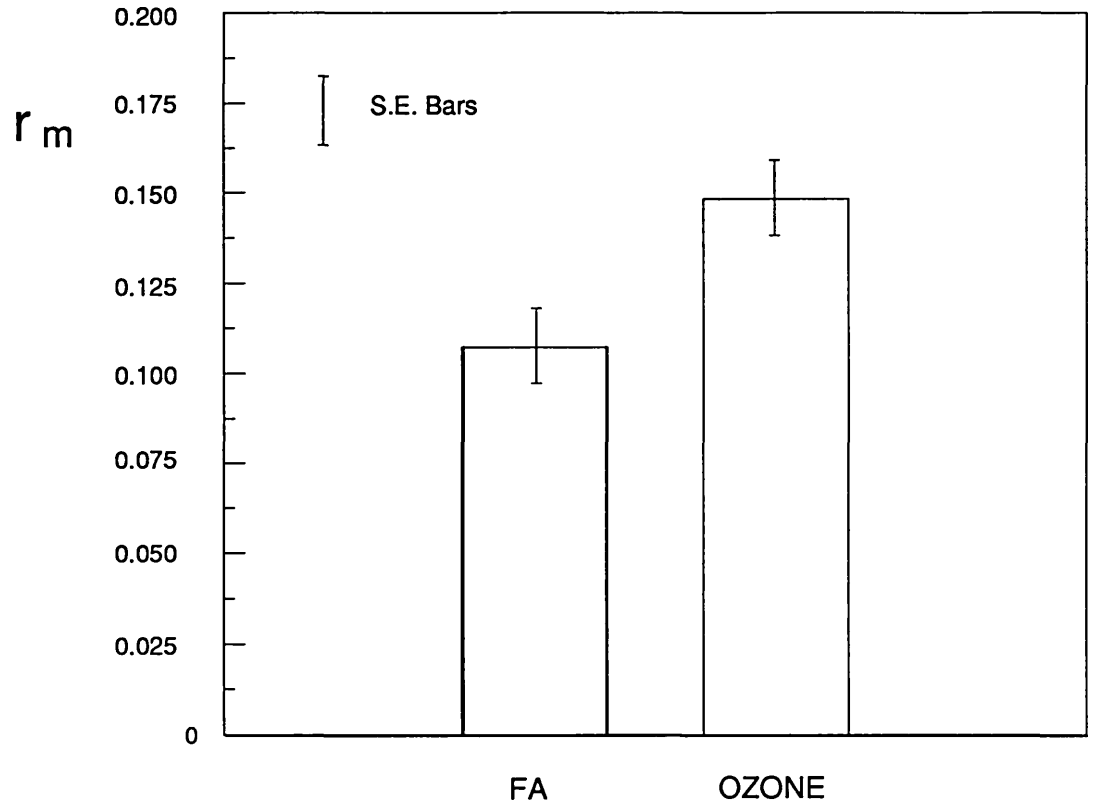


**Figure 2.3** **The Intrinsic Rate of Natural Increase of Aphids on Wheat Following Fumigation with either Ozone or Filtered Air for 72 Hours.**

A significant increase ($n = 35$, $p < 0.021$) in $r_m$ is seen following fumigation with 100 ppb ozone for 72 hours compared with filtered air. This is consistent with the MRGR result.

## 2.4.2 Nitrogen Dioxide Fumigation

### 2.4.2.1 Barley

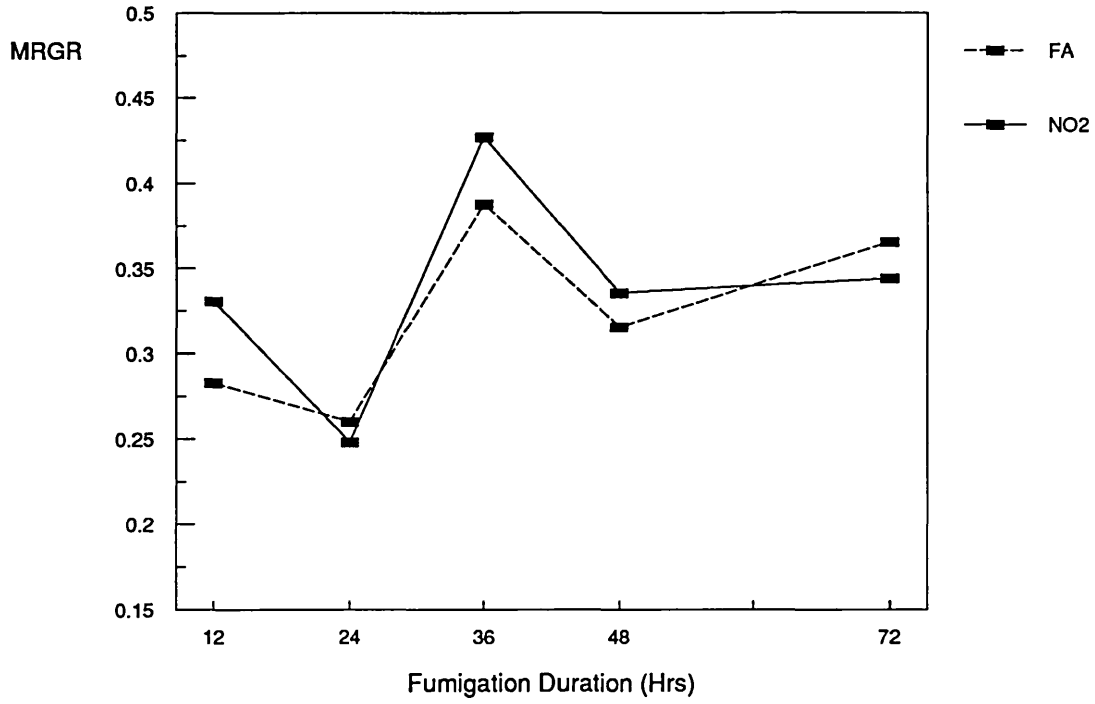


**Figure 2.4** **MRGR of Aphids on Barley Following Fumigation with $NO_2$ for the Durations Shown.**

There is no consistent trend in alteration of MRGR in $NO_2$ and none of the differences are significant.

**Analysis of Combined Data Set**

**(i) Covariate Analysis**

When the effects of maximum temperature, mean temperature, and fumigation duration were considered as covariates in the analysis, the MRGR difference due to air quality was non-significant.

**(ii) Regression Analysis**

The regressions revealed a significant increase in MRGR with increase in maximum and mean temperatures ($p < 0.010$ and $p < 0.006$ respectively). However, the r squared values associated with these regressions were low (0.330 and 0.364 respectively),

indicating that the temperature effects did not explain the majority of the variation in growth rates. The duration of the experiment did not significantly alter the subsequent growth rate of the aphid.

Table 2.2 Results of the Ozone/Wheat Fumigation Series.

| DUR | NO. OF EXPS | NO. OF PLANTS | MRGR FA | MRGR $O_3$ | S.E. FA | S.E. $O_3$ | % +/- IN $O_3$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **24** | 1 | 18 | 0.342 | 0.378 | 0.040 | 0.042 | + 9.5 | 0.38 | 0.55 | 20.0 | 20.0 |
| **36** | 1 | 17 | 0.414 | 0.308 | 0.032 | 0.044 | - 25.6 | 3.82 | 0.07 | 22.1 | 20.0 |
| **48** | 2 | 28 | 0.313 | 0.279 | 0.027 | 0.028 | - 11.1 | 0.91 | 0.35 | 20.0<br>20.0 | 20.0<br>20.0 |
| **72** | 1 | 22 | 0.251 | 0.349 | 0.025 | 0.025 | + 28.1 | 7.49 | 0.01 | 25.5 | 20.0 |
| **96** | 1 | 24 | 0.271 | 0.307 | 0.029 | 0.027 | + 11.6 | 1.20 | 0.28 | 28.1 | 20.0 |

Table 2.3 Results of the $NO_2$/Barley Fumigation Series.

| DUR | NO. OF EXPS | NO. OF PLANTS | MRGR FA | MRGR $NO_2$ | S.E. FA | S.E. $NO_2$ | % +/- IN $NO_2$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **12** | 1 | 22 | 0.283 | 0.331 | 0.030 | 0.028 | + 14.4 | 1.36 | 0.257 | 20.8 | 20.0 |
| **24** | 2 | 24 | 0.260 | 0.248 | 0.024 | 0.020 | - 4.6 | 0.18 | 0.676 | 27.8<br>28.5 | 24.2<br>24.1 |
| **36** | 1 | 36 | 0.388 | 0.427 | 0.019 | 0.020 | + 9.2 | 2.02 | 0.164 | 34.6 | 29.9 |
| **48** | 5 | 69 | 0.315 | 0.336 | 0.020 | 0.018 | + 6.0 | 0.56 | 0.463 | 20.8<br>28.2<br>20.0<br>20.0<br>24.7 | 20.0<br>23.5<br>20.0<br>20.0<br>20.9 |
| **72** | 1 | 23 | 0.366 | 0.344 | 0.020 | 0.020 | - 5.8 | 0.57 | 0.458 | 34.6 | 27.0 |

### 2.4.2.2 Wheat

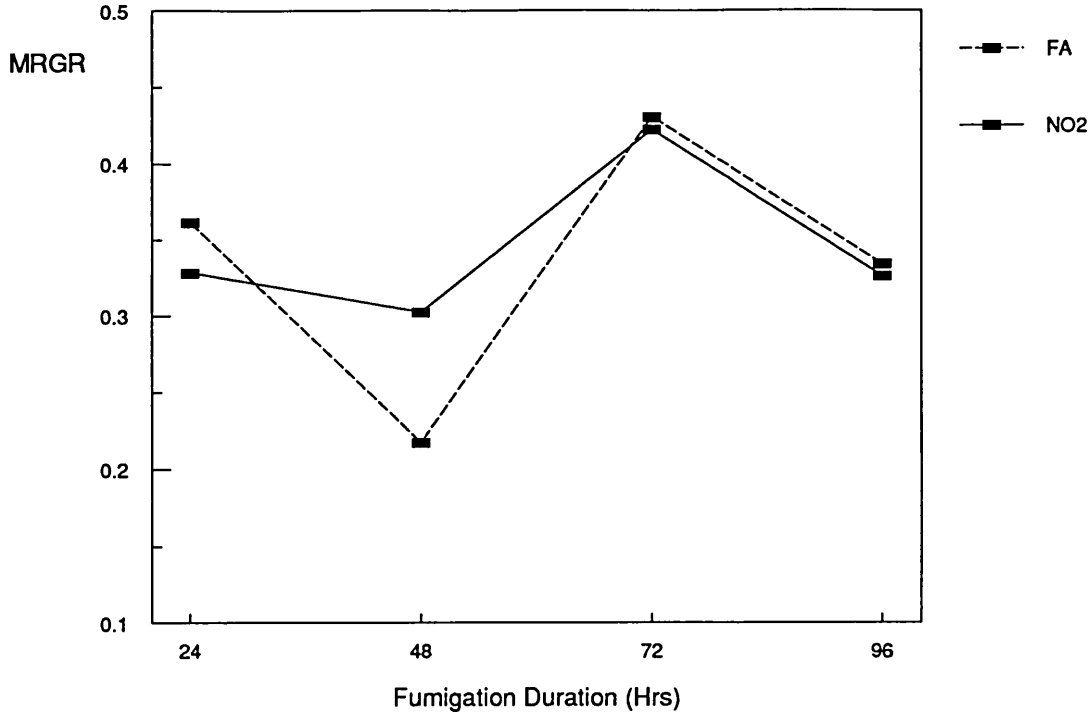


**Figure 2.5** **MRGR of Aphids on Wheat Following Fumigation with $NO_2$ for the Durations Shown.**

Non-significant decreases in MRGR were seen in $NO_2$ for each fumigation duration except 48 hours, when a significant increase ($p < 0.042$) was observed. This was further investigated by conducting an intrinsic rate of natural increase study.

**Combined Analysis**

**(i) Covariate Analysis**

It can be seen from Table 2.4 that the temperatures in the chambers remained close to 20°C for most of the fumigations. When the temperature effects were considered in the covariate analysis the MRGR differences due to the air quality were non-significant. Inclusion of the experimental duration covariate also produced no significant difference in MRGR due to air quality.

### (ii) Regression Analysis

The regressions revealed no significant difference in MRGR with increase in maximum temperature in the chambers. The regression of mean temperature was not undertaken, and there was no significant effect of fumigation duration.

## Intrinsic Rate of Natural Increase Study

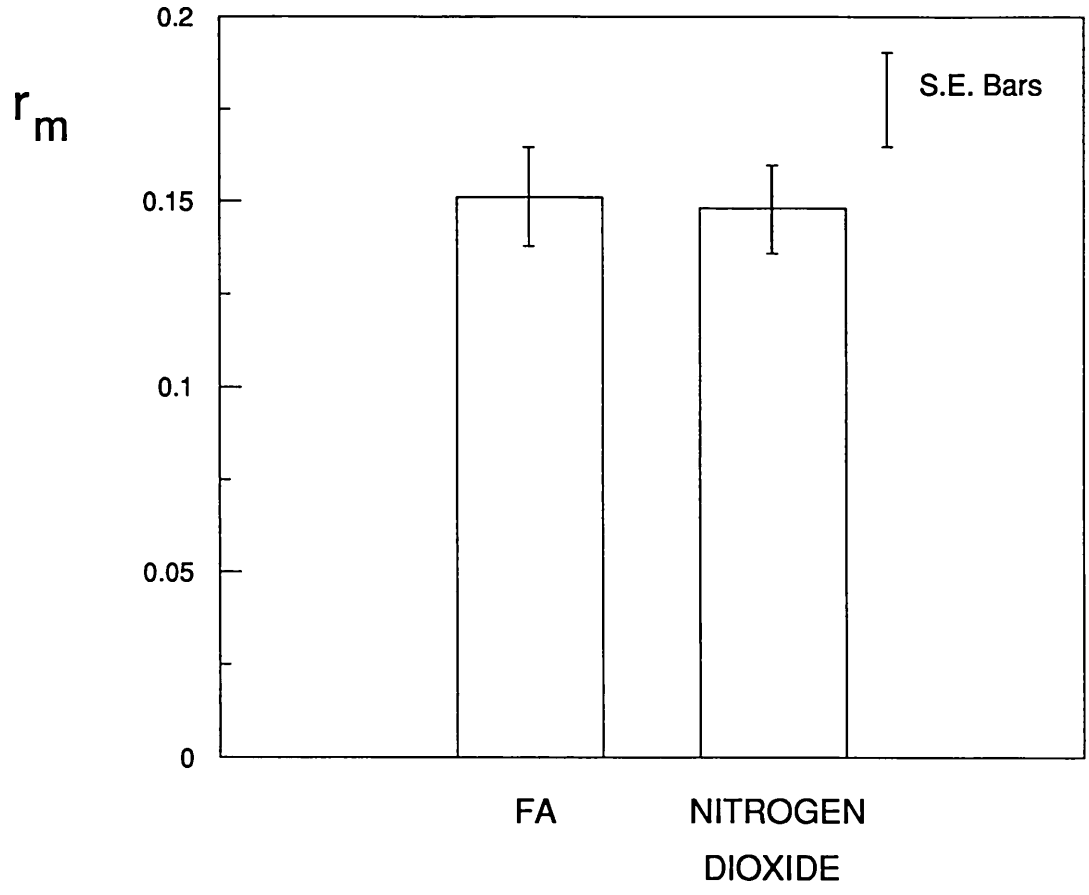


**Figure 2.6** **The Intrinsic Rate of Natural Increase Following $NO_2$ Fumigation of Wheat for 48 Hours.**

There is no significant difference in the $r_m$ value observed in the filtered air or the filtered air plus nitrogen dioxide. This is contrary to the MRGR result, but may possibly be explained by the elevated temperatures in the chambers during the $r_m$ fumigation. The maximum temperature experienced was 28.4°C and the mean temperature was 24.8°C. Temperatures during the fumigation for the MRGR study did not exceed 20°C (see Table 2.4). However the reason for the different results could only be ascertained by further experimentation.

## 2.4.3 Nitric Oxide Fumigation

### 2.4.3.1 Wheat

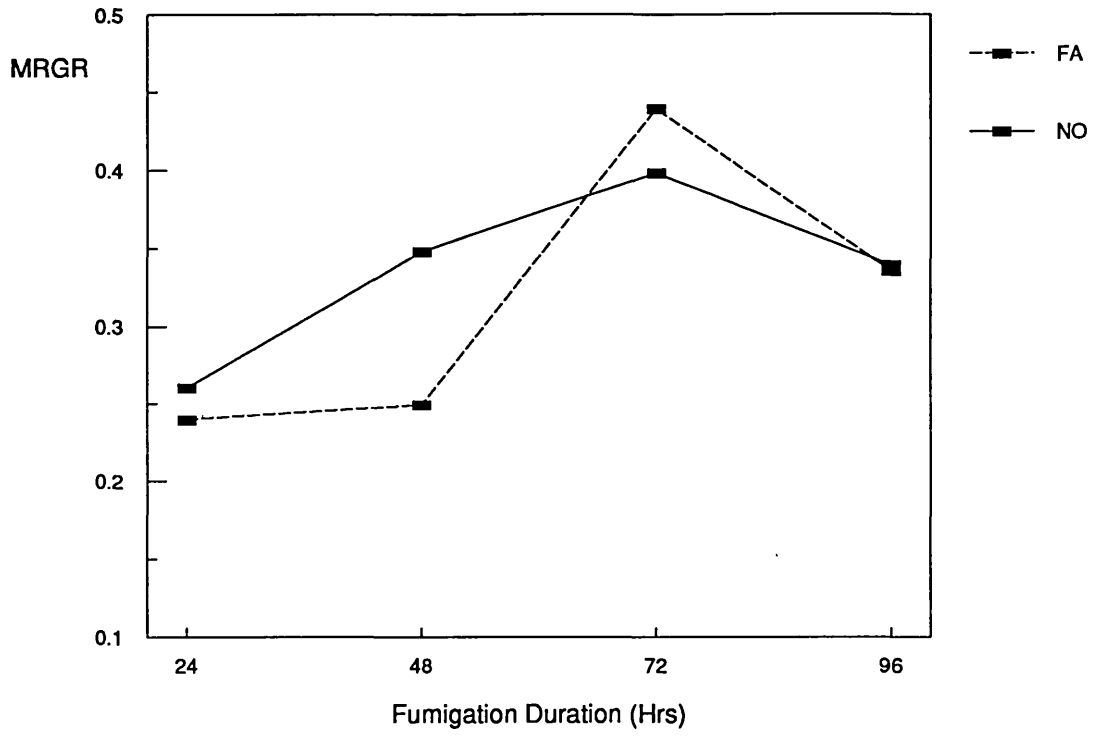


**Figure 2.7** **MRGR of Aphids on Wheat Following Fumigation with NO for the Durations Shown.**

Increases in MRGR were seen in NO for each fumigation duration except 72 hours. The increase observed following 48 hours was significant ($p < 0.015$), but all other differences were non-significant.

**Combined Analysis**

**(i) Covariate Analysis**

When the effects of temperature and fumigation duration were considered in the analyses of covariance there was no significant difference in MRGR due to air quality.

**(ii) Regression Analysis**

The regressions of the four fumigations together revealed no significant differences in aphid growth rate with maximum and mean temperatures, or with fumigation duration.

Table 2.4 Results of the $NO_2$/Wheat Fumigation Series.

| DUR | NO. OF EXPS | NO. OF PLANTS | MRGR FA | MRGR $NO_2$ | S.E. FA | S.E. $NO_2$ | % +/- IN $NO_2$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 1 | 20 | 0.362 | 0.329 | 0.038 | 0.037 | - 9.1 | 0.38 | 0.54 | 20.0 | 20.0 |
| 48 | 2 | 29 | 0.217 | 0.303 | 0.028 | 0.029 | + 28.1 | 4.58 | 0.04 | 20.0<br>20.0 | 20.0<br>20.0 |
| 72 | 1 | 29 | 0.431 | 0.422 | 0.019 | 0.019 | - 1.9 | 0.09 | 0.76 | 25.7 | 21.1 |
| 96 | 1 | 22 | 0.334 | 0.326 | 0.025 | 0.025 | - 2.3 | 0.05 | 0.83 | 20.7 | 20.0 |

Table 2.5 Results of the NO/Wheat Fumigation Series.

| DUR | NO. OF EXPS | NO. OF PLANTS | MRGR FA | MRGR NO | S.E. FA | S.E. NO | % +/- IN NO | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 1 | 20 | 0.239 | 0.260 | 0.023 | 0.021 | + 7.9 | 0.43 | 0.52 | 32.4 | 28.2 |
| 48 | 1 | 18 | 0.249 | 0.348 | 0.027 | 0.023 | + 28.3 | 7.45 | 0.01 | 39.4 | 31.3 |
| 72 | 1 | 24 | 0.439 | 0.398 | 0.016 | 0.016 | - 9.3 | 3.40 | 0.08 | 36.5 | 32.5 |
| 96 | 1 | 19 | 0.335 | 0.339 | 0.027 | 0.023 | + 1.1 | 0.01 | 0.92 | 20.4 | 20.0 |

### 2.4.4 Relationship Between RGR and $r_m$.

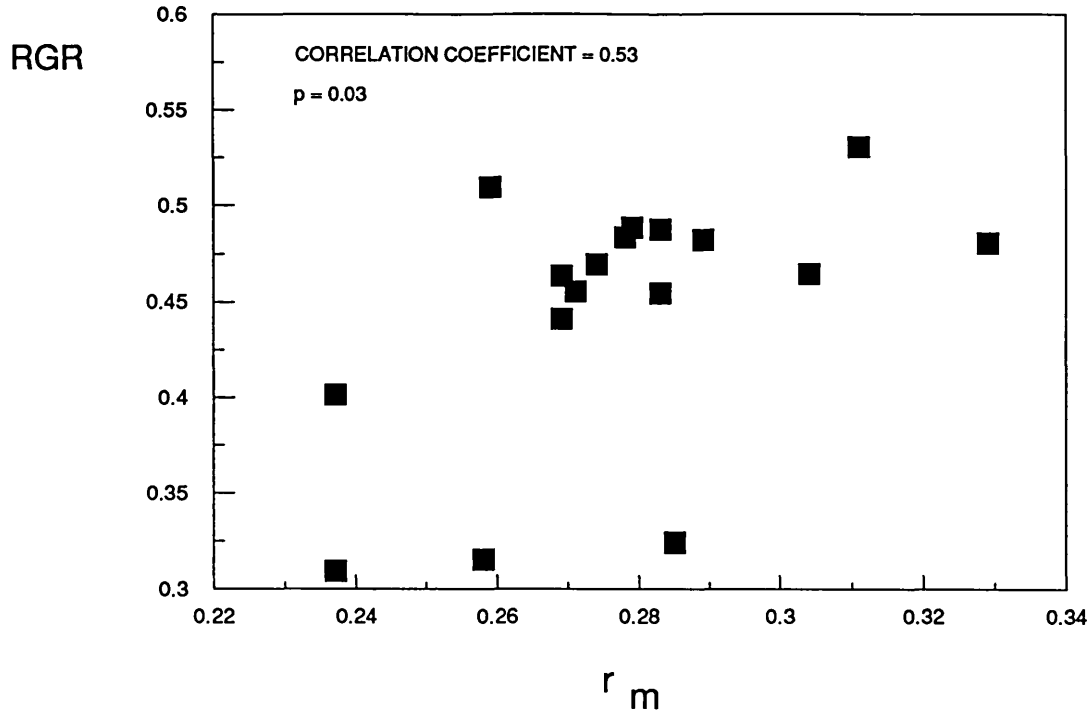


**Figure 2.8 Relationship Between RGR and $r_m$.**

This shows a significantly positive relationship between the two parameters.

## 2.5 Conclusions

The results from the five combinations of pollutant and cereal do not produce any general pattern of change in aphid MRGR due to pollution of the plant. However, following particular fumigation durations, significant increases in MRGR on wheat were observed. There were no significant increases on barley, and no significant decreases in growth rate on either wheat or barley. This suggests that the variety of barley chosen, Atem, may be relatively resistant to air pollution. This is particularly true when the results of Houlden *et al.* (1990) are considered. They found a large significant increase in the MRGR of *Sitobion avenae* on the barley variety 'Igri', following fumigation with $NO_2$ at 100 ppb, for seven hours (see literature review). Although they used a different aphid species a similar response might have been expected. Atem was chosen for its resistance to powdery mildew and it is possible that it is also resistant to other stresses.

Significant increases in MRGR were observed on wheat following 72 hours of ozone fumigation and 48 hours of $NO_2$ and NO fumigation, compared with filtered air controls. The ozone and nitrogen dioxide results were further investigated by conducting the intrinsic rate of natural increase studies. These endorsed the MRGR result for ozone, but produced conflicting results for nitrogen dioxide. This appears anomolous but the differing temperatures in the chambers during the two fumigations with $NO_2$ may provide an explanation. Further work is therefore required to ascertain the effects of this pollution regime at different temperatures.

Following this potentially anomalous $r_m$ result the relationship between MRGR and $r_m$ was investigated in the absence of pollution and temperature fluctuations. This showed that the relationship was not tight but that it was significantly positive. The degree of scatter in the results was expected since the $r_m$ study takes about three weeks to complete, during which time plant and aphid physiological changes occur. The daily removal of the nymphs during the last 10 to 12 days is also stressful to the adult aphid, and may alter the rate of nymph production.

The pollution regimes which produced significant increases in MRGR also produced large percentage increases in growth rate (see tables). This is potentially very important since the rapid reproductive rate of aphids can lead to escape from natural enemy control and therefore pest outbreaks. Dixon *et al.* (1982) demonstrated that a 13% increase in MRGR can lead to a 60% increase in aphid numbers after 10 days. Very large percentage differences were observed for some fumigations which were not significantly different. This may be a problem of insufficient replication, or may be due to the genuine and random variation inherent in the system.

When the individual fumigations were considered together in the five pollutant/cereal combinations, the analyses of covariance with maximum temperature, mean temperature and fumigation duration produced no significant differences, due to air quality, for any of the combinations. The use of these variables in an attempt to explain the differences in MRGR in the polluted or filtered air was therefore unsuccessful. The regressions of the duration of plant time in the chambers only produced a significant effect (a decrease) in the subsequent aphid MRGR for the ozone/wheat series of fumigations. This indicates that the fumigation chambers normally present the plants with no different stress levels, measured in terms of subsequent aphid MRGR, than the growing-up greenhouse, but that exceptions to this do exist. The temperature vs. MRGR regressions showed that temperature had an important effect for the barley fumigations.

A significant decrease in MRGR was observed with increasing temperatures following ozone fumigation, but a significant increase was observed following fumigation with nitrogen dioxide. This result is difficult to explain but could be a function of the different chambers used, or perhaps that the range of temperatures experienced during the $NO_2$ fumigations was greater than for the ozone fumigations. Low r squared values were associated with each result, which indicates that temperature does not explain the majority of variation in MRGR, and that a factor, or combination of factors, and not simply temperature alone, affects the MRGR of the aphids. The humidity of the ozone and $NO_2$/NO chambers, for example, may be different at different temperatures, but unfortunately humidity was not measured during the fumigations.

No significant effects of temperature on subsequent aphid MRGR were observed for any of the wheat fumigations. For the ozone and nitrogen dioxide fumigations this is because the temperatures were all very close to 20°C, rather than the wheat being necessarily more resistant than the barley. However, the nitric oxide fumigations were conducted at higher temperatures and produced MRGRs comparable with the ozone and nitrogen dioxide fumigations results. The one nitric oxide fumigation conducted at 20°C also produced no noticeable difference in growth rate compared with the other three NO fumigations. This may indicate that the wheat is in fact less susceptible than this variety of barley to elevated fumigation temperatures.

Interpretation of this series of experiments would have been greatly simplified if the temperatures in the chambers were constant. However, the significant increases and decreases in growth rates on barley due to the different temperatures at which fumigations were conducted indicate the importance of consideration of this variable for potential aphid success. Tingey and Singh (1980) proposed three mechanisms whereby temperature can induce changes in host plant suitability to insects;

(i) Temperature induced stress can cause changes in plant physiology that affect the expression of genetic resistance, resulting in changes in the levels of allelochemicals and/or morphological defenses and/or nutritional quality of the host.

(ii) Temperature induced stress can directly affect plant physiology, resulting in altered plant growth and development and so changing plant response to insect injury.

(iii) Temperature induced stress can directly affect insect behaviour and physiology.

The third mechanism is obviously irrelevant for these experiments since the insects did not experience the elevated temperatures, however, the other two mechanisms could clearly have an important role in this experimental system. Benedict and Hatfield (1988) suggested that increased rates of metabolism occur in plants exposed to warm temperatures due to an increase in enzyme reaction rates. They went on to say that this leads to decreases in temporary storage compounds (e.g. starches), and if the high temperatures are prolonged, increases may occur in amino acid concentration due to disruption of cellular function and cellular membrane function. Such plant responses to high temperatures are probably crucial to the insect response in this experimental system, but further experimentation at differing temperatures is again needed to elucidate changes in levels of metabolite pools.

A further limitation in the analysis of the results was the use of only one treatment and one control chamber for each experiment. This experimental design was due to the nature of existing facilities, but is probably a problem more of precise statistical interpretation rather than actually misleading results. Blank experiments were conducted in the $NO_2$/NO chambers (see Houlden *et al*, 1990) which demonstrated that no 'chamber effects' were apparent. The ozone chambers were capable of receiving either ozone or filtered air, and in all the experiments in which fumigations were repeated with the chambers exchanged, no significant differences in result were obtained.

Studies cited in the literature concerning the relationship between ozone fumigated plants and aphids have demonstrated no general aphid response. Increases, decreases and no change in aphid performance have been observed on ozone polluted plants, compared with controls (see literature review). The work presented here also does not produce a clear response, but indicates that only following certain fumigation durations and under certain environmental conditions are significant differences in aphid performance found. The previous work undertaken with aphids and nitrogen dioxide produced clear increases in aphid performance on polluted plants. The results of this chapter show that this response is not general and that each aphid/host plant interaction must be investigated before universal conclusions can be drawn. The nitric oxide work shows that this pollutant is of potential importance in mediating altered aphid performance, but that this is also dependant on fumigation conditions.

# Chapter 3
# The Direct Effect of Ozone and Nitrogen Oxides on *Metopolophium dirhodum*

## 3.1 Introduction

To ensure that any direct effect on the aphid of exposure to ozone and nitrogen oxides is not mediated via the plant, the aphid and the plant must be separated. Aphids, however, can only tolerate short periods of separation since they feed almost constantly. A method of aphid feeding which does not involve a plant was therefore required. Initial attempts to artificially feed plant sucking insects were undertaken by Carter (1927) who successfully separated a leaf hopper from its host plant. The first studies with aphids were conducted by Hamilton (1930) working with *Myzus persicae* (Sulzer), and have since been extensively refined by various workers for example Maltais (1952), Mittler and Dadd (1962 and 1963) and Mittler (1967). The success of the technique depends both on the apparatus used to simulate the host plant and the quality of the diet itself. The medium now generally used is a sucrose solution in which amino acids, vitamins, and minerals are dissolved, enclosed by an artificial membrane. The diets have been modified by various workers to suit different aphid species and morphs. The one used in this study was formulated by Aminu-Kano (1987) to suit the cereal aphid, *Sitobion avenae*, and is a modification of the original formulated by Dadd and Krieger (1967), for *Aphis fabae*. The best possible diet formulation is required so that the physiological state of the aphid is as similar as possible to that on a plant.

### 3.1.1 Direct Fumigation of Insects with Ozone

Fumigation of various insect species has been undertaken, but with no consistent response. The most common result is no change in performance following fumigations with realistic to moderately high concentrations, but both increases and decreases in performance have been observed. Levy *et al* (1972) investigated the direct effect of a high (unspecified) level of ozone fumigation on three species of Diptera, and found no observable differences between control and experimental insects during the larval and pupal stages. However, the treatments significantly stimulated oviposition of the adults, resulting in a dramatic increase in the number of eggs laid. This produced an increase in the adult population when compared with the controls. A 15% inhibition of egg hatching was observed in one species (*Musca domestica* L.). Levy *et al* (1974) also found no direct deleterious effects of ozone (at ca. 300 ppb) on two species of Cockroaches or on Fire Ants. Beard (1965) on the other hand found that prolonged exposures to high levels of ozone (ca. 100 ppb) were lethal to house flies and caused

them to lay fewer eggs per female. Feir and Hale (1983b) found reduced growth, smaller size at maturity, and reduced egg production in the large milkweed bug *Oncopeltus fasciatus* in an ozone atmosphere typical of that in an urban environment. They also found that the effects were less apparent if the insects had been reared in a polluted environment, indicating that there might be a type of ozone adaptation, which might influence the active concentrations absorbed and distributed within the adult and immature insect. The direct effect of ozone on plant sucking insects, particularly aphids, so far appears to be unstudied.

### 3.1.2 Direct Fumigation of Insects with Nitrogen Oxides

Few studies on the direct effect of nitrogen oxides on insects have been undertaken. Feir and Hale (1983a) found that $NO_2$ at very high levels (500 to 1000 ppb) increased the mortality of the milkweed bug *Oncopeltus fasciatus*, but that the surviving individuals had a significantly higher growth rate than the controls. The average number of eggs per female was also reduced by this regime. Dohmen *et al* (1984) compared the mean relative growth rate (MRGR) of *Aphis fabae* feeding on artificial diets in $NO_2$ at 200 ppb, or in charcoal filtered air, and found no significant difference. Bolsinger and Fluckiger (1989) exposed *Aphis fabae* on diets to either ambient air or to charcoal filtered air for seven days by the side of a motorway, and also found no significant difference in MRGR. Houlden *et al* (1990) used the same technique and found no significant difference in the growth rate of *Aphis fabae* following fumigation for seven hours with $NO_2$ at 100 ppb. The same artificial diet technique is used in this study to separate *Metapolophium dirhodum* from its host plant to determine the direct effects of ozone and nitrogen oxides on its survival and MRGR.

## 3.2 Materials and Methods

### 3.2.1 Diet Formulation and Method of Use

The diet used in this study is that of Aminu-Kano (1987). He altered the Dadd and Kreiger (1967) diet to the formulation which gave the highest mean relative growth rates for *Sitobion avenae*. He increased the sucrose concentration from 15 to 20%, and also increased each individual amino acid concentration by a third, which increased the total amino acid concentration from 2.41% to 3.23%. The solutes were all dissolved in pure water produced using de-ionised distilled water, which was oxidised with potassium permanganate to kill the microorganisms, and then redistilled over charcoal to remove other organic molecules and to act as a filter for impurities.

**Table 3.1 Composition Of Artificial Diet**

| Amino Acids | Weight (mg/100ml diet) | Vitamins, Minerals and Sucrose | Weight (mg/100ml diet) |
|---|---|---|---|
| Alanine | 130 | Ascorbic Acid | 100.00 |
| Arginine | 360 | Thiamin Hydrochloride | 2.5 |
| Asparagine | 730 | Riboflavin | 0.5 |
| Aspartic Acid | 190 | Nicotinic Acid | 10.0 |
| Cysteine | 50 | Pyridoxin Free Base | 2.5 |
| Glutamic Acid | 190 | Folic Acid | 0.5 |
| Glutamine | 200 | Calcium Pantothenate | 5.0 |
| Glycine | 110 | Inositol | 50.0 |
| Histidine | 110 | Choline Chloride | 50.0 |
| Isoleucine | 110 | Biotin | 0.1 |
| Leucine | 110 | $KH_2PO_4$ | 500.0 |
| Lysine | 160 | $MgCl_2.6H_2O$ | 200.0 |
| Methionine | 50 | Fe Sequestrene | 1.5 |
| Phenylalanine | 50 | Zn Sequestrene | 0.8 |
| Proline | 110 | Mn Sequestrene | 0.8 |
| Serine | 110 | Cu Sequestrene | 0.4 |
| Threonine | 190 | Sucrose | 20,000 |
| Tryptophan | 110 | | |
| Tyrosine | 50 | | |
| Valine | 110 | | |
| Total amino acids | 3230 | | |

The diet solution was adjusted to pH 7 using potassium hydroxide, and stored in syringes in the freezer, at -20°C, until needed.

The diet was then enclosed in sachets, according to the method of Ho (1980). The syringes were allowed to thaw and were gently shaken to mix the contents. They were fitted with a 0.2μm pore diameter 'acrodisc' filter to remove any bacteria or other contaminants in the diet. A sheet of parafilm M was thinly stretched across a 2.5cm diameter curtain ring, on a rubber bung, and approximately 0.4ml of artificial diet was expelled from the syringe onto the centre of the parafilm. Suction was applied to the under side of the ring, via a mouth operated 'vacuum' line, connected to the bung, causing a well to form, and a second sheet of stretched parafilm was placed over the

top. The suction was then released, the edges of the parafilm pressed together and the excess cut away. This process was conducted under sterile conditions using a flow hood, with all apparatus regularly swabbed with alcohol. The aphids were placed on the under side of the sachet and therefore fed upside down. In all experiments a green/yellow acetate filter was placed above the sachet to simulate the colour of the plant, to encourage the aphids to settle.

### 3.2.2 Initial Experiments

The experiments were carried out in the same greenhouse fumigation chambers as in chapter 2. They were conducted during the summer months and therefore the temperatures were often greater than 20°C and the humidity was often low. The temperatures for all the fumigations are tabulated in the results section. Standardised two day old nymphs were produced on a plant from the aphid culture (as detailed in chapter 2), in the controlled temperature (C.T.) room. Five nymphs of a similar size were chosen, and weighed individually onto each diet sachet. The sachets were placed on muslin boards, which permitted free air circulation to the aphids. Ten sachets were placed on each of two boards and one board was placed in the charcoal filtered air chamber, and one in the filtered air plus ozone chamber. Ozone was the only pollutant for which this method was used. It was administered continuously and the concentration for all durations was 100 ppb. The durations used were 6, 12, 24, and 72 hours, and each experiment was conducted separately. For durations of less than 72 hours the aphids were returned to the C.T. room until this time had elapsed. The intention was to calculate the MRGR but it was found that this exposure method led to high mortality and this calculation was therefore imprecise. Instead the proportion survival for each five aphid sachet was calculated. The results for each fumigation duration were initially analysed as one way ANOVAs, using air quality as the factor. The four fumigations were then analysed together using a two way ANOVA with air quality and time in the chambers as factors. All analyses were carried out in GLIM using binomial errors.

### 3.2.3 Improved Experiments

These experiments were designed to improve the survivorship of the nymphs. Adult aphids were placed on artificial diet sachets (as opposed to a plant in the previous experiments) and left in the C.T room for 24 hours to produce nymphs. The adults were then removed and the nymphs left for a further 24 hours. In this way the standardised two day old nymphs were born onto the artificial diet, which was intended

to reduce the stress associated with transfer from a plant to the diet. The nymphs were transferred from these diet sachets and weighed individually, one per sachet, onto new diet. One aphid per sachet was used which allowed a precise MRGR to be calculated.

Between 20 to 30 diet sachets were used for each experiment. They were placed on two muslin boards, one of which was placed in the charcoal filtered air chamber, and the other in the filtered air plus pollutant chamber. Both boards were suspended approximately 3cm above trays of water to maintain humidity around the aphids. The aphids were left in the chambers for durations ranging from 12 to 96 hours, and all the experiments were conducted separately. Following fumigations of less than 72 hours the aphids were returned to the C.T. room until 72 hours had elapsed, and then weighed off the sachets, and three day mean relative growth rates (MRGRs) calculated. Following fumigations of 72 and 96 hours the aphids were not transferred to the C.T. room, but were immediately weighed off the sachets at the end of the fumigation. Three day MRGRs were calculated for the 72 hour fumigations and four day MRGRs were calculated for the 96 hour durations. The pollutants used were:

1) ozone
2) nitrogen dioxide
3) nitric oxide. (Only one fumigation of 96 hours was undertaken.)

They were all administered continuously at 100 ppb, and for the durations shown on the graphs. The results for each fumigation duration were analysed as one way ANOVAs in SX. The results for all ozone fumigations were then considered together as were the results for all nitrogen dioxide fumigations. They were analysed as two way ANOVAs in GLIM, using air quality and time in the chambers as factors.

### 3.2.4 Direct Fumigation of Aphids on Plants.

In two cases the aphids and plants were fumigated together. The pollution exposure regimes were chosen as a consequence of results from both chapter 2 and this chapter. The fumigations were both conducted with wheat and ozone, and were for 24 and 72 hours. Immediately before the start of the fumigation two, two day old standardised aphid nymphs (produced on plants in the C.T. room) were weighed onto five week old plants (as in chapter 2). The plants plus aphids were then transferred to the fumigation chambers and exposed continuously, at 100 ppb, for the required time. Following the 24 hour fumigation the plants were transferred to the C.T. room for a further 48 hours, the aphids then weighed off the plants, and the three day MRGR calculated. Following the 72 hour fumigation the aphids were immediately weighed off the plants and the

MRGR calculated. The results were analysed as one way ANOVAs in GLIM, using air quality as the factor, and weighting a MRGR composed of two aphids twice as strongly as for one aphid.

## 3.3 Results

### 3.3.1 Initial Experiments

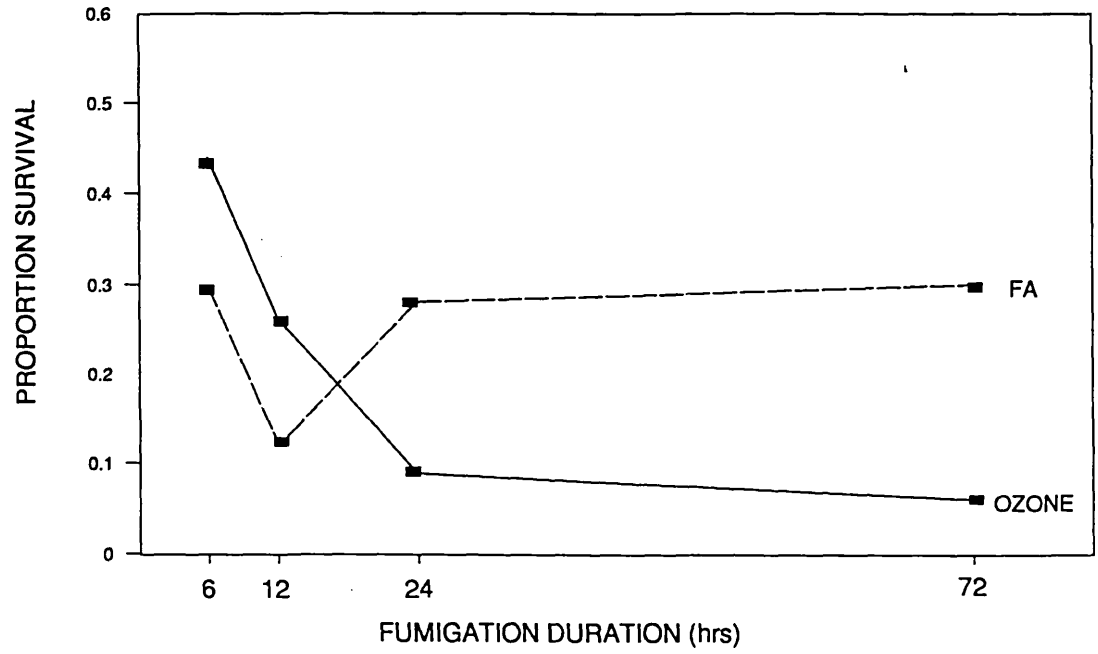


**Figure 3.1** **The Proportion Survival of Aphids Following Fumigation with Either Filtered Air or Ozone.**

The reduction in the proportion surviving in ozone following the 24 and 72 hour fumigations approached significance ($p < 0.059$ and $p < 0.06$ respectively); however, the other durations were not significantly different. When the fumigations were analysed together there was a significant interaction ($p < 0.009$) between the time the sachets were in the chambers and the air quality received. This indicated that the ozone fumigation and the time in the chambers together reduced the proportion surviving. Neither the air quality or the duration of the sachets in the chambers alone produced a significant difference in the proportion surviving. The temperature in the chambers was approximately 25°C for all fumigations (see Table 3.2).

**Table 3.2 Proportion of Aphids Surviving During Ozone Fumigation**

| DUR | NO. OF SACH'S | SUV. IN FA | SUV. IN $O_3$ | S.E. FA | S.E. $O_3$ | % +/- in $O_3$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|
| **6** | 20 | 0.299 | 0.440 | 0.129 | 0.140 | + 32.0 | 0.70 | 0.41 | 29.1 | 24.9 |
| **12** | 20 | 0.120 | 0.260 | 0.093 | 0.124 | + 53.8 | 1.34 | 0.26 | 31.9 | 27.5 |
| **24** | 20 | 0.280 | 0.089 | 0.127 | 0.288 | - 68.2 | 4.23 | 0.06 | 31.6 | 26.4 |
| **72** | 20 | 0.300 | 0.060 | 0.129 | 0.069 | - 80.0 | 4.03 | 0.06 | 28.3 | 24.3 |

**KEY (For tables 3.2 to 3.5)**

| | |
|---|---|
| DUR | Duration of Fumigation (Hours). |
| NO. OF SACH'S | Number of Sachets in the Experiment. |
| SUV IN FA | Proportion of Aphids Surviving in Filtered Air (Table 3.2). |
| SUV IN $O_3$ | Proportion of Aphids Surviving in Ozone (Table 3.2). |
| PROP. SUV. | Proportion of Aphids Surviving in FA + $O_3$ (Tables 3.3, 3.4 and 3.5). |
| MRGR FA | Mean Relative Growth Rate in Filtered Air. |
| MRGR $O_3$ | Mean Relative Growth Rate in Ozone. |
| S.E. FA | Standard Error of either Survival (Table 3.2) or MRGR (Tables 3.3, 3.4 and 3.5) in FA. |
| S.E. $O_3$ | Standard Error of either Survival (Table 3.2) or MRGR (Tables 3.3, 3.4 and 3.5) in $O_3$. |
| % +/- in $O_3$ | Percentage increase or decrease in Prop. Suv. (Table 3.2) or MRGR (Tables 3.3, 3.4 and 3.5). |
| F | F Ratio. |
| p | Probability. |
| MAX TEMP | Maximum Temperature Experienced in the Chambers. |
| MEAN TEMP | Mean Temperature in the Chambers (0900 to 2100) |

### 3.3.2 Improved Experiments

**1) For Ozone.**

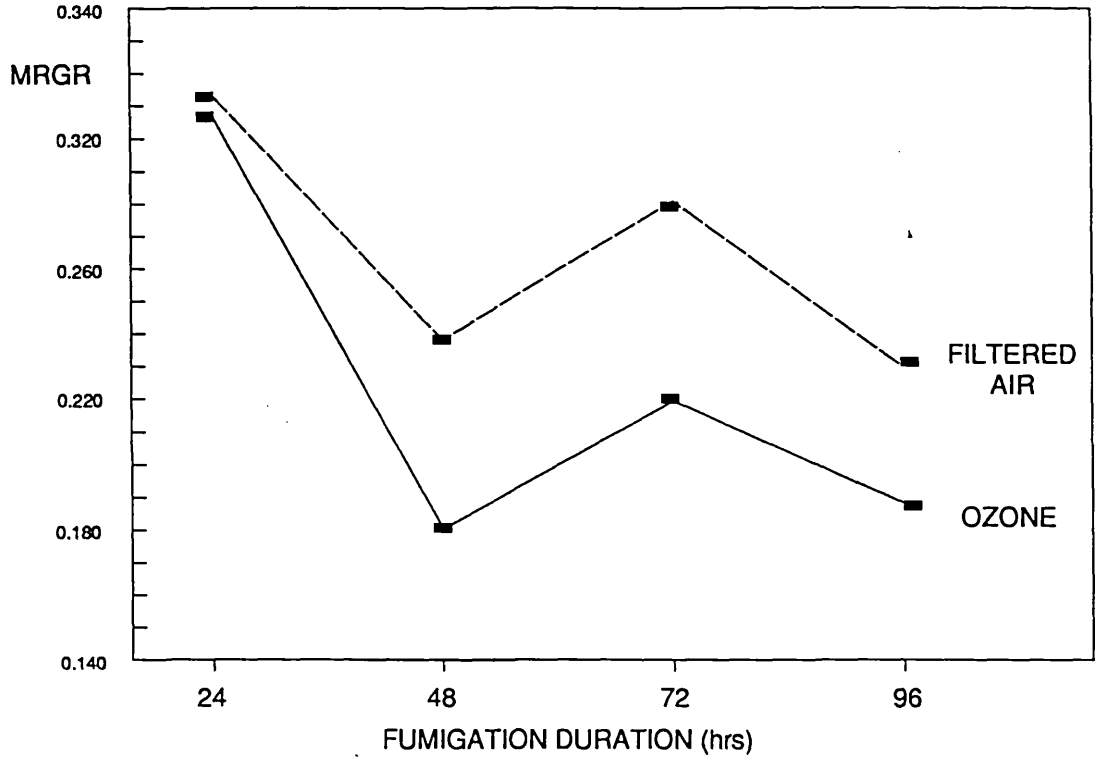


**Figure 3.2** **The MRGR of Aphids Following Fumigation With Either Filtered Air or Filtered Air plus Ozone.**

A decrease in MRGR was seen in ozone for each fumigation duration, but none of the differences were statistically significant. When all the fumigations were analysed together there was a significant decrease in the MRGR as the duration of the sachets in the chambers increased ($p < 0.006$), and the difference due to ozone approached a significant decrease ($p < 0.14$). There was no significant interaction between time in the chambers and air quality received. The temperatures in the chambers were lower than for the initial experiments (approximately 20°C - see Table 3.3), and this in conjunction with the improved experimental technique, enhanced the aphid survival. The proportion surviving was not affected by increased time in the chambers, and there was no difference in the survival rates in filtered air or ozone.

**2) For Nitrogen Dioxide.**

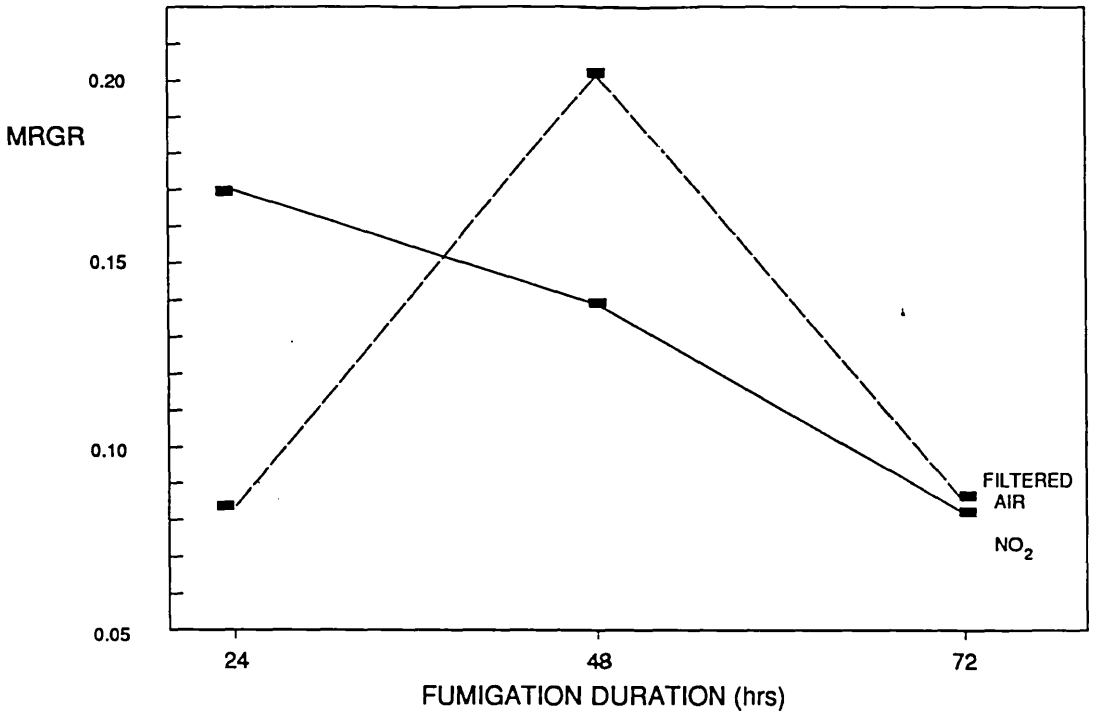


**Figure 3.3** **The MRGR of Aphids Following Fumigation with either Filtered Air or Filtered Air plus Nitrogen Dioxide.**

There was no trend in the difference between MRGR in $NO_2$ or filtered air and none of the individual fumigations were significantly different. When the three fumigations were analysed together there was a significant decrease in the MRGR as the duration of the sachets in the chambers increased ($p < 0.05$). This was because of the greatly reduced MRGR found for the 72 hour exposure period, due to the high temperatures in the chambers - see Table 3.4. The overall difference due to $NO_2$ was not significant ($p < 0.51$), and there was no significant interaction with this and duration of exposure in the chambers. It is not possible to determine if the proportion survival was reduced by increased time in the chambers due to the high mortality produced by the elevated temperatures during the 72 hour exposure duration, however, there was no difference in the survival rates in filtered air or nitrogen dioxide.

**3) For Nitric Oxide.**

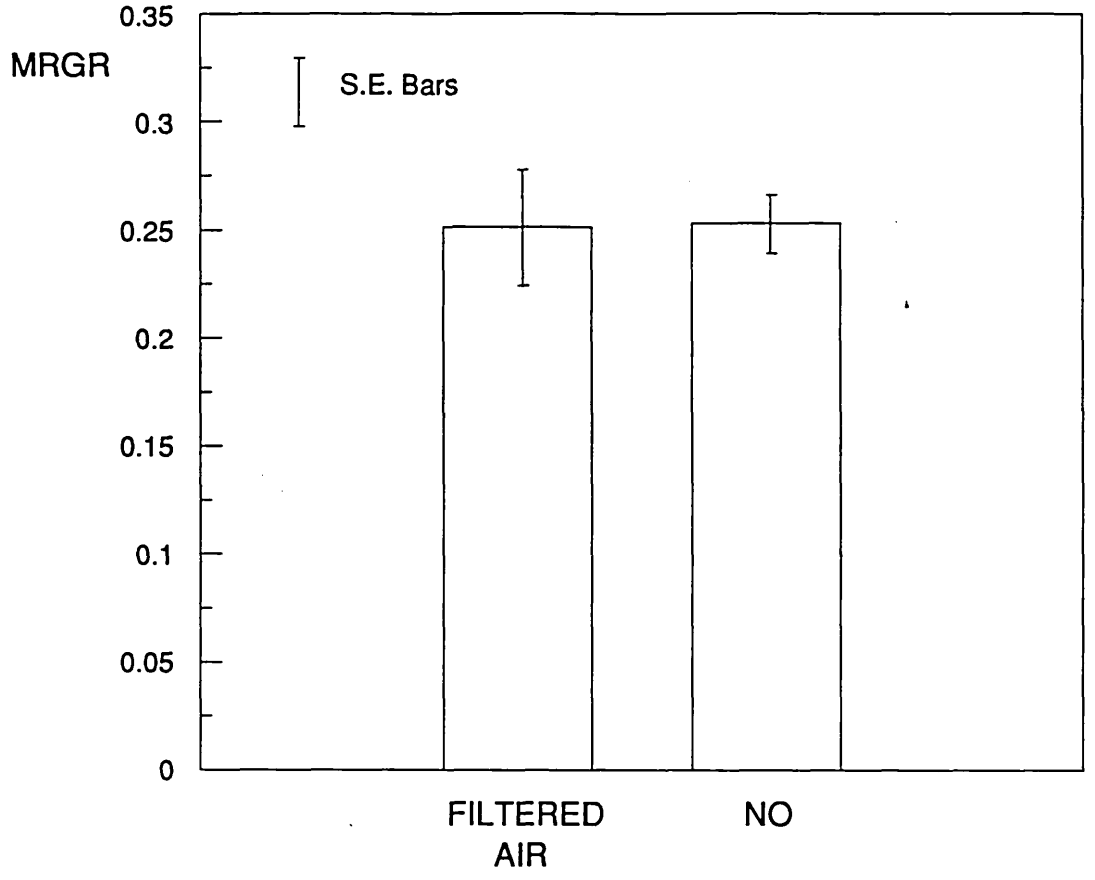


**Figure 3.4** **The MRGR of Aphids Following Fumigation for 96 hours with either Filtered Air or Filtered Air plus Nitic Oxide.**

Very little difference ($p < 0.95$) was seen in the growth rate in the polluted or filtered air. The lack of difference at this duration suggests there would be no difference at shorter durations and these were therefore not undertaken. The proportion of aphids surviving was 0.90 and the maximum and mean temperature experienced was 20°C.

### 3.3.3 Direct Fumigation of Aphids on Plants.

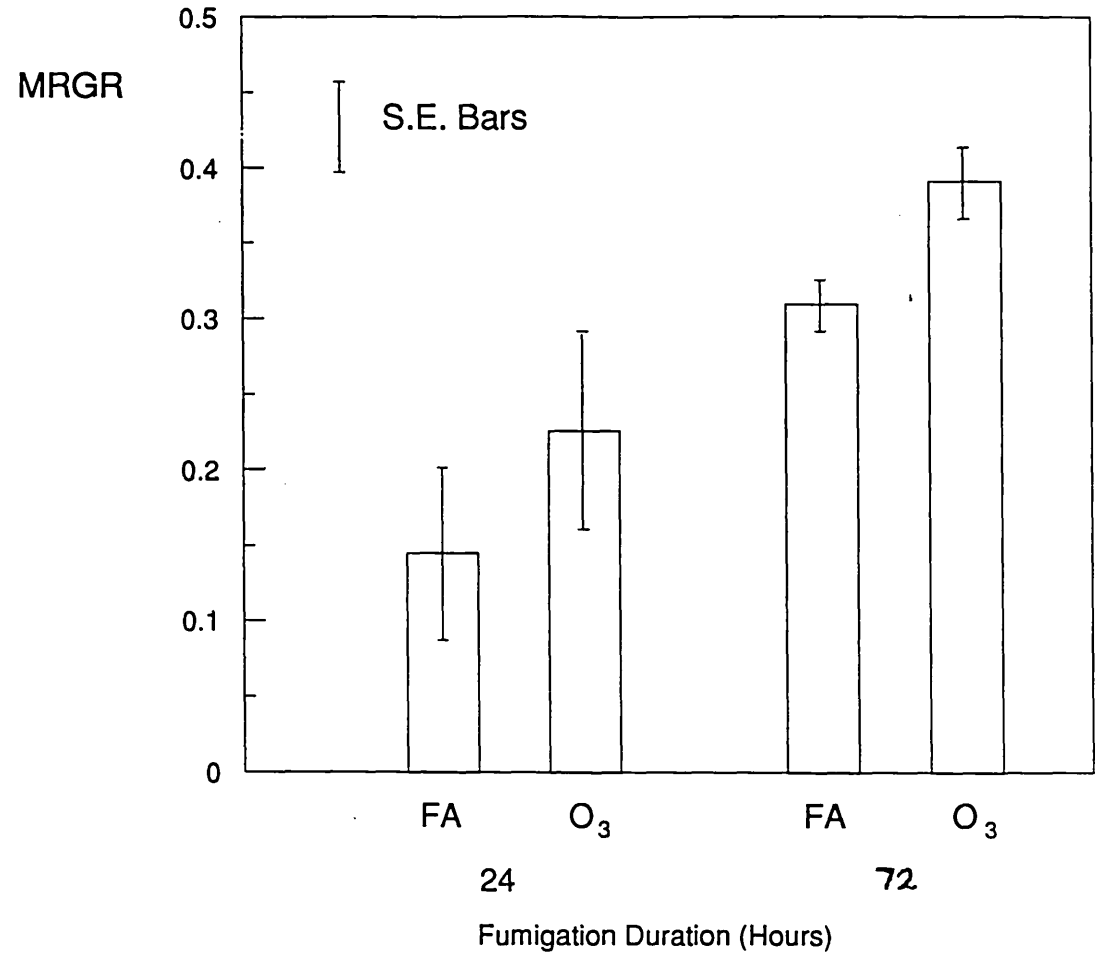


**Figure 3.5** **The MRGR of Aphids on Wheat During Fumigation with Ozone for 24 and 72 hours.**

This shows that there was no direct deleterious effect of ozone on the aphid when it is on a plant, indeed increases in MRGR in $O_3$ are seen for both fumigation durations. A large significant increase in growth rate is seen following 72 hours of fumigation, probably indicating a significant improvement of food quality for the aphid during the experiment. The high temperatures in the chambers during the 24 hour fumigation (see Table 3.5) are probably the cause of the lower MRGRs observed in this experiment.

Table 3.3 MRGRs on Artificial Diet During Ozone Fumigation.

| DUR | NO. OF SACH'S | PROP. SUV. | MRGR FA | MRGR $O_3$ | S.E. FA | S.E. $O_3$ | % +/- in $O_3$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 30 | 0.67 | 0.313 | 0.307 | 0.036 | 0.031 | - 1.9 | 0.02 | 0.89 | 22.8 | 20.8 |
| 48 | 20 | 0.50 | 0.238 | 0.180 | 0.057 | 0.076 | - 24.4 | 0.29 | 0.60 | 21.7 | 20.0 |
| 72 | 30 | 0.83 | 0.281 | 0.219 | 0.026 | 0.028 | - 22.1 | 2.59 | 0.12 | 22.2 | 20.0 |
| 96 | 27 | 0.85 | 0.230 | 0.188 | 0.026 | 0.024 | - 18.3 | 1.37 | 0.25 | 20.0 | 20.0 |

Table 3.4 MRGRs on Artificial Diet During Nitrogen Dioxide Fumigation.

| DUR | NO. OF SACH'S | PROP. SUV. | MRGR FA | MRGR $NO_2$ | S.E. FA | S.E. $NO_2$ | % +/- in $NO_2$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 30 | 0.57 | 0.081 | 0.170 | 0.053 | 0.031 | + 52.3 | 2.37 | 0.14 | 20.0 | 20.0 |
| 48 | 30 | 0.70 | 0.201 | 0.138 | 0.034 | 0.036 | - 31.3 | 1.61 | 0.22 | 20.0 | 20.0 |
| 72 | 30 | 0.27 | 0.089 | 0.081 | 0.020 | 0.021 | - 8.3 | 0.05 | 0.82 | 38.0 | 25.0 |

Table 3.5 MRGR of Aphids on Wheat During Fumigation with Ozone.

| DUR | NO. OF PLANTS | PROP. SUV. | MRGR FA | MRGR $O_3$ | S.E. FA | S.E. $O_3$ | % +/- IN $O_3$ | F | p | MAX TEMP | MEAN TEMP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 11 | 0.61 | 0.144 | 0.226 | 0.058 | 0.065 | + 35.9 | 0.86 | 0.376 | 26.4 | 22.7 |
| 72 | 22 | 0.90 | 0.310 | 0.391 | 0.016 | 0.016 | + 20.8 | 13.1 | 0.002 | 24.2 | 20.0 |

## 3.4 Conclusions

The results of the artificial diet work indicate that both nitrogen dioxide and nitric oxide have no direct effect on aphid MRGR, which endorses the work of Dohmen *et al* (1984) for *Aphis fabae*. However the results for the effects with ozone on diets are more difficult to interpret. It appears that at longer fumigation durations the proportion surviving in ozone decreases, but that this is partly due to longer exposure durations in the fumigation chambers. The results from the MRGR experiments demonstrate a consistent decrease in growth rate for all the fumigation durations, although no single fumigation produces a significant decrease.

Although the measures taken in the improved experiments did enhance the aphid survival (c.f. Tables 3.2 and 3.3), the aphids were still stressed, as shown by the significant reduction in MRGR for the ozone fumigations, with increased time in the chambers. The stress probably arises from the reduced humidity and increased temperature and light intensity in the chambers compared with conditions in the C.T. room. The aphids are also stressed by the artificial diet technique compared with feeding on a plant, as seen by reduced growth rates on the diet compared with on a plant (c.f. Tables in chapter 2 with results of this chapter). This, however, does not explain the reduction in MRGR with increasing fumigation duration, since the aphids were always on the diet for 72 hours (except for the 96 hour fumigations).

To allow a precise MRGR to be calculated only one aphid per sachet was used in the improved experiments. If 5 aphids per sachet are used and high levels of mortality are encountered assumptions are required concerning the probable final weight of the dead aphids, based on the weight of the surviving individuals. In the initial experiments, despite the aphids being grouped together as a five due to their similar initial weights, it is not accurate to assume that they will attain the same final weight. Hence, the MRGR was not calculated. Although the improved experiments were conducted when conditions in the chambers were more favourable, the death of individuals made the use of one aphid per sachet a more precise technique.

Fumigation of the aphids on the plants showed that there was no direct deleterious effect of ozone on the aphid. The 72 hour fumigations of both the initial experiments and the improved experiments showed almost significant *decreases* in the proportion of aphids surviving and the MRGR, respectively. However, experiments in chapter 2 demonstrated significant *increases* in MRGR and $r_m$ following 72 hours of ozone fumigation of the plant only. A possible result of the fumigation of the plant plus aphid was therefore a negation of these opposite effects, so producing no significant difference in MRGR. However, this did not occur, which indicates that the result of direct

fumigation of insects on artificial diets should be interpreted with caution. Any possible direct deleterious effect of ozone to the aphid was removed by the improvement of the ozone fumigated plant as a host for the insect.

The value of the diet technique is in the complete separation of the direct effect to the aphid from effects mediated via the plant, which inevitably occur when aphid and plant are fumigated together. The technique is also useful as a first investigation, since if no direct deleterious effects are observed when the aphid is on the diet, there are unlikely to be any effects when on the plant. This is due to the stronger physiological state of an aphid on a plant, as evidenced by higher MRGRs, and explains the lack of experiments in this chapter fumigating plant and aphid together with nitrogen oxides. However, such experiments could be undertaken to investigate any possible beneficial effect of the fumigation.

# Chapter 4
# Pollution Mediated Changes in Plant Biochemistry

## 4.1 Introduction

Experiments performed in chapter 2 demonstrated that under certain circumstances the relative growth rate of aphids was significantly increased as a result of prefumigation of the host plant. Since the insect was introduced to the plant after fumigation, the effect must be mediated via the host plant, and since the aphid does not have the opportunity to select the plant, the observed increases in growth rate are probably due to biochemical factors. It is obviously impossible to characterise all the biochemical changes in the plant due to the pollutant gas, and changes in the quantity and quality of secondary chemicals, contributing to host defense mechanisms, are not investigated here. Changes in surface morphology and phloem morphology, which both may have an effect on the aphid, are also not considered. In an outdoor polluted system, depending on the soil type and properties, changes in the availability of mineral nutrients would also alter the status of the plant for the insect. Changes in the short term, such as for the experiments conducted in this study, only produce changes in the phloem constituents, which cannot be assessed accurately in this system. Only those measurable changes likely to affect aphid performance are of interest, and some well known modulators of aphid growth rate can be assessed. The soluble and total nitrogen levels (and amino acid levels in one case only), the reducing and total soluble sugar levels, and levels of glutathione were investigated.

### 4.1.1 What is Glutathione ?

Reduced glutathione is the major non-protein sulphur containing constituent of plants. The amino acids cysteine and methionine contain approximately 90% of the total sulphur in most plants, but almost all of these are found in protein (Rowland *et al* 1988). Grill *et al* (1979) demonstrated that glutathione was the most abundant SH compound, in the leaves of four tree species, and that it usually comprises more than 95% of the total SH content. Glutathione is a tri-peptide and is synthesized from glutamine, cysteine and glycine. It is predominantly found in the chloroplasts and is normally in the reduced form (GSH), with most of its proposed function related to the thiol group and its use as a reductant (Foyer and Halliwell, 1976). It is thought to be involved in the removal

of $H_2O_2$ and superoxide, and also functions in sulphur transport and metabolism (De Kok *et al*, 1986), in amino acid transport (Meister and Anderson, 1983), and in the stabilisation of enzyme systems (Higgins and Payne, 1982).

Glutathione is also common in animal systems, and plant glutathione is thought to be utilized directly by herbivorous insects. It is known to function in the detoxification of insecticides and some naturally occurring antibiotics and toxins (in Chiment *et al*, 1986).

### 4.1.2 Methods of Assessing Differences in Host Plant Food Quality

Aphids feed on phloem sap, and it is changes in its composition which affect the aphid performance. Any studies of plant biochemistry related to changes in aphid growth rate should ideally therefore look at the composition of the phloem sap. This has been undertaken successfully using aphids as a tool. The aphid is allowed to settle and commence feeding but is then rapidly removed from the plant using a razor blade, or in later studies using laser beams. If the aphid is surprised sufficiently it does not have time to remove its stylets, and these remain embedded in the phloem. They continue exuding sap for a few hours, due to the pressure of the phloem. However, this technique has only proved possible for a limited number of aphid species, and these have been for large aphids feeding on woody host plants (e.g. Mittler, 1958). The small size of *M. dirhodum* and soft tissues of wheat and barley precluded the use of this technique in these studies.

A second technique occasionally employed to determine the composition of phloem sap involves the use of chelating agents such as EDTA (e.g. Braun and Fluckiger, 1989, and Bolsinger and Fluckiger, 1989). However this is also not without its problems since there is no way to ascertain if the exuded liquid is phloem sap or merely extra- or intra-cellular fluid, and only a very small quantity of liquid is exuded which makes very sensitive analysis necessary.

In the light of the above limitations to studying only the phloem sap constituents, the most pragmatic alternative is to look at the biochemical composition of the whole leaf. This is the method employed in this study. Van Emden (1966) defended the use of the whole leaf since amino acids formed in the mesophyll of green leaves are either translocated fairly rapidly or converted into insoluble protein. This implies that if amino acids are detected in whole plant material they were probably largely situated in the phloem. He also pointed out that the concentration of solutes in the phloem, at the

position where the aphid is feeding, are probably not in the same proportion as elsewhere in the phloem. He concluded that analysis of whole leaf material gives a guide to levels of soluble nitrogen in the phloem. No justification for the analysis of the whole leaf to elucidate changes in phloem sugar or glutathione concentration could be found, but it seems likely that, at least for the sugar analyses, similar arguments would apply.

### 4.1.3 Alteration of Insect Performance as Related to Host Plant Biochemistry.

McNeill and Southwood (1978) suggested that an altered plant metabolism and therefore nutritional status of a plant may be the basis of increased insect abundance. The result of their synthesis of the role of nitrogen in the development of insect/plant relationships is that the biochemical status of a plant must always be considered. They proposed specifically that - "Growth and reproduction of phytophagus insects should be influenced by the quantity and quality of the proteins and amino acids in their food."

It has been widely reported that there is a positive correlation between leaf soluble nitrogen and aphid success (see reviews by Auclair, 1963, and van Emden *et al*, 1969). For example a lower amino acid content was found in winter wheat cultivars resistant to *M. dirhodum* compared with susceptible cultivars (Kowalski and Visser, 1983). The leaf soluble nitrogen is mainly in the form of free amino acids (Pate, 1968 and Beevers, 1976) and low molecular weight peptides (van Emden, 1973). The results of various studies indicate that it is an increase in particular amino acids or groups of amino acids which affects aphid performance (e.g. van Emden and Bashford, 1971, van Emden, 1972, and Prestidge and McNeill, 1983). However, more work needs to be done with particular aphid species, to identify which amino acids have stimulatory effects and to find out which, if any, have inhibitory effects.

The level of total nitrogen within the plant is unimportant in terms of aphid nutrition since they are unable to utilize this component. It is of interest, however, since comparison of soluble and total nitrogen levels could provide information concerning the resulting levels of soluble nitrogen. A reduction in total nitrogen may also indicate that nitrogen is being translocated to other parts of the plant. Total nitrogen includes nitrogen in structural proteins, non-protein amino acids, and nitrogen incorporated into defensive compounds (Hare, 1983). It is theoretically assumed, though not proven, that the level of sugar within the phloem sap is unlikely to be a limiting factor in aphid nutrition since this is its largest component (Kennedy, 1958 and van Emden, 1972). Aphids excrete most of the sugar they imbibe as honeydew (van Emden, 1973), which

further suggests it is not limiting, but does not consider that individual sugars may be of importance. Sugar levels may therefore only be important if they are decreased, which would produce an increase in the carbon : nitrogen ratio within the plant.

Insects and plants are interdependent as a result of coevolution, and environmental stresses can alter the normal susceptibility or resistance of vegetation to phytophagus insects (Jeffords and Endress, 1984). Air pollution is a recent phenomenon to which insects and plants have not yet coevolved, and as such, altered levels of insect attack are found. In a stable environment, as evolution of both insect and plant occurs, a new equilibrium level for the rate of insect attack may be achieved. Alteration of levels of plant metabolites affecting insect performance have been found for all the major air pollutants, and the changes relating specifically to ozone and nitrogen oxides will be reviewed here.

### 4.1.3.1 Alteration of Plant Biochemistry due to Ozone

A large body of literature has built up recently and this is reviewed by Riemer and Whittaker (1989), Koziol, Whatley and Shelvey (1988) (carbohydrates) and Rowland, Borland and Lea (1988) (nitrogen and amino acids), amongst others.

## 1a Effect of Ozone on Nitrogen Levels

Many studies have investigated this question, but extremely diverse exposure regimes make comparison of results difficult. Early studies frequently used very high ozone concentrations, for short periods of time, but recently more realistic levels have been used. Tingey *et al* (1973) showed that ozone fumigation of soybean (*Glycine max*), at concentrations atypical of natural environments (490 ppb for 2 hours), initially produced a decrease in the concentration of amino acids and total protein, followed by a large increase 24 hours later. Later work by Tingey *et al* (1976) showed that ozone exposure of *Pinus ponderosa* seedlings at 100 ppb per 6 hour day, for 7 days a week, for periods of up to 20 weeks, produced no significant difference in the total nitrogen content of the above ground parts of the seedlings, but produced a significant increase in the nitrogen content in the roots. Similar quantities of amino acids were observed in the control and treatment trees, in the above ground part of the seedlings, but increased quantities were found in the roots of the exposed plants.

In contrast to this, Tomlinson and Rich (1967) found an increase in the levels of free amino acids when they fumigated beans (*Phaseolus*), beets (*Beta*), corn (*Zea*), barley (*Hordeum*) and rye (*Secale*) with ozone at 100 ppb for one hour or until visible damage

was apparent. They concluded that ozone inhibited protein synthesis as well as photosynthesis. Japanese workers (Ito *et al* 1986) also found increased levels of amino acids in *Phaseolus vulgaris* following short term exposure (up to 8 hours) to either ozone (at 400 ppb), nitrogen dioxide (at 4000 ppb), or their combination. Craker and Starbuck (1972) showed that the amine concentration in *Phaseolus vulgaris* steadily increased over the course of 24 hours following an ozone exposure of 250 ppb for 2 hours. The protein concentration, however, was shown to decrease following exposure, which they postulate to be caused by an induction of protease activity or decreased protein synthesis. Protein concentration was also shown to decrease following fumigation of *Ulmus americana* seedlings for 5 hours with 900 ppb ozone. The effect was detected within 24 hours of fumigation and persisted in old leaves for at least 5 weeks after fumigation (Constantinidou and Kozlowski, 1979). In contrast, the protein content of *Phaseolus vulgaris* fumigated with ozone at 150 ppb for 6 hours on five days was shown to increase, compared with controls (Beckerson and Hofstra, 1979).

Heath (1984) summarised the results of four studies and found that triose phosphate derived amino acids (cysteine, glycine and serine) frequently increased following ozone treatment, with the late TCA-derived family (aspartate, asparagine, lysine, methionine, threonine and isoleucine) also showing some increase. The other groups of amino acids had not been well monitored but showed no obvious common change of pattern. Ting and Mukerji (1971) found increases in most amino acids in cotton (*Gossypium hirsutum*), following one hour of ozone fumigation at 80 ppb. The effect was most apparent at a susceptible period in its growth when a minimum concentration of free amino acids and soluble sugars were found. The soluble protein levels decreased following ozone exposure, but not to the same extent as the increase in the free pool of amino acids. They suggest that visible ozone damage occurs where there is a depletion of soluble reserves, since this may reduce the ability of the plant to repair damage. In this way ozone can produce an acceleration of senescence. For example Grandjean and Fuhrer (1989) found that senescence of spring wheat (*Triticum aestivum* L. cv. Albis) in open top field fumigation chambers with 1.5 and 2 times the ambient ozone levels, was accelerated, compared with controls. This was probably due amongst other things, to an earlier decrease in soluble protein content in flag leaves, which resulted in significant yield reductions. Bennett *et al* (1977) parallelled the metabolic changes occurring due to ozone with those seen due to water stress. They conducted work on bean varieties but no details of ozone exposure regimes were given. They found increases in amino acids related to photosynthesis and stress metabolism.

Mudd *et al* (1969) showed in vitro, that ozone preferentially oxidized certain amino acids, the order of susceptibility in aqueous solution being cysteine, methionine, tryptophan, tyrosine, histidine, cystine and phenylalanine. Within the cell this would result in the breakdown of structural proteins into their component parts, and produce significant increases in free amino acid concentrations, and in some cases soluble protein levels. An action of ozone on foliar tissues may therefore be the apparent release of large amounts of soluble nitrogenous compounds from plant structural forms. (Trumble *et al*, 1987). This may explain the above results.

Price *et al* (1990, in press) fumigated two week old barley (*Hordeum vulgare* cv. Atem, the same variety used in this study) seedlings, with 200 ppb ozone, and found significant reductions in the concentration of soluble protein in all leaves. They transversely sectioned the leaves into quarters and found that most protein loss occurred in the tips of the leaves (the oldest section), and also in the oldest leaves.

It appears from the above that a general response to ozone exposure is an increase in the levels of soluble amino acids with either an increase or decrease in protein content. Changes in the proportions of amino acids occur in some cases, but no clear consensus of change is apparent.

## 1b Relationship Between Nitrogen Level and Insect Performance

In the above studies no attempt was made to correlate nitrogen levels, induced by ozone exposure, with insect performance. This has been undertaken in few studies and, as expected, an increase in soluble nitrogen levels has led to an improved insect performance. For example, Trumble *et al* (1987) found a transient but significant increase in the soluble protein concentration following ozone fumigation of tomato plants (*Lycopersicon esculentum)* for 3 hours at 280 ppb. This fumigation period was repeated four times with an interval of three days between each treatment. The increase was still significant two days after the final fumigation ceased, but was insignificant seven days later. These results were associated with improved survival and decreased developmental time of *Keiferia lycopersicella* (tomato pinworm). The concentration of the total free amino acids in ozonated foliage did not alter significantly, but significant changes were observed in at least 10 individual amino acids.

## 2a Effects of Ozone on Sugar Levels

Tingey *et al* (1973) showed that fumigation of soybean (*Glycine max*) with a single 2 hour exposure of ozone (at 490 ppb) caused an initial decrease in the levels of soluble sugars followed by a subsequent return to control levels. They later found (Tingey *et al*, 1976) that ozone exposure of *Pinus ponderosa* seedlings, increased the levels of soluble sugars in the above ground parts of the seedlings, but decreased their levels in the roots. Asada (1980, cited in Treshow, 1984) pointed out that sugars act as scavengers of free radicals, and it is possible that an increase in their levels can partly overcome the phytotoxic effects of the free radicals produced by ozone. Lee (1965) found an illustration of this effect with *Nicotiana tabacum* leaves. He found that the normal variability between leaves produced differential levels of visible damage due to ozone, and that damage was more severe when reducing sugar and sucrose levels were lower. Dugger *et al* (1962) also showed that a high sugar level in *Phaseolus vulgaris* leaves could provide protection from ozone fumigation. Dugger *et al* (1966) later investigated the effect of ozone (at 250 ppb, 8 hours per day, five days a week for nine weeks) on lemon (*Citrus limon*) seedlings and found significant increases in both reducing sugars and sucrose concentrations, and significant decreases in starch levels, compared with control plants. Barnes (1972) fumigated five species of pine seedlings with 50 ppb ozone for up to twenty two weeks, and found significant increases in total soluble carbohydrates and reducing sugars in primary and secondary needles. He suggested that this may be due to an impairment in polysaccharide synthesis, and noted the possible value of this in resistance to damage.

Several studies cited here report that ozone fumigation causes a decrease in the levels of soluble carbohydrates. Miller *et al* (1989) fumigated cotton (*Gossypium hirsutum*) in open top field chambers for 12 hours a day at five ozone concentrations, for the duration of the growing season, and found significant reductions in levels of total non-structural carbohydrates, plus some hexose sugars and starch, in stems, leaves and roots. Other studies which show suppression of soluble carbohydrates and starch include Dugger and Palmer (1969), and Tingey, Fites and Wickliff (1973). Fumigation of *Ulmus americana* seedlings with 900 ppb ozone for 5 hours produced a decrease in total non-structural carbohydrates within 24 hours of fumigation, with the effect persisting in older leaves for at least five weeks (Constantinidou and Kozlowski, 1979). Some studies also showed no effects of ozone on carbohydrate metabolism, for example Blum, Smith, and Fites (1982) and Ito *et al* (1985b), although in the latter study the level of carbohydrates significantly decreased in the roots.

From the above it appears that the response of carbohydrate levels to ozone is unpredictable and no general conclusions can be drawn.

## 2b Relationship Between Sugar Level and Insect Performance

The only study which relates ozone induced sugar content to insect performance was conducted by Braun and Fluckiger (1989). They found increased population growth of *Phyllaphis fagi* on *Fagus silvatica* exposed to ambient air, at an average ozone concentration of 40 ppb, (other pollutants were at very low levels), compared with filtered air. This was associated with a significantly increased amino acid to sugar ratio of phloem exudate, largely due to a decreased sugar content. They also found a significant decrease in population growth of *Aphis fabae* on *Phaseolus vulgaris* associated with no significant difference in the amino acid and sugar content of the phloem exudate. This implies that other biochemical changes important for the aphid may occur.

## 3a Ozone Effects on Glutathione Levels

Many studies exist documenting an increase in glutathione levels in plants following sulphur dioxide fumigation (for example Grill *et al*, 1979), but interestingly increases in levels are also observed following exposure to ozone. Melhorn *et al* (1986) found a significant increase in the glutathione content of *Abies alba* and *Picea abies* following exposure to ozone at 37 ppb for 12 hours on every sunny day during one summer. The initial level of glutathione was less in *Abies alba* which they suggest could be the reason for its observed increased sensitivity to $SO_2$ compared with *Picea abies*. They suggest that elevated glutathione levels may protect the tree by preventing the accumulation of $H_2O_2$ and free radicals. The enzyme glutathione reductase converts oxidised glutathione (GSSH) to its reduced form (GSH). An increase in its activity was observed by Guri (1983) following exposure of two ozone insensitive cultivars of *Phaseolus vulgaris* to 280-320 ppb ozone for 8 hours. They found that the reduced glutathione content of two sensitive cultivars was significantly lower than that of two resistant cultivars, following fumigation, despite all the cultivars initially containing similar quantities. Decreased ozone sensitivity was also noted in *Phaseolus vulgaris* following exogenous artificial treatment of the foliage with glutathione (Dass and Weaver, 1968).

Hughes and Chiment (unpubl., in Hughes 1988) found initial decreases in GSH followed by marked increases in ozone fumigated radish, bean and soybean. Tanaka *et al* (1988) also found increases in the levels of glutathione reductase in spinach (*Spinacia oleracea*)

following fumigation with ozone for 2 days at 70 ppb, but no mention was made of the resulting glutathione concentration. In contrast, the glutathione reductase activity of spinach following exposure to $H_2S$, at 250 ppb for two days, was not affected despite a four fold increase in the reduced glutathione content (De Kok *et al*, 1986). This suggests that glutathione may also function in the transport and storage of reduced sulphur. Grill *et al* (1979) proposed that increasing levels of glutathione itself, due to $SO_2$, was at least in part responsible for $SO_2$ phytotoxicity. They suggested that changes in the balance of reduced to oxidised sulphur compounds occurs which induces a general disruption of cellular processes.

Price *et al* (1990, in press) found that ozone fumigation of barley resulted in a net loss of glutathione in the leaves, which was due to a loss from the youngest sections of the leaf. There was a slight increase in levels in the oldest sections of the leaf which may indicate that glutathione transport was occurring.

### 3b Relationship Between Glutathione Level and Insect Performance

Much of the work concerning insect response to altered glutathione levels, induced by air pollution, has been conducted by Hughes and his co-workers (see review, Hughes 1988) and has been conducted with sulphur dioxide. For example Chiment *et al* (1986) found that the glutathione levels in soybean (*Glycine max*) varied with $SO_2$ in the same way as did the response of Mexican bean beetle (*Epilachna varivestis*). When the foliar glutathione level was artificially increased by allowing the leaves to imbibe a solution of glutathione through their petioles, all of the changes related to insect growth and reproduction observed on fumigated plants were also produced (Hughes and Chiment, 1988). This, however, is not a general effect since the foliar glutathione concentration increased in *Phaseolus vulgaris* exposed to $SO_2$, but the success of the Mexican bean beetle was not affected on these plants (Hughes *et al*, 1981). The growth, rate of development, and survivorship of cabbage loopers (*Trichoplusia ni*) was also unaffected when larvae were reared on soybean leaves artificially augmented with glutathione (Hughes, 1988). No studies could be found which directly investigated the response of insects to altered glutathione levels due to ozone fumigation.

## 4.1.3.2 Alteration of Plant Biochemistry due to Nitrogen Oxides

In the environment it is difficult to attribute effects of nitrogen oxide pollution to either NO or $NO_2$ since they frequently co-occur (see chapter 1). Most studies either consider the effects due to $NO_2$ alone, or to $NO_2$ and NO together. Far fewer studies exist, than

for ozone, concerning the effects of $NO/NO_2$ pollution on plant biochemistry and related effects on insect populations. No studies could be found which related nitrogen oxide pollution to levels of glutathione, and as Rowland *et al* (1988) stated: 'The effects of nitrogen oxides on sulphur metabolism is an area about which virtually nothing is known.'

## 1a Effects of Nitrogen Oxides on Nitrogen Levels

Nitrogen oxide pollution differs from ozone pollution since under certain situations it can have a beneficial role in plant metabolism, especially where the nitrogen supply is limiting (Hughes 1988, Wellburn *et al*, 1980). Rowland (1986) demonstrated that barley exposed to $NO_2$, at 300 ppb for 9 days, had a higher nitrogen and amino acid content than controls when under nitrogen stress, but a lower content when the nitrogen supply to the roots was adequate. The water extractable protein levels were, however, unaffected by exposure to $NO_2$. The implication is that the extra nitrogen arises from the atmosphere, but the possibility that it is the result of stimulation of uptake of endogenous nitrate, or increased rate of translocation of nitrogen compounds from roots to above ground parts, must not be discounted (Troiano and Leone, 1977). However, Rowland (1986) showed that $^{15}N$ was present in barley 24 hours after exposure of the leaves to $^{15}NO_2$, indicating that it can indeed be absorbed through the aerial parts. Yoneyama *et el* (1980) demonstrated that $NO_2$ can be incorporated through the leaves of sunflower, kidney bean and corn, and that a small proportion is taken up through the roots, once the soil is saturated. Utilisation of atmospheric $NO_2$ arises since it can react in extracellular water to form nitrite and nitrate ions, which can be used by the plant in some of the normal reactions of nitrate metabolism. Srivastava and Ormrod (1984) also demonstrated that nitrogen oxides were assimilated into organic compounds when *Phaseolus vulgaris* plants had a shortage of inorganic nitrogen from the soil. They demonstrated an increase in organic nitrogen level following fumigation with 20, 100 and 500 ppb for 7 days, in seedlings supplied with no external nitrogen. Troiano and Leone (1977) found an increase in total nitrogen levels in the leaves of tomato (*Lycopersicon esculentum*) plants, following exposure to 365 ppb for 164 hours. The effect persisted for at least 48 hours following the end of the fumigation, but was less apparent for plants which had received a sub-optimal supply of nitrate to the roots. Okano and Totsuka (1986) found a similar effect with sunflower plants (*Helianthus annus L.*) fumigated with $NO_2$ for 7 days with 300 ppb.

Takeuchi *et al* (1985) found that the leaf content of rapidly metabolized amino acids (e.g. aspartic acid, glutamic acid and alanine) was increased in squash (*Cucurbita maxima* Duch.) seedlings following exposure to $NO_2$ for 4 hours at 400 ppb. Murray and Wellburn (1985) found that the total leaf content of amino acid was unchanged for two varieties of tomato (*L. esculentum*), and two varieties of pepper (*Capsicum annuum*) following fumigation with $NO_2$ at 150 ppb for 18 hours. However, the amine (asparagine, glutamine and serine) content for both species was significantly increased. Rowland (1988) pointed out that such increases may be the result of increased protein degradation, an increase in nitrate assimilation or a decrease in transport out of the leaf.

An increase in total nitrogen is however, not a general effect. Elkiey and Ormrod (1981) fumigated petunia (*Petunia hybrida* Vilm.) with 80 ppb $NO_2$ for 4 hours for 4 days, and found a significant decrease in the total nitrogen content of the leaves. They could offer no explanation for this effect. Taylor and Eaton (1966) reported that the total nitrogen content of tomato (*L. esculentum*) seedlings was unaffected by $NO_2$ treatment. This followed continuous fumigation for 19 days at very high levels (up to 520 ppb) and was accompanied by visible damage. Rowland (1986) stated that it appears that the beneficial effect of atmospheric $NO_2$ on plant growth can be reversed if the $NO_2$ concentration is too high.

Only two studies could be found which directly investigated the effects of NO on plant biochemistry. Wellburn *et al* (1980) found that certain cultivars of tomato (*L. esculentum*) may benefit from low levels of NO, since it can be reduced and incorporated into the plant tissues. Work conducted in the same laboratory (Murray and Wellburn, 1985) also demonstrated that the amine (asparagine, glutamine and serine) content of one variety of tomato (cv. Eurocross BB) was significantly increased following fumigation for 18 hours at 150 ppb. However, the levels remained unchanged in a second variety of tomato and in a variety of pepper (*Capsicum annuum*). The total level of amino acid remained unchanged in both species. At high levels of NO deleterious effects occur, such as reductions in photosynthesis and crop yield.

### 1b Relationship Between Nitrogen Level and Insect Performance

Almost all the work relating the response of insects to changing nitrogen levels in the host plant, induced by nitrogen oxides, has been conducted by Fluckiger and his co-workers. The studies have been conducted at motorway verges, where nitrogen oxides are the primary pollutants. The studies are unable to separate the effects due to NO and $NO_2$, or to other vehicle pollutants. Braun and Fluckiger (1985) demonstrated an increased infestation of *Aphis pomi* on *Crataegus* species in ambient motorway air (exact pollutant levels were not given), and related this to a significant increase in glutamine relative to sugar content, in phloem exudate. The total level of free amino acids was not increased, but there was a significantly higher level of total nitrogen in the ambient air plants following 10 weeks of exposure. Bolsinger and Fluckiger (1987) demonstrated an increased population of *Aphis fabae* on two different host plants, *Viburnum opulus* and *Phaseolus vulgaris*, in ambient roadside air, compared with filtered air controls. They found increases in the total foliar nitrogen levels in the ambient air plants, especially for bean seedlings which had been grown on nitrogen deficient vermiculite. Phloem exudate also contained significant increases in the amount of free amino acids, for both hosts, in the plants growing in ambient air. They later repeated this work (Bolsinger and Fluckiger, 1989) and found similar results. The analysis of phloem sap for individual amino acids demonstrated increases for all amino acids (except tryptophan and serine in bean plants). They translated these changes into artificial diets and found similar increases in aphid performance (see introduction to chapter 5). These results strongly suggest that nitrogen oxides are responsible for the increases in foliar nitrogen, but the authors point out the possibility that the plants in the ambient air were prematurely senesced, due to the pollution stress, resulting in an increased protein degradation, remobilisation of organic nitrogen, and hence increased content of free amino acids in the phloem exudate. Port and Thompson (1980) conducted similar work in Britain and found slight increases in soluble amino acids, and considerable increases in total protein content, of leaves close to a motorway. This was associated with outbreaks of Buff Tip Moth, *Phalera bucephala* on *Fagus sylvatia*, and Gold Tail Moth, *Euproctis similis* on *Crataegus monogyna.*

### 2a Nitrogen Oxide Effects on Sugars Levels

Little work appears to have been undertaken concerning the direct effect of nitrogen dioxide fumigation on sugar levels. Ito *et al* (1985) found increases in glucose, fructose and sucrose in *Phaseolus vulgaris* following fumigation with $NO_2$ at 2000 ppb.

### 2b Relationship Between Sugar Level and Insect Performance

The only study which could be found was conducted by Fluckiger *et al* (1978). He correlated increasing aphid numbers on hawthorn with increasing levels of soluble sugars along a gradient of increasing vicinity to a motorway.

## 4.2 Materials and Methods

In chapter 2 a significant difference in aphid relative growth rate was observed following three different pollutant regimes;

1) Wheat fumigation with $O_3$, at 100 ppb, for 72 hours,
2) Wheat fumigation with $NO_2$, at 100 ppb, for 48 hours,
3) Wheat fumigation with NO, at 100 ppb, for 48 hours.

In this study plants were grown, exactly as in chapter 2, and exposed to these regimes again. All the fumigations were conducted in the winter months, so the temperature within the chambers was 20°C during the day and 15°C at night. The other fumigation conditions were as in chapter 2. Approximately half of the plants were harvested immediately following fumigation, and the other half after a delay of four days ($O_3$ fumigation) or two days ($NO_2$ and NO fumigations). The plants were returned to the growing-up greenhouse for the duration of this delay. The total above ground parts were harvested. They were freeze dried for at least 3 days and then ground in a Glen Creston ball mill for 10 minutes. This powder was then analysed for the following constituents:

1) Soluble Nitrogen
2) Total Nitrogen
3) Reducing Sugars
4) Total Soluble Sugars
5) Glutathione
6) Amino Acids (immediately following ozone fumigation only).

The carbon : nitrogen (C : N) ratio was calculated using the reducing sugar and soluble nitrogen values.

| Fumigation Regime | No. of Plants Used | |
|---|---|---|
| | Immediate Harvest | Delayed Harvest |
| Ozone | 30 | 26 |
| Nitrogen Dioxide | 30 | 23 |
| Nitric Oxide | 20 | 20 |

### 4.2.1 Nitrogen Analysis

Two methods were used in the determination of total nitrogen content since new improved equipment was purchased and therefore used in later determinations. The old method of analysis was used for all the soluble nitrogen determinations. In both methods a Kjedahl digest was first prepared and then analysed.

**a) Total Nitrogen**

**Old Method**

Approximately 60mg of dried plant material was accurately weighed into a Kjedahl flask. 2ml of concentrated nitrogen-free sulphuric acid and a selenium catalyst tablet were added, and the flask heated over a gas burner, gently at first, and later more strongly. The flask was gently shaken at intervals, and when the liquid retained a permanent straw colour it was allowed to cool. The digest was transferred to a volumetric flask and made up to 50ml with distilled water. About 10ml was retained for analysis, and the rest discarded. The analysis was undertaken using a Technicon Auto-analyser (Model no. AA1), as described by Broodbank (1984). This measured the concentration of ammonium ions (in ppm) using the indo-phenol technique described in detail in Varley (1966). The standards were derived from a solution of ammonium sulphate ($(NH_4)_2SO_4$). Two blanks were made, comprising acid and catalyst tablet only, and the results were subtracted from the sample values.

**New Method**

Approximately 60mg of dried plant material was accurately weighed in a large boiling tube. 2ml of nitrogen-free concentrated sulphuric acid and a selenium catalyst tablet were added. This was digested using a heating block, (Tecator 1016 Digestion System) initially at a low heat, for about half an hour, and then at approximately 400°C. Digestion was complete when the samples retained a straw yellow colour when shaken. The

samples were then allowed to cool and were analysed using a Tecator Kjeltec Auto 1030 Analyser. Two blanks were made, and ammonium sulphate standards were again used.

**Cross Calibration of the Two Methods**

The total nitrogen determination was carried out twice for one set of samples. This was the the delayed harvest of the ozone fumigated plants.

**b) Soluble Nitrogen**

An extract of the soluble nitrogen fraction was prepared according to the method of van Emden and Bashford (1969): Approximately 100mg of the plant powder was accurately weighed into a 5cm x 2.5cm polytop tube. 2ml of a solution of 0.02% phenol and 2.5% trichloroacetic acid was added and the mixture shaken for 10 hours. This was transferred to an Eppendorf tube and centrifuged for 5 minutes in a Quickfit microcentrifuge at 5000 r.p.m. 1ml of the supernatant was then transferred to a Kjedahl flask, and the determination of soluble nitrogen content was then conducted as for the total nitrogen. 2 blanks were digested using 1ml of the phenol and trichloroacetic acid solution and the results subtracted from the samples.

## 4.2.2 Sugar Analysis

**a) Reducing Sugars**

This is the method used by Aminu-Kano (1987) which is a modification of the method of Kozoil and Cowling (1980) and is evaluated in detail by Kozoil (1981). Approximately 20mg of dried plant material was accurately weighed into a test-tube. 8ml of a 50% ethanol/water solution was added, and the tube placed in a water bath, at 80°C for 2 hours. The tubes were then centrifuged at 4000 r.p.m for 15 minutes and the supernatant retained in a 2.5 x 5cm polytop tube. 8ml of distilled water was added to the plant material in the tubes, and returned to the 80°C water bath for a further hour. They were then centrifuged in the same way and the supernatant added to the previous one. 1ml of this extract was added to 2ml of PAHBAH reagent (made with 2% w/v p-hydroxybenzoic acid hydraxide, 0.5M hydrochloric acid and 3M sodium hydroxide solution), in a test-tube, agitated on a vortex mixer, and placed in a boiling water bath for exactly 5 minutes for the colour reaction to occur. The reaction was stopped by placing the tubes in ice for about 2 minutes, and the absorbances of the solutions read at 410nm, on a spectrophotometer (Cecil

Instrument 588 Microcomputer scanning spectrophotometer). Reducing sugar values were calculated with reference to glucose standards. Three blanks were prepared which received the same treatment, without the inclusion of plant material.

**b) Total Soluble Sugars**

The same sugar extraction procedure was used as above. 3ml of the 16ml of extract was added to 3ml of 0.2M hydrochloric acid in a test-tube. This was transferred to a 40°C water bath for a further 2 hours. 1ml of this solution was added to 2ml of PAHBAH reagent and the analysis continued as above.

### 4.2.3 Glutathione

The technique used was that of Sandman and Gonzales (1989), with modifications by Sandman (1989, pers. comm.) The determination is for the total quantity of free SH groups, but since at least 95% of this is comprised by reduced glutathione (Grill *et al*, 1979) the term 'glutathione' is used to mean 'total free SH groups'. 20mg of dried plant powder was mixed in an Eppendorf tube with 1ml of a solution of 3% m-phosphoric acid and 3% sodium acetate, adjusted to pH 5. This was vortexed for 20 seconds, left for 5 minutes, and vortexed for a further 20 seconds. The sample was then centrifuged in a Quickfit microcentrifuge at 5000 r.p.m for 5 minutes. 50µl of the supernatant was transferred to a second Eppendorf tube containing 950µl of 0.1M sodium phosphate buffer, adjusted to pH 7, containing 30µg $ml^{-1}$ of 5,5-dithiobisnitrobenzoic acid. This was vortexed for 10 seconds and left for 60 seconds before the absorbance was read at 412nm using the spectrophotometer, as above.

### 4.2.4 Amino Acids

The extraction and analysis procedures used here are as described by van Emden and Bashford (1971), although different quantities of reagents are used. Approximately 150mg of dried plant material was accurately weighed into a 2.5 x 5cm polytop tube and 2ml of pH 2.2 citric acid buffer added. This consisted of the following constituents, made up to 1000ml with distilled water:

21g citric acid
8.4g sodium hydroxide
16ml concentrated hydrochloric acid
0.1g sodium octoate
5ml thiodiglycol

The tube was shaken for 12 hours, the contents transferred to an Eppendorf tube, and centrifuged for 5 minutes in a Quickfit microcentrifuge. The supernatant was transferred to a 1 x 5cm polytop tube and stored at -20°C, before analysis using an amino acid analyser (Model Mk.4, Locarte Company, London). Asparagine and glutamine could not be separated because they are eluted simultaneously from the Zeocarb resin column, and they were therefore designated as 'amides'. The results were compared with a standard amino acid solution of known composition and concentration, obtained from Sigma.

### 4.2.5 Carbon : Nitrogen Ratio

This was calculated by dividing the reducing sugar values by soluble nitrogen values. This is of potential importance to the aphid.

## 4.3 Results

All the bars shown on all the figures in these and subsequent diagrams are standard errors (plus and minus 1).

### 4.3.1 Nitrogen Content

#### 1) Following Ozone Fumigation

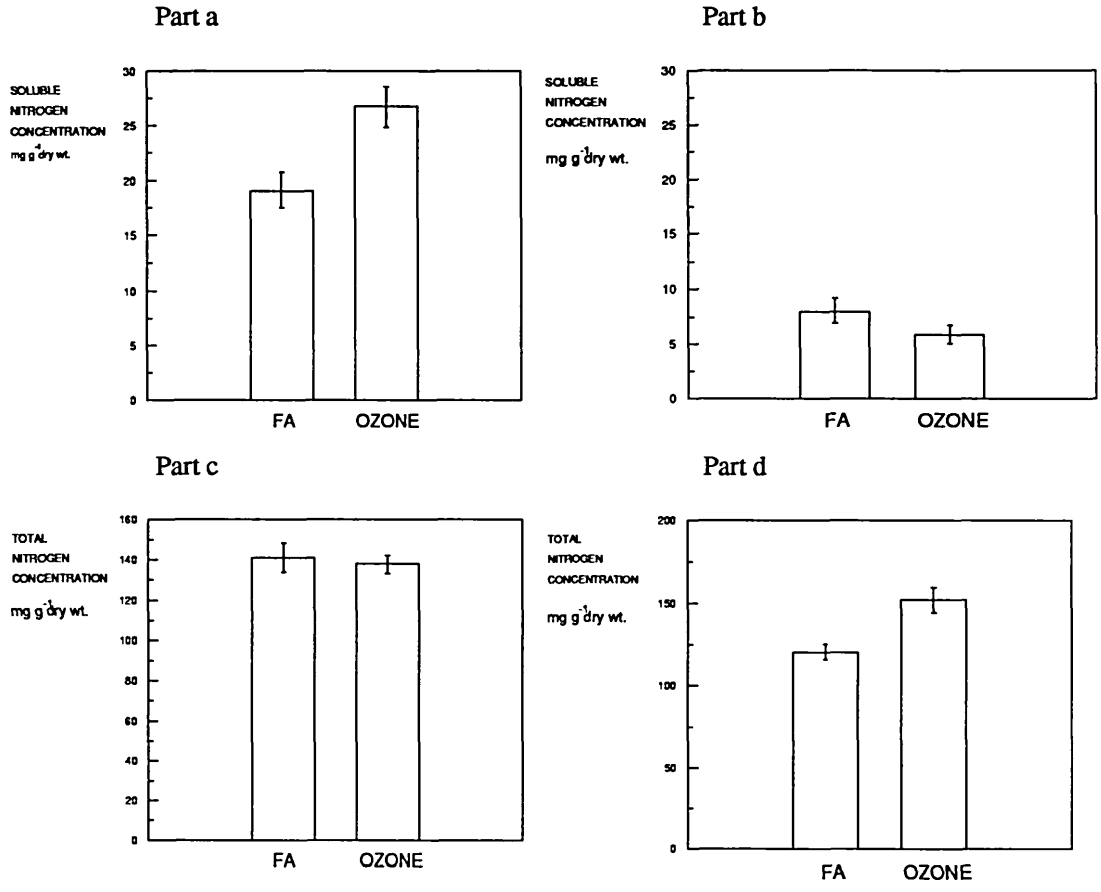


**Figure 4.1 Nitrogen Content Following Ozone Fumigation for 72 hours:**

**Part a** **Soluble Nitrogen Content Immediately Following Fumigation**
**Part b** **Soluble Nitrogen Content Four Days after end of Fumigation**
**Part c** **Total Nitrogen Content Immediately Following Fumigation**
**Part d** **Total Nitrogen Content Four Days after end of Fumigation**

A significant increase in soluble nitrogen concentration is seen immediately following ozone fumigation, ($p < 0.036$, 28.8% increase), but this is not apparent four days later, when the levels are very much reduced, and a non-significant decrease is observed. The total nitrogen levels are not significantly different immediately following

fumigation, but are significantly increased four days later ($p < 0.0002$). These delayed harvest total nitrogen values were determined using the new method of calculation. They were also determined using the old method, and the ozone and filtered air values were then found to be not significantly different. A comparison of the results from the two methods demonstrated, however, that they were not significantly different (see below).

## Cross Calibration of the Old and New Analysis Methods

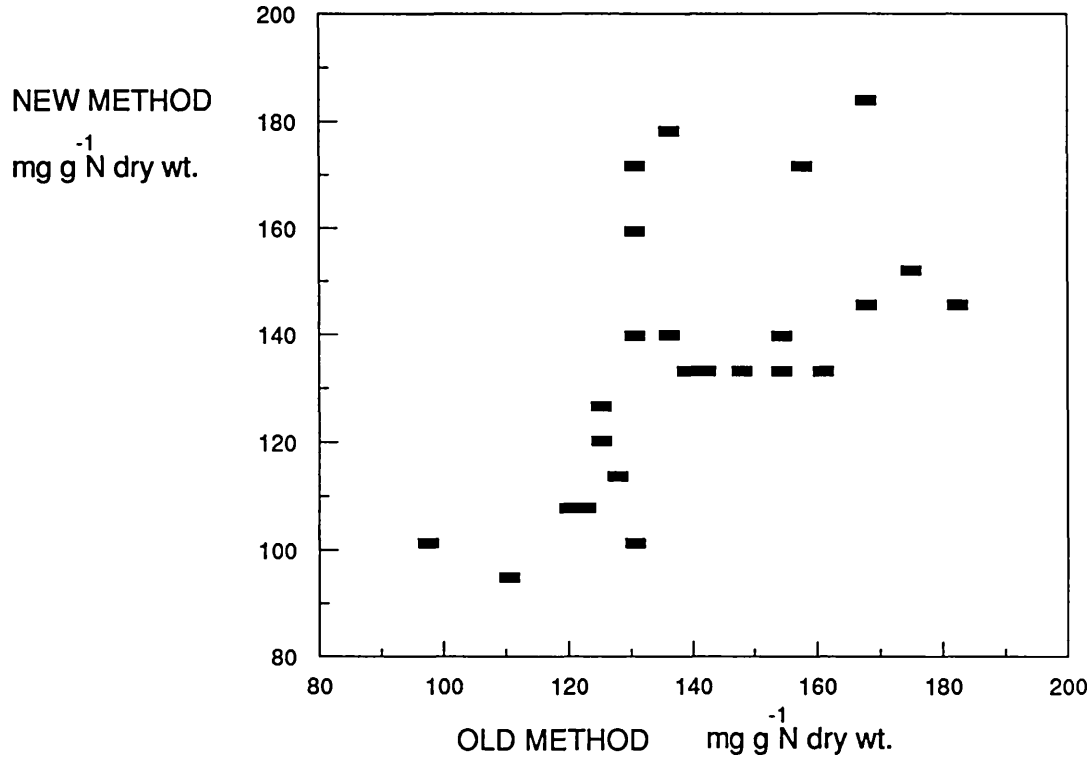


**Figure 4.2** **Correlation of the Old and New Analysis Methods. This Shows the Total Nitrogen Values for the Delayed Harvest Ozone Fumigation.**

There is a significantly positive correlation coefficient between the two methods, with $p < 0.001$. There is also no significant difference between the results obtained from the two methods ($p < 0.55$).

## 2) Following Nitrogen Dioxide Fumigation

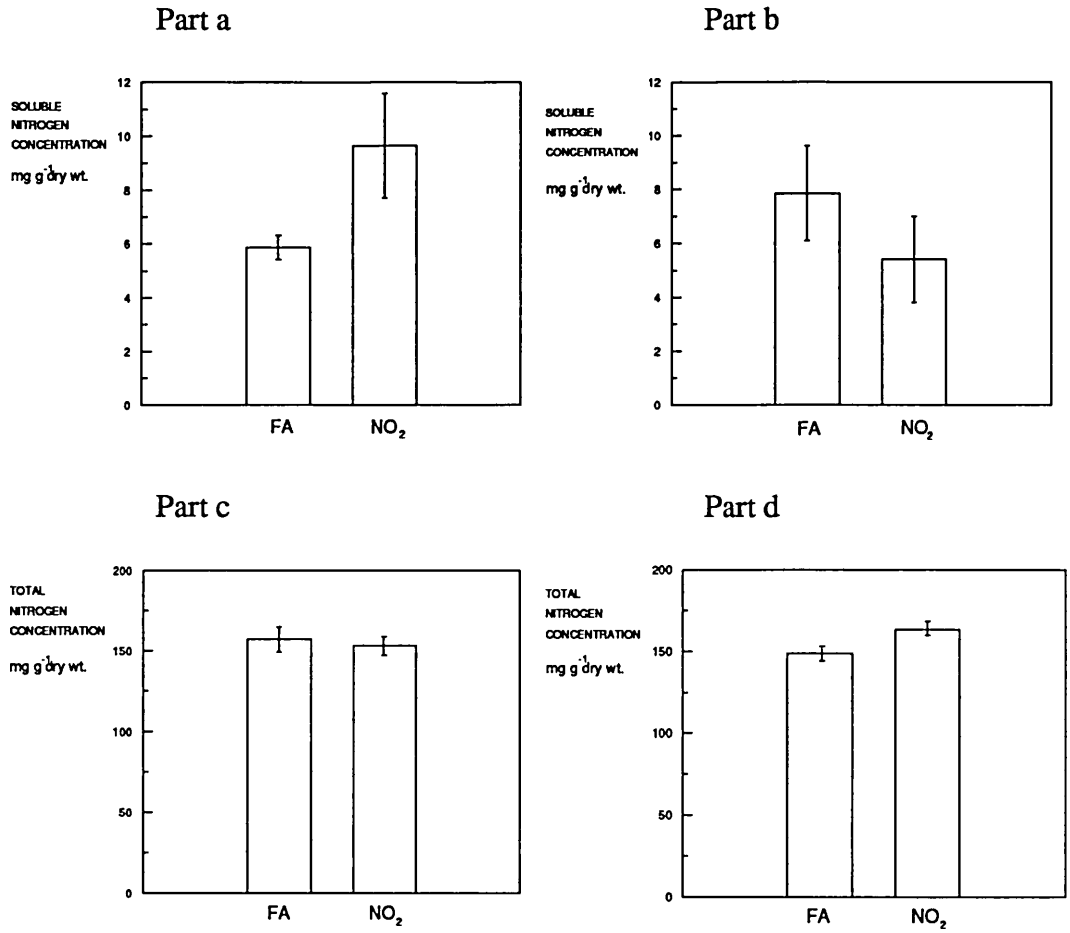


**Figure 4.3** **Nitrogen Content Following Nitrogen Dioxide Fumigation for 48 Hours:**

**Part a** **Soluble Nitrogen Content Immediately Following Fumigation**

**Part b** **Soluble Nitrogen Content Two Days after end of Fumigation**

**Part c** **Total Nitrogen Content Immediately Following Fumigation**

**Part d** **Total Nitrogen Content Two Days after end of Fumigation**

An almost significant increase in soluble nitrogen concentration is seen following $NO_2$ fumigation, ($p < 0.07$, 39.1% increase), but this is not apparent just two days later. There is no significant difference in total nitrogen concentration. The total nitrogen value two days following fumigation was calculated using the new method.

## 3) Following Nitric Oxide Fumigation

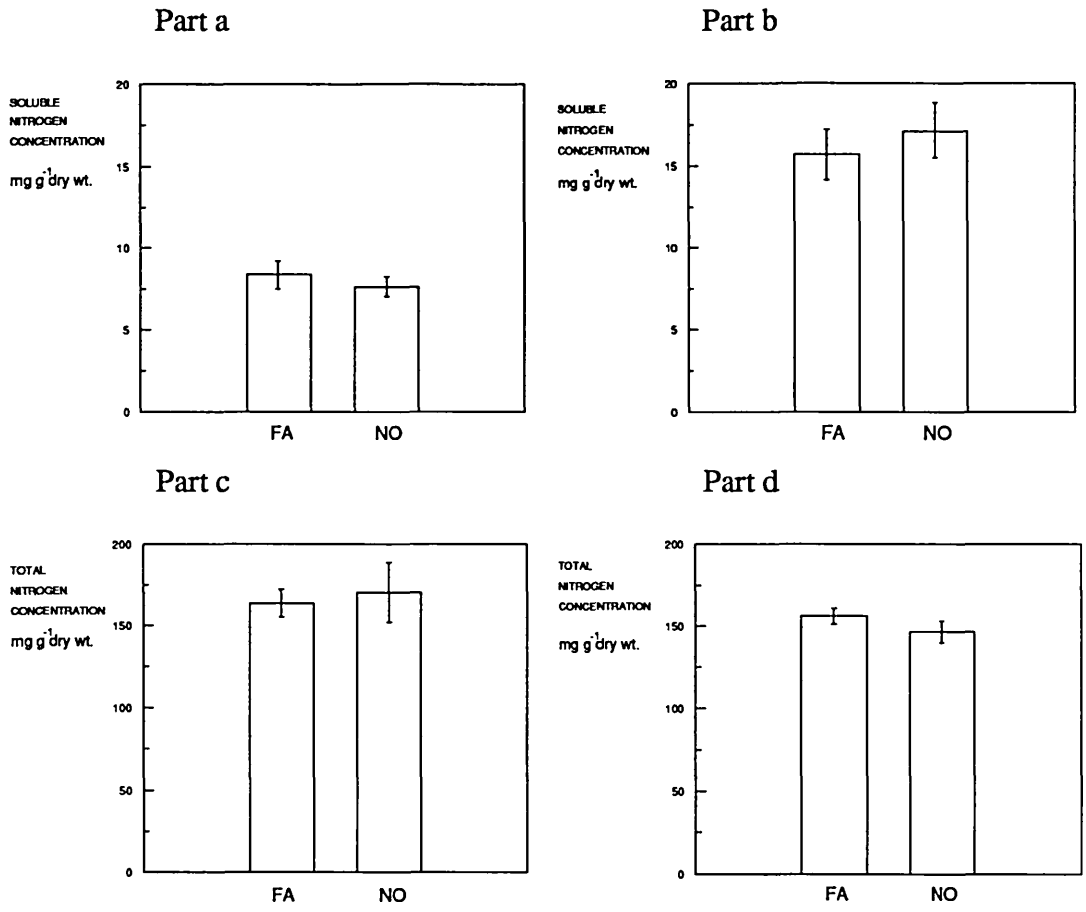


**Figure 4.4** **Nitrogen Content Following Nitric Oxide Fumigation for 48 Hours:**

**Part a** **Soluble Nitrogen Content Immediately Following Fumigation**
**Part b** **Soluble Nitrogen Content Two Days after end of Fumigation**
**Part c** **Total Nitrogen Content Immediately Following Fumigation**
**Part d** **Total Nitrogen Content Two Days after end of Fumigation**

There is no significant difference in either the soluble or total nitrogen content both immediately following or two days after fumigation. The total nitrogen value two days following fumigation was calcul ated using the new method.

## 4.3.2 Sugar Content

### 1) Following Ozone Fumigation

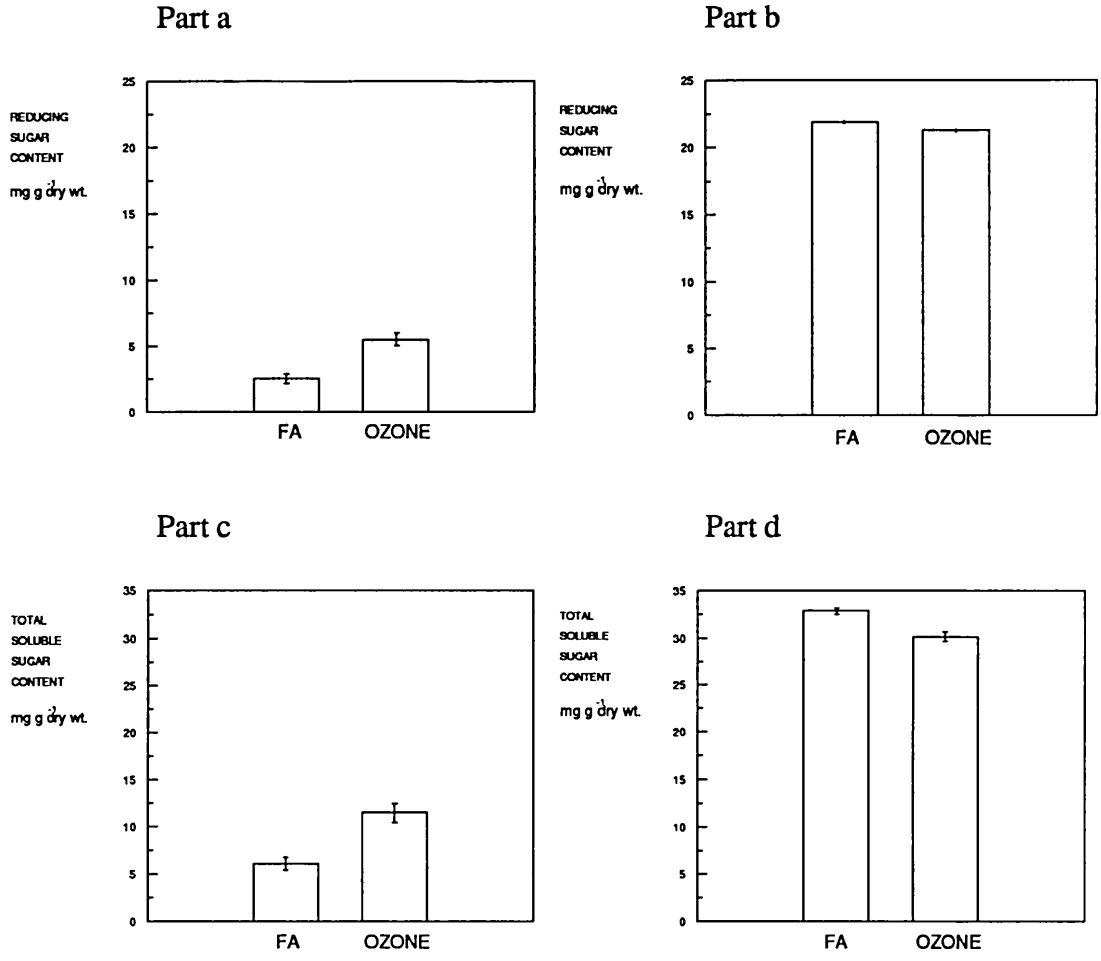


**Figure 4.5 Sugar Content Following Ozone Fumigation for 72 hours:**

**Part a** **Reducing Sugar Content Immediately Following Fumigation**
**Part b** **Reducing Sugar Content Four Days after end of Fumigation**
**Part c** **Total Sugar Content Immediately Following Fumigation**
**Part d** **Total Sugar Content Four Days after end of Fumigation**

Both the reducing and total soluble sugar levels are low immediately following fumigation, but there is a significant increase in levels in both parameters ($p < 0.0001$, and $p < 0.0003$, respectively) in plants exposed to ozone. Four days after the end of fumigation the levels were substantially increased and the effect reversed. Significant decreases in ozone were observed for both parameters, ($p < 0.0084$ for reducing sugars and $p < 0.0001$ for total soluble sugars).

## 2) Following Nitrogen Dioxide Fumigation

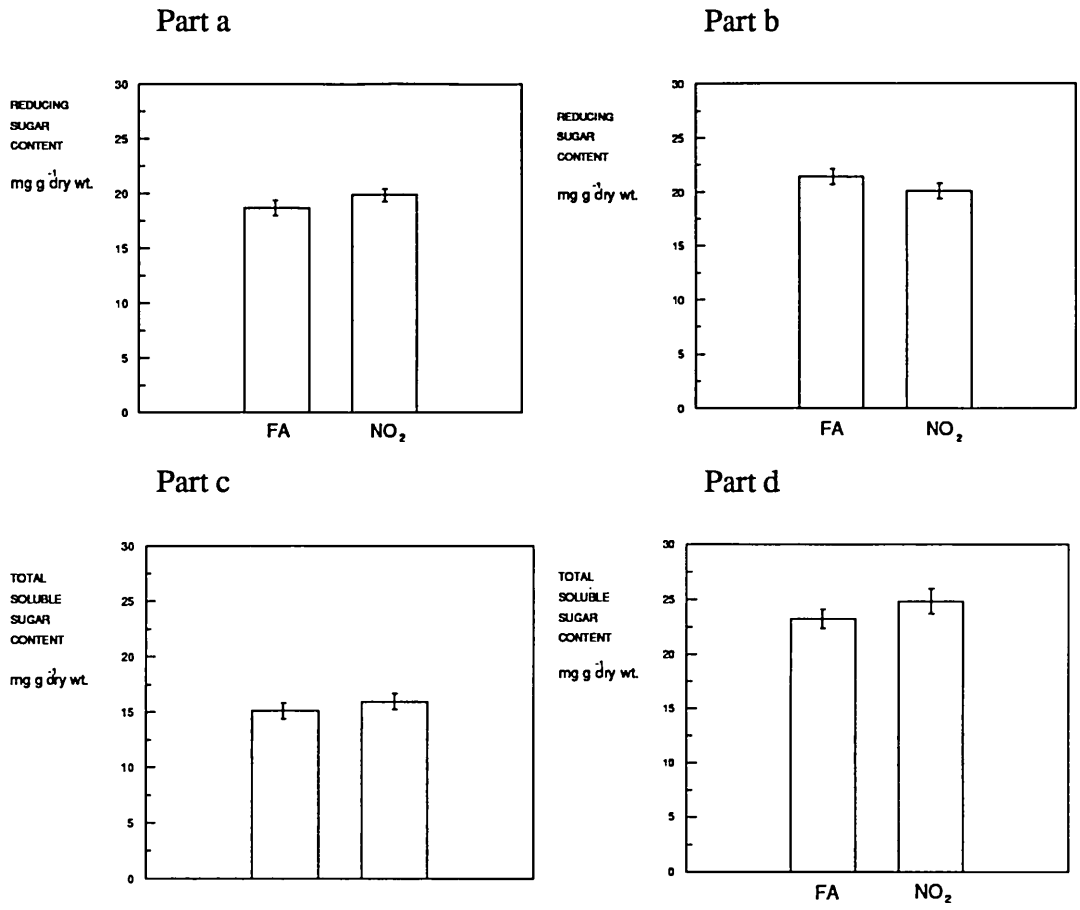


**Figure 4.6** **Sugar Content Following Nitrogen Dioxide Fumigation for 72 hours:**

**Part a** **Reducing Sugar Content Immediately Following Fumigation**
**Part b** **Reducing Sugar Content Two Days after end of Fumigation**
**Part c** **Total Soluble Sugar Content Immediately Following Fumigation**
**Part d** **Total Soluble Sugar Content Two Days after end of Fumigation**

A non-significant increase in reducing sugar levels was seen immediately following fumigation, which became a non-significant decrease two days later. A non-significant increase in levels of total soluble sugar was observed, both immediately following and four days after the end of the fumigation. The levels of total soluble sugar immediately following fumigation were, however, recorded as lower than the levels of reducing sugars alone. This anomaly must be a result of experimental error such as incomplete hydrolysis of the non-reducing sugars. Since the technique used in this analysis is precisely as the others it may indicate that less than complete hydrolysis also occurred

in the other analyses. Further investigations into the ideal acid concentration and duration of hydrolysis are therefore required, and the total soluble sugar results should be viewed as comparisons only.

### 3) Following Nitric Oxide Fumigation

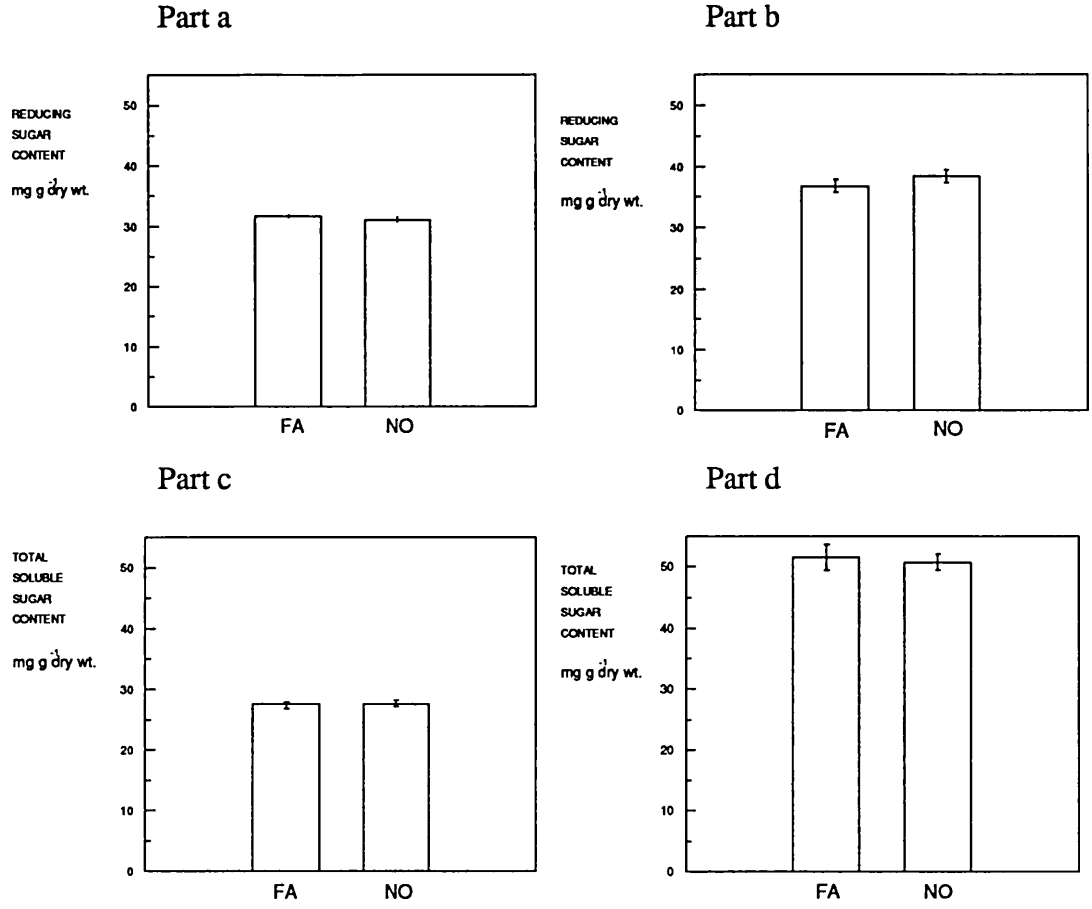


**Figure 4.7 Sugar Content Following Nitric Oxide Fumigation for 72 hours:**

**Part a** **Reducing Sugar Content Immediately Following Fumigation**
**Part b** **Reducing Sugar Content Two Days after end of Fumigation**
**Part c** **Total Soluble Sugar Content Immediately Following Fumigation**
**Part d** **Total Soluble Sugar Content Two Days after end of Fumigation**

No significant differences were observed both immediately following fumigation and two days later, in both reducing and total soluble sugar levels. A similarly reduced level of total soluble sugars compared with reducing sugars was observed immediately following fumigation which again indicated that the total soluble sugar levels should be viewed as comparisons only.

## 4.3.3 Glutathione

### 1) Following Ozone Fumigation

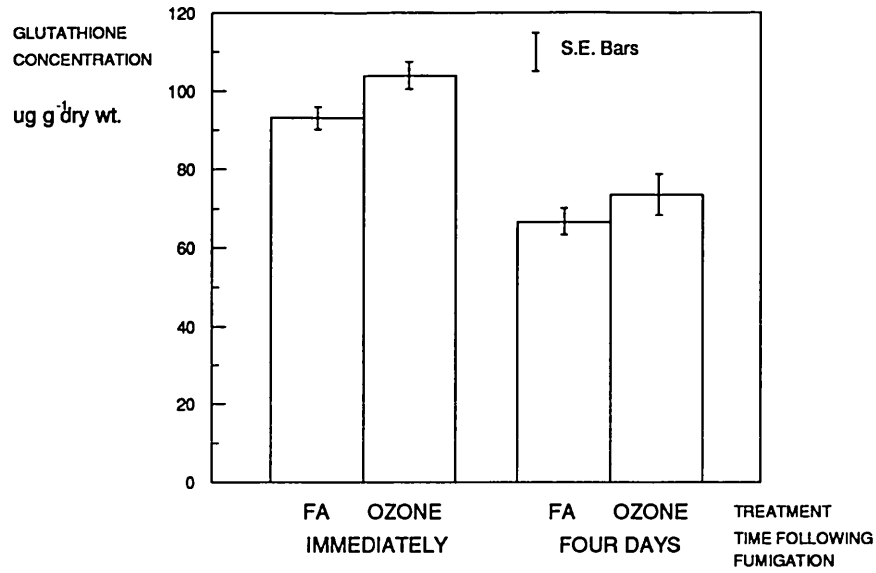


**Figure 4.8** **Glutathione Concentration in Plants both Immediately Following and Four Days after Fumigation with Ozone (at 100 ppb) or Filtered Air (FA) for 72 hours.**

A significant 10.4% increase ($p < 0.029$) in glutathione concentration in plants fumigated with ozone is seen immediately following fumigation, compared with plants in filtered air. Four days later the overall levels are decreased but the increase in levels in ozone is still apparent (9.7%), although this is not significant.

## 2) Following Nitrogen Dioxide Fumigation

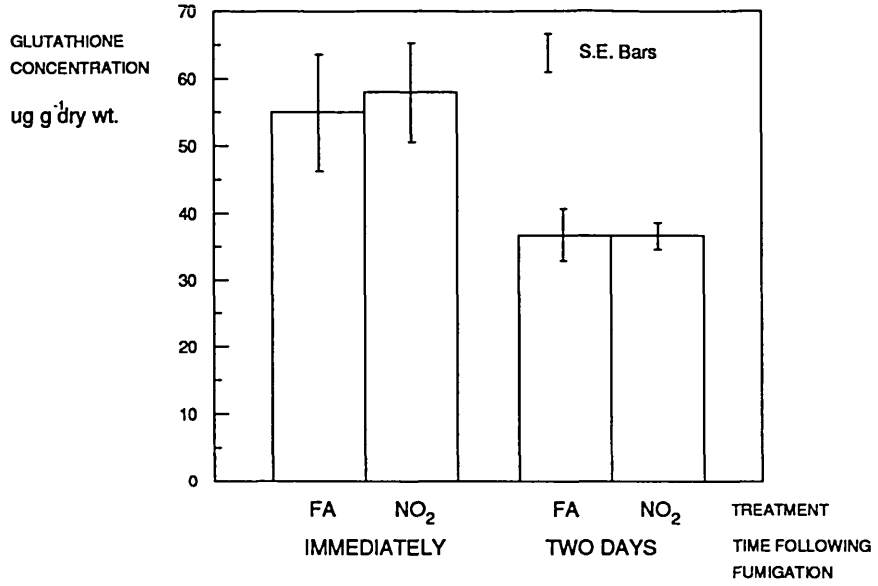


**Figure 4.9** **Glutathione Concentration in Plants both Immediately Following, and Two Days after Fumigation, with $NO_2$ (at 100 ppb) or Filtered Air (FA) for 48 hours.**

There is no significant difference in the glutathione levels either immediately following or two days after the end of the fumigation. A 5.4% increase in levels immediately following fumigation is not apparent two days later.

### 3) Following Nitric Oxide Fumigation

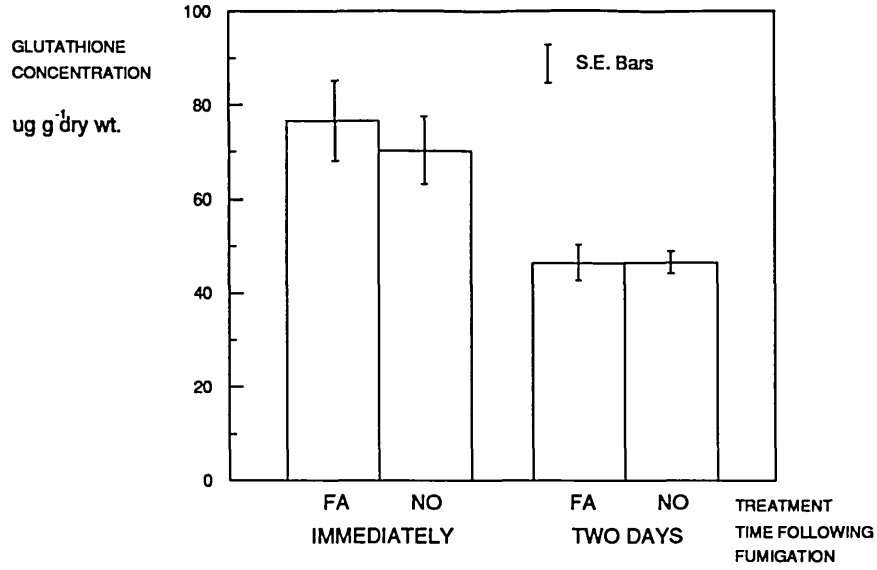


**Figure 4.10** **Glutathione Concentration in Plants both Immediately Following, and Two Days after Fumigation, with NO (at 100 ppb) or Filtered Air (FA) for 48 hours.**

There is no significant difference in the glutathione levels either immediately following or two days after the end of the fumigation. A 6.3% decrease in levels immediately following fumigation is not apparent two days later.

### 4.3.4 Amino Acids

**Table 4.1** **Free Amino Acid Content Immediately Following Fumigation with Either Ozone or Filtered Air for 72 hours.**

| Amino Acid | Weight (nM/mg) | | % + or - in $O_3$ | Sig. Level |
|---|---|---|---|---|
| | FA | $O_3$ | | |
| Aspartic Acid | 0.802 | 0.794 | - 1.0% | NS |
| Threonine | 1.268 | 1.254 | - 1.1% | NS |
| Amides | 1.899 | 2.087 | + 9.0% | NS |
| Glutamine | 2.175 | 2.358 | + 7.8% | NS |
| Proline | 0.307 | 0.343 | + 10.5% | NS |
| Glycine | 0.188 | 0.249 | + 24.5% | * |
| Alanine | 1.579 | 1.924 | + 17.9% | NS |
| Cysteine | 0.104 | 0.202 | + 48.6% | *** |
| Valine | 0.733 | 0.966 | + 24.1% | NS |
| Methionine | 0.308 | 0.641 | + 51.9% | *** |
| Isoleucine | 0.348 | 0.526 | + 17.8% | * |
| Leucine | 0.100 | 0.073 | - 40.0% | NS |
| Tyrosine | 0.377 | 0.428 | + 11.8% | NS |
| Phenylalanine | 0.377 | 0.628 | + 40.0% | NS |
| GABA | 0.415 | 0.476 | + 12.8% | NS |
| Histidine | 0.326 | 0.467 | + 30.1% | NS |
| Lysine | 0.238 | 0.392 | + 39.3% | NS |
| Arginine | 0.290 | 0.518 | + 43.9% | NS |
| Total weight amino acid per sample (n = 24) | 12.60 | 16.00 | + 21.1% | * |

Key: NS Non significant
* Significant at $p < 0.05$
*** Significant at $p < 0.001$

An overall significant increase in levels of amino acids was observed in plants exposed to ozone ($p < 0.04$). Non-significant decreases in levels of three amino acids were

observed (aspartic acid, threonine and leucine), but all others were increased. Significant increases in levels were observed for glycine, cysteine, methionine and isoleucine.

### 4.3.5 Carbon : Nitrogen Ratio

#### 1) Following Ozone Fumigation

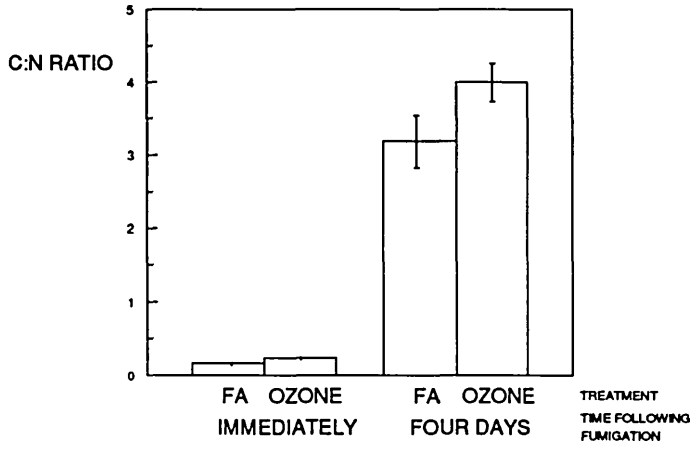


**Figure 4.11** **The Carbon to Nitrogen Ratio both Immediately Following and Four Days after Fumigation with either Ozone or Filtered Air.**

This shows a very large increase in the overall C : N ratio four days following fumigation, compared with levels immediately after fumigation. This indicates the large fluxes in metabolites possible following fumigation. There is an indication of an increase in the ratio in the ozonated plants, both immediately ($p < 0.083$) and four days after the end of the fumigation ($p < 0.080$).

### 2) Following Nitrogen Dioxide Fumigation

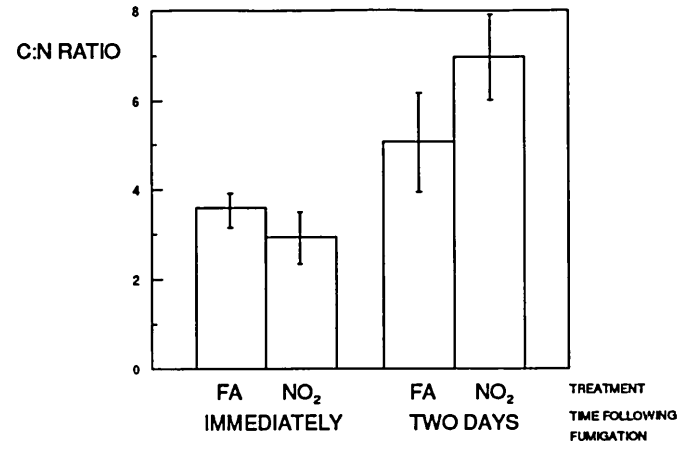


**Figure 4.12** **The Carbon to Nitrogen Ratio both Immediately Following and Two Days after Fumigation with either Nitrogen Dioxide or Filtered Air.**

This shows a non-significant decrease in the ratio immediately following fumigation, which becomes a non-significant increase two days later.

### 3) Following Nitric Oxide Fumigation

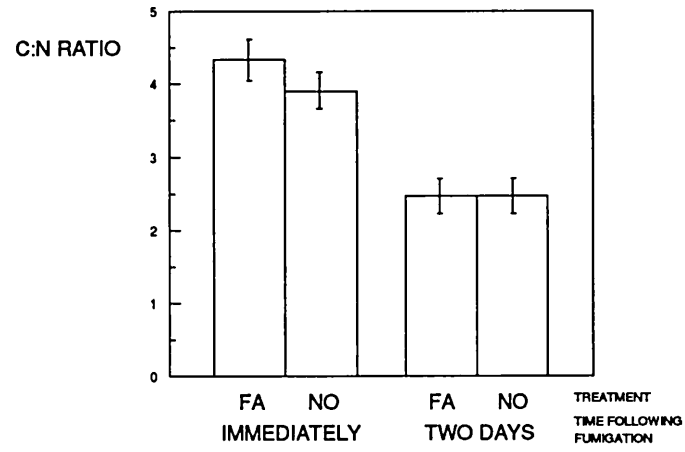


**Figure 4.13** **The Carbon to Nitrogen Ratio both Immediately Following and Two Days after Fumigation with either Nitric Oxide or Filtered Air.**

This shows a non-significant decrease immediately following fumigation, but levels were found to be exactly the same two days later.

## 4.4 Conclusions

The results for ozone are broadly consistent with the effects described in the literature. An increase in soluble nitrogen and amino acid levels immediately following fumigation

is a common theme in the literature, and is also found here. There is little consensus as to which particular amino acids are specifically altered following fumigation, but in this study the increase in the sulphur amino acids (methionine and cysteine) is notable. This is accompanied by the significant increase in glutathione (free SH groups) immediately following fumigation and is the basis of the investigations undertaken in the following chapter. The glutathione levels are still increased four days later (9.7% compared with 10.3% immediately following), but they are no longer significantly different.

The levels of soluble nitrogen immediately following ozone fumigation were larger than those observed for the other fumigations, which may be due to the stress imposed by the longer exposure period in the chambers. Four days after fumigation, the levels of soluble nitrogen had returned to levels comparable with results for the other pollutants. The increase in levels in ozone fumigated plants is no longer apparent, and there is in fact a non-significant decrease in levels. There is a slight decrease in total nitrogen levels immediately following ozone fumigation, which may indicate that the soluble nitrogen level increase is due to breakdown of the total nitrogen, perhaps through oxidation of the ozone sensitive amino acid residues. This effect is reversed four days later, when there is a significant ($p < 0.0002$) increase in levels in ozone. This is difficult to interpret in the light of the explanations offered in the literature. These values were also calculated using the old method, where although there was an increase in levels in ozone, this was not significant. This was probably because of the increased variance attributable to the residual error found for the old method of calculation.

The reducing and total soluble sugars are markedly decreased immediately following fumigation, compared with levels for the other pollutants. The reason for this is not immediately obvious, but may be due to a longer exposure period in the chambers, though this is only a possibility. There is a significant increase in reducing sugar levels in ozone which produces an overall increase (though non-significant) in the carbon : nitrogen ratio. This would be expected to be detrimental to the aphid and since this is not so, it appears that this parameter is either genuinely a non-significant result, or the parameter is unimportant in this case. It may also be that the increase in soluble nitrogen levels is sufficient to stimulate increased feeding and increased extraction efficiency, irrespective of the sugar levels, leading to the observed increase in aphid growth rate observed at this pollution regime.

The total soluble sugar levels immediately following fumigation are also significantly increased in ozone. The trend in sugar levels is reversed four days later, when significant decreases in both reducing and total soluble sugars are observed. This indicates that the pollution is still affecting the metabolism of the plant. It is possible to speculate that structural carbohydrates are initially broken down but over the course of four days are able to re-synthesise, but there is no evidence for this.

It is difficult to determine which of the above changes in metabolites promotes the significant increases in aphid growth rate. It may be that all the factors operating together are required, or it may be that one factor such as the increase in sulphur containing soluble molecules is sufficient. This is investigated in the following chapter.

A large but non-significant ($p < 0.07$) increase in soluble nitrogen level was observed immediately following $NO_2$ fumigation. This may be due to breakdown of total nitrogen, or absorption and metabolism of the pollutant through the leaves. This increase is not sustained two days later, when a non-significant decrease in levels is seen. This may be accounted for in the increase in total nitrogen level seen two days following fumigation, perhaps indicating incorporation of the soluble nitrogen. No difference in the total nitrogen level is seen immediately following fumigation.

The reducing sugar levels immediately and two days after fumigation appear not to be affected by the $NO_2$. The total soluble sugar levels are similarly unaffected. The trend is, however, similar to that found by Koziol (1984) in his review paper. He noted that a frequent response was an initial increase in carbohydrate levels, followed by a subsequent decrease, as observed here. The total soluble sugar levels immediately following fumigation were recorded as lower than the reducing sugar levels. This is obviously impossible and must be the result of an unexplained experimental error (see results). There is a non-significant ($p < 0.13$) 18% decrease in the C : N ratio immediately following fumigation which may explain the increase in aphid growth rate seen at this regime. However, this effect is not apparent 2 days later. There is no significant difference in the glutathione concentration either immediately following or two days after fumigation.

No significant differences were observed following NO fumigation for any of the parameters tested. The C : N ratio was non-significantly decreased by 10% immediately

following fumigation, but this was not sustained 2 days later. The biochemistry of the plants on which the aphids grew would probably have been different from the plants under investigation here, since the original fumigation was conducted at elevated temperatures - see chapter 2. This possibility should be investigated further before any conclusions can be drawn. The previous ozone and $NO_2$ fumigations were both conducted at temperatures comparable to those in chapter 2.

The increases in aphid growth rate following the $NO_2$ and NO fumigations may have been as a result of alterations in the proportions of amino acids. However, analysis was unfortunately not possible due to technical difficulties.

A result common to all three fumigations is the increased glutathione concentration immediately following fumigation in both polluted and filtered air, compared with the concentration two or four days later. This may be a stress effect due to exposure time in the chambers, which could be tested by analysis prior to exposure.

The marked changes in levels of metabolites following the delay before the second harvest, especially for the ozone fumigation, indicates the dynamic nature of the metabolite pools. A time course investigating biochemical changes at shorter intervals after the end of fumigation would provide important information. Since the disruption of metabolism appears to extend until at least four days after the end of the ozone fumigation (as seen clearly by the sugar levels), it would also be interesting to conduct delayed aphid growth rate studies. A critical change in metabolite levels at some stage following fumigation may produce a highly significant alteration in insect performance.

# Chapter 5
# Simulating Host Plant Biochemical Changes.

## 5.1 Introduction

Experiments reported in chapter 2 showed that aphid growth rates were increased on wheat plants which had been fumigated with ozone at 100 ppb for 72 hours. Later experiments, reported in chapter 4, showed that this treatment caused a significant increase in leaf contents of compounds containing sulphur i.e glutathione, cysteine and methionine. In this study a possible causal link between these results is investigated by simulating the plant biochemical changes. Two techniques are used:
1) Manipulation of the composition of artificial diet, as used in chapter 3.
2) Manipulation of the composition of a nutrient solution in the hydroponic growth of plants.

The artificial diet technique has previously been used only once to simulate plant biochemical changes due to air pollution, although many alterations have been made to diets to favour the growth of aphids on them. The work to simulate changes due to air pollution was done by Bolsinger and Fluckiger (1989). They observed an increase in almost all of the phloem sap amino acids in *Viburnum opulus* L. and *Phaseolus vulgaris* L. due to vehicle emissions from a motorway. These authors chose an artificial diet best suited to *Aphis fabae* and halved its amino acid composition to represent a 'filtered air' diet. To simulate the 'ambient air' diet they proportionally added to, or subtracted from, the 50% diet according to the proportions of amino acids in the polluted plants. They found a significantly higher weight at birth, a significantly increased MRGR, and a significantly shorter development time on the ambient air diets compared with filtered air diets. They did not, however, attain a MRGR on either diet as large as that on the original diet. This indicates that the 50% diet was inadequate, and consequently it is difficult to distinguish between the increased performance being due to the particular proportions of amino acids in the ambient air diet, or simply to the increased quantities of amino acids in this diet. A further consideration is the role of the midgut symbionts of the aphid. Studies have shown (e.g Mitler (1971a)) that aphids can grow on diets lacking a number of essential amino acids, but if the aphids are treated with antibiotics they lo se this ability, indicating that the symbionts play a role in the amino acid metabolism of these insects. The actual proportions of specific amino acids in artificial diets may therefore be of less importance than their total quantity.

Studies of the incorporation of sulphate and sulphur amino acids into artificial diets, and its effect on aphid performance have been made previously. Markkula and Laurema (1967) found that methionine significantly promoted *Acyrthosiphon pisum* development, and that sodium sulphate could be substituted in place of methionine and cysteine with no change in the results. They also found that the sulphur containing amino acids accelerated *A. pisum* development in the presence of sulphate. This ability to use sulphate has been connected with the activity of intracellular symbionts (Haines *et al*, 1960). Mittler *et al* (1970) added sulphate and doubled the methionine content for *Myzus persicae* and found increased growth. Akey and Beck (1972) tested three levels of inorganic sulphate in combinations with cholesterol, plus 32mg/100ml of cysteine and methionine in all diets. They found mean adult weight and fecundity of *A. pisum* were significantly higher at all levels regardless of the quantity of cholesterol used. However, Retnakaran and Beck (1968) found no difference in growth in *A. pisum* reared on diets with or without inorganic sulphur. This was possibly because the diet was deficient in trace metals. Many other studies have looked at the dietary requirements for methionine and cysteine. Mitler *et al* (1970) replaced L-ascorbic acid with reduced glutathione, since they are both antioxidants. They found that reduced glutathione could not replace L-ascorbic acid, but did not look at the effects of additional glutathione with an optimal L-ascorbic acid concentration.

No studies could be found which directly manipulated the levels of a biochemical metabolite by incorporating it into the nutrient solution of hydroponically growing plants. Hausladen and Kunert (1990) hydroponically produced high intracellular concentrations of glutathione in spinach (*Spinacia oleracea)* using OTC (L-2-oxothiazolidin-4-carboxylate) and also using the herbicide 'acifluorfen'. Hughes and Chiment (1988) succesfully enhanced glutathione levels in soybeans (*Glycine max* L.) following the results of Chiment *et al* (1986), who found an increase in the levels of glutathione in $SO_2$ fumigated foliage. They dipped detached petioles in either deionised water or 0.33mM reduced glutathione, and found that larval growth, rate of development, survivorship, adult fecundity and longevity of the Mexican bean beetle (*Epilachna varivestis* Mulsant), were all significantly higher on leaves dipped in the glutathione solution. This method of enhancing levels was not possible here since it is necessary for the turgor pressure of the phloem to remain high for a long period of time to permit aphids to feed. The experiment described in this study aims to enhance the levels of glutathione within the plant and compare the MRGR of aphids on these plants with unenhanced controls.

## 5.2 Materials and Methods

### 5.2.1 Artificial diet technique

The same basic diet formulation as used in chapter 3 was adapted to produce three series of diet solutions:

Diet A) The ratio of glutathione to other amino acids was increased but the total quantity of amino acids and therefore nitrogen was kept constant. This was achieved using two solutions, one of which was the basic diet formulation, and other was the required concentration of glutathione which when added to the basic diet, in the correct proportions, produced the final diet concentration. Six concentrations were produced.

Diet B) Glutathione was added to the standard diet solution, so increasing the total quantity of nitrogen and sulphur in the diet. Six concentrations were produced.
(Reduced glutathione was used for both diets A and B.)

Diet C) Sulphate, in the form of sodium sulphate, was added to the standard diet, so increasing only the level of sulphate, but maintaining the same total quantity of amino acids and therefore nitrogen. Five concentrations were produced.

The diets were stored until required in the same way as in chapter 3, and the diet sachet production technique was also the same. Two day old aphid nymphs were produced on standard diet sachets in the C.T. room (- conditions as described in chapter 2). For each diet series a minimum of six diet sachets per concentration were made, and one nymph was weighed onto each sachet. The sachets were returned to the C.T. room for three days, the nymphs weighed off the sachets and the Mean Relative Growth Rate (MRGR) calculated. The result for each series was analysed as a one way ANOVAs in SX.

### 5.2.2 Hydroponic Plant Growth Technique

The aim of this technique was to enhance the levels of glutathione in the plant tissues by growing the plants hydroponically in a nutrient solution containing glutathione. Wheat seeds were germinated in the greenhouse in petri-dishes on filter paper moistened with distilled water. The same greenhouse as in chapters 2 and 4 was used, and the photoperiod conditions and temperature regime was the same. Eighteen seedlings were chosen and transferred to individual plastic pots which were filled with 280ml of nutrient

solution. The nutrient solution consisted of 0.14ml of 'Baby Bio' (made by Pan Britannia Industries Limited) in distilled water. This commercially available product was used in initial trials of the apparatus as no recognised hydroponic solution was available. Since the plants grew very well its use was continued in this experiment. Its exact formulation will not be released by the company but it contains nitrogen, phosphorous pentoxide and potassium oxide in the ratio 10.6 : 4.4 : 1.7, which produces concentrations of 10.6%, 1.9%, and 1.4% for N,P and K repectively. It also contains humus and seaweed to supply trace elements. Half of the pots, in addition, contained 93mg of reduced glutathione.

Each seedling was held in place by carefully wrapping the 'stem' in cotton wool and placing it between two halves of a cork bung. This was inserted in a hole cut in the lid of the plastic pot and allowed the roots to hang down into the nutrient solution. The lid also contained a second hole through which a length of narrow plastic tubing dipped into the solution. This was connected, via a syringe needle fitted to its end, to a larger piece of rubber tubing attached to an aquarium pump which ran continuously throughout the experiment to aerate the roots and nutrient solution. The walls of the pots were covered in black PVC self adhesive tape and its lid covered with black filter paper, to exclude all light to the roots. After two weeks the nutrient solution was replaced with a fresh solution containing exactly the same formulation as before, and the extensive root growth around the walls of the pot was noted. Five weeks after the germination of the seeds had been started, a maximum of five standardised two day old aphid nymphs (produced as in chapter 2) were weighed onto the second oldest leaf of each plant. The whole experiment was then transferred to the C.T. room for three days after which the aphids were weighed off the plants and the MRGR calculated. A mean of the MRGR values was taken for each plant, and differentially weighted according to the number of aphids used to calculate the mean. This was analysed as a one way ANOVA in GLIM.

To ensure the technique had enhanced the levels of glutathione, as intended, the plants were harvested for analysis. The shoots were cut at the point level with the lid of the pot, placed in paper bags and freeze dried for 5 days. They were then finely ground in the ball mill for ten minutes, and analysed for glutathione as in chapter 4.

## 5.3 Results

### 5.3.1 Artificial Diet Technique

Diet A)

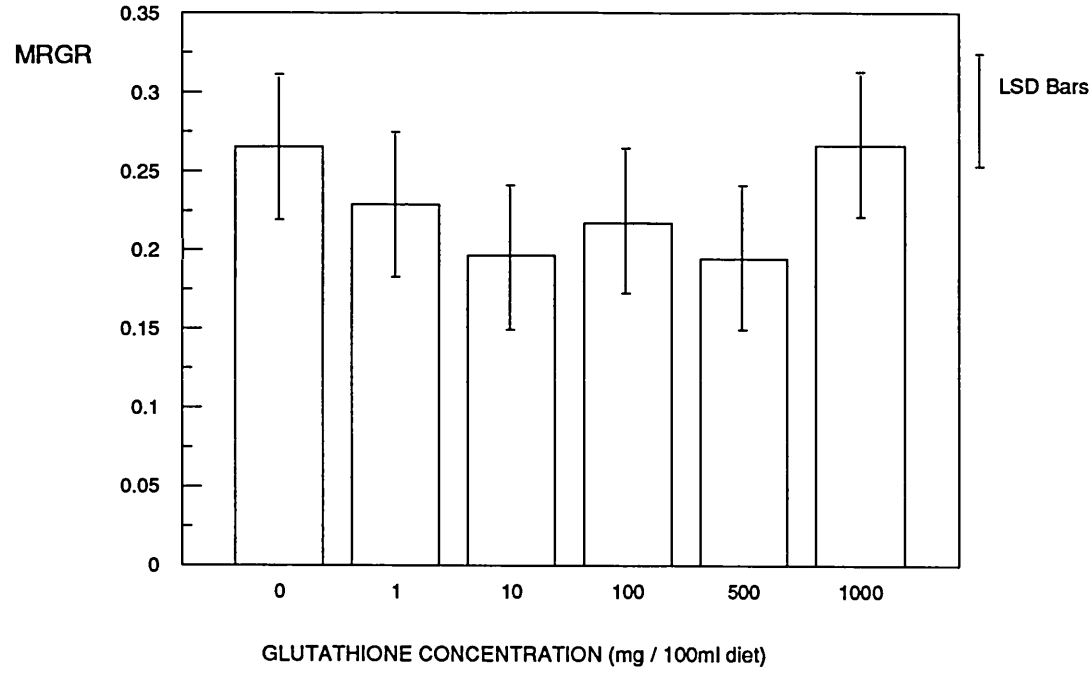


**Figure 5.1** **MRGR of Aphids on Diet with an Enhanced Ratio Of Glutathione but a Constant Total Quantity of Amino Acids.**

There was no significant difference in the Mean Relative Growth Rate of aphids feeding on any of the diet concentrations. The increased glutathione ratio to total quantity of all other amino acids, in the diet, therefore had no effect on the aphid growth rate.

DIET B)

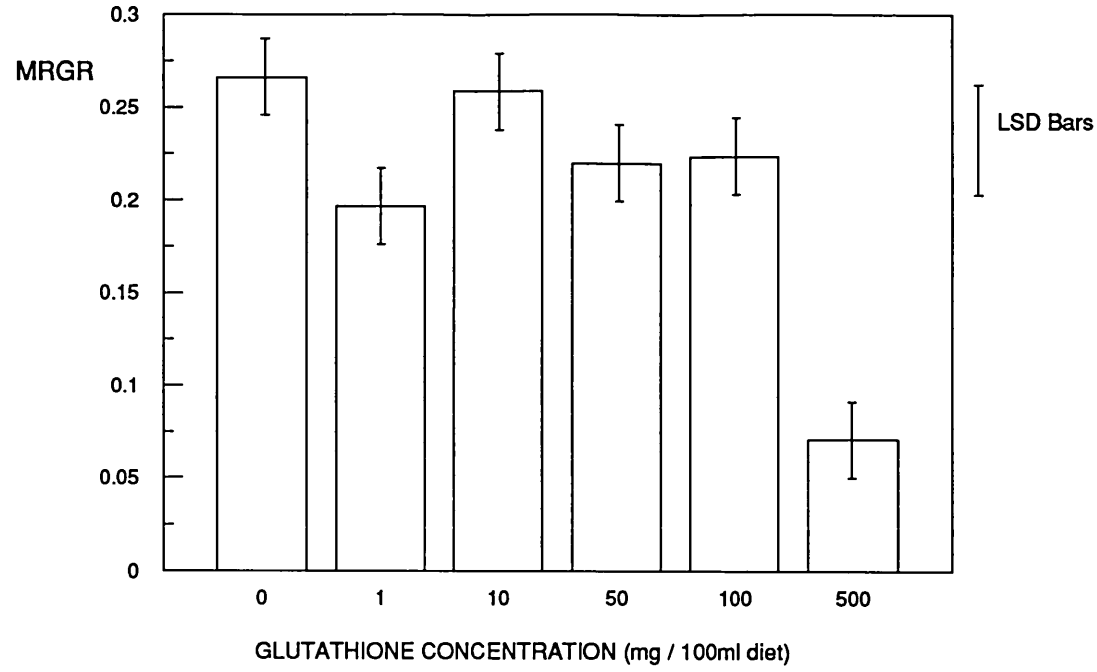


**Figure 5.2 MRGR of Aphids on Diet With an Enhanced Glutathione Concentration.**

There is also no significant increase in growth rate at the enhanced levels of glutathione used here. The significant decrease in growth rate observed at 500 mg/100ml of diet might be a toxic effect of the high levels of nitrogen and sulphate present at this concentration.

DIET C)

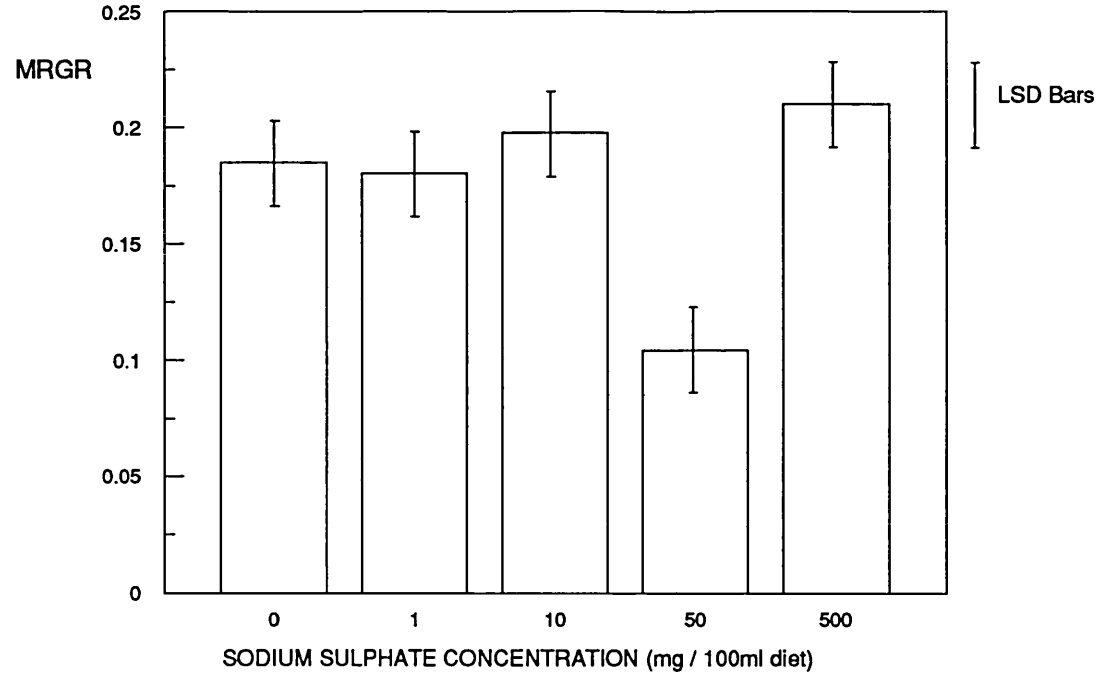


**Figure 5.3 MRGR of Aphids on Diet Enhanced with Sodium Sulphate.**

Again there is no trend in difference in growth rate due to the increased levels of sulphate. There was a significantly reduced growth rate at sulphate concentration 50mg/100ml diet which can not be explained.

### 5.3.2 Hydroponic technique

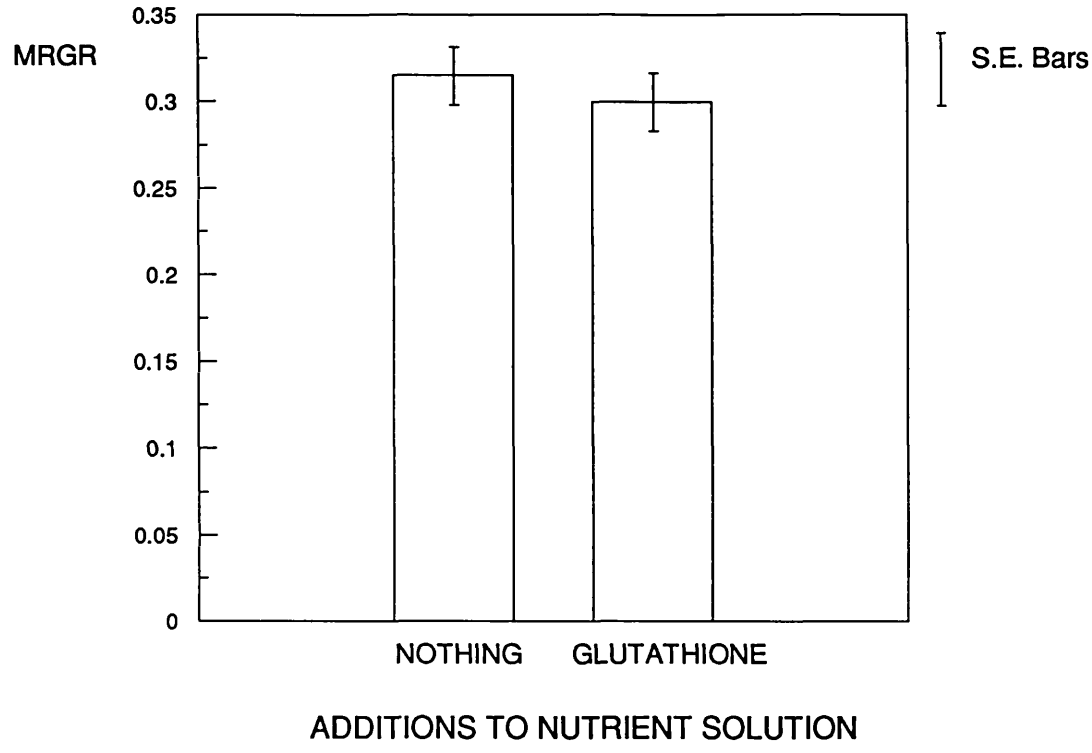


**Figure 5.4** **MRGR of Aphids on Hydroponically Grown Plants with a Nutrient Solution Enhanced with Glutathione, Compared with an Unenhanced Control**

There is no significant difference in MRGR of the aphids on either set of plants. However, the results from the glutathione analysis of the plant material showed a 32.6% increase ($p < 0.0007$) in the levels of glutathione in the plants grown in the enhanced nutrient solution. This is a clear indication that this method of enhancing the levels of glutathione within the shoots of the wheat plant was successful.

## 5.4 Conclusions

The diet manipulation data appears to show that glutathione and sulphate levels in general are unimportant to the MRGR of *Metopolophium dirhodum*, and that this is not the factor leading to the observed increases in MRGR on the plant. In the light of the many previous papers demonstrating improved performance of aphids with additional sulphur amino acids or inorganic sulphur, it appears that the Aminu-Kano (1987) diet is either maximally sufficient in sulphur, or is lacking in an essential component required for the overall growth rates to be increased. Since the growth rates on the diet were less than those observed on the plant (see chapter 2) it is more likely that the second

possibility is the case. This was the case with the Retnakaran and Beck (1968) diet where growth rates were not increased with the addition of sulphur until sufficient quantities of trace metals were added by Akey and Beck (1972) - see introduction. However, it may be that no increase in growth rate following the addition of glutathione and/or sulphate would be observed, even if the diet was improved by the addition of a further unknown component, or levels of existing components were altered. This could only be tested by isolating the unknown component or components.

The results from the diet study actually show that an increase in glutathione or sulphate levels *in the diet* do not produce an increase in growth rate. Van Emden (pers. comm.) has pointed out that; 'If an aphid performs well on artificial diet this suggests that the diet is balanced in relation to its nutrient content and therefore any change - even for example, increasing the concentration of a nutrient supposed to be beneficial - is likely to affect the aphid in a deleterious manner.' This therefore is a further possibility to explain the lack of improved performance. Indeed for diets A and B the highest MRGRs are observed on the original diet solutions, which may indicate that these are the most balanced in relation to the nutrient content. For diet C slightly higher MRGRs are observed on the diet concentrations containing increased sulphate which at least indicates it is not toxic and may indicate some utilisation.

The problems inherent in using the artificial diet technique to prove a causal link between plant biochemical changes and aphid MRGR were apparent before the start of the study. The only result which would have provided proof would have been a positive stimulation of growth rate, and this then would only have proved that alteration of a component benefits the aphid when it is on the artificial diet. However since the component, in this case glutathione, is also increased in the plant a stimulation of growth rate if observed on the plant and on the diet, would have suggested that the mechanism behind the improved performance was the same. Since the results were not positive it was necessary to attempt to prove a causal link by directly manipulating the levels within the plant.

The method chosen to directly increase the levels of glutathione within the plant was successful, although no reference to its incorporation into a hydroponic solution in this way could be found. The results found here do not indicate an increase in growth rate of *Metapolophium dirhodum* with an increase in levels of glutathione. There is a possibility that there was no increase in the levels of glutathione in the phloem, the place in which the aphid feeds, although this seems unlikely. If this is not the case then

it must be concluded that there is no proof from either method employed in this chapter, that the elevated levels of sulphur containing compounds found in the plants following ozone fumigation can alone lead to the increase in aphid growth rate. The increase in these compounds may merely be a correlating factor with the increase in aphid growth rate, or may be a determining factor but one which is only effective in conjunction with the other observed biochemical changes.

# Chapter 6

# The Distribution of Radio - labelled Sulphur Within Polluted Wheat Plants and Physiological Responses of *M. dirhodum* Thereupon.

## 6.1 Introduction

This study further considers the results described in chapter 4 which demonstrate an elevation in soluble sulphur containing compounds (glutathione, cysteine and methionine), following ozone fumigation. The experiments described below investigate the ratio of water insoluble bound sulphur molecules, to soluble sulphur molecules, capable of uptake by aphids, following ozone fumigation. This is done using radio-labelled sulphate, $^{35}SO_4$, which is incorporated into the plant tissues via application to the leaves (Experiment 1) and via the roots (Experiment 2). Experiment 1 further studies the incorporation of $^{35}S$ into aphids, and its subsequent quantity in honeydew. The amino acid composition and total quantity of the honeydew is also considered. Experiment 3 compares the quantity of honeydew and its amino acid composition, produced by aphids on plants to which no sulphate is added, and which are fumigated with either polluted or filtered air.

## 6.2 Experiment 1

### 6.2.1 Materials and Methods

Eighty wheat plants (cv. Ralle) were planted and grown in the greenhouse for five weeks, as described in chapter 2. At weekly intervals 37KBq of carrier free $^{35}SO_4$ was applied in 2µl of distilled water to the second youngest leaf of each plant, approximately two centimeters from the plant axis. The first application was when the plants were exactly 1 week old, and there were four applications. On the third application twice as much activity was added (74KBq) in an attempt to increase the total activity of the plant. A total activity of 185KBq was therefore added which was equivalent to 17.5µg of sulphur. After 5 weeks the plants were randomly and equally distributed between the eight ozone fumigation chambers (see figure 7.1). Half were exposed to ozone at 100 ppb for 72 hours, and half to filtered air. Immediately following fumigation three studies were carried out:-

### 1) Measurement of Bound $^{35}S$ Within the Plant Material.

Four plants from each chamber (32 in total) were freeze dried for three days, and then ground in a Glen Creston Ball Mill for 10 minutes as described in chapter 4. The powder was placed in a polytop tube, 5ml of distilled water added and the tube shaken for 12 hours. The contents were then transferred to an Eppendorf tube and microcentrifuged for 5 minutes at 5000 r.p.m. The supernatant was removed and discarded and the pellet resuspended and transferred to a second polytop tube. 5ml of distilled water was again added and the contents shaken for 12 hours, centrifuged as before and the pellet dried overnight in an oven at 70°C. 20mg of the powder was transfered to a glass scintillation vial, moistened with a few drops of distilled water, and 1ml of 0.3N NCS (Amersham), a tissue solubilising agent, was added. After digesting for 24 hours 10ml of the scintillant Cocktail T was added, and the samples left for a further 24 hours in the dark, to reduce chemiluminesence. The radioactivity was recorded in a Beckman LS 250 liquid scintillation counter. Samples were counted for 50 minutes or until the 2 $\sigma$ error (equivalent to the 95% confidence interval) was reached. The counts were corrected for background and for counting efficiency using the external standard channels ratio method. The data were analysed as a two way ANOVA in SX using air quality and chamber as factors.

### 2) Measurement of (a) MRGR and (b) $^{35}S$ of the Aphids.

Four plants from each chamber (32 in total) were transferred to the C.T. room (as in chapter 2) and two, two day old *M. dirhodum* nymphs were weighed onto each. After three days they were reweighed and the MRGR calculated. Each aphid was placed into a 5ml plastic scintillation vial and 1ml of 0.3N NCS added and left for 24 hours. 5ml of Cocktail T was added to each vial, and left in the dark for 24 hours, before the activity was recorded in the scintillation counter, as above. The results of the MRGR assessment were analysed in Glim, weighting the survival of two aphids per plant twice as strongly as one (as in chapter 2). The radioactivity data were analysed in SX. The analyses were by two way ANOVA using air quality and chamber as factors.

### 3) Measurement of (a) Honeydew Quantity, (b) Amino Acid Composition and (c) $^{35}S$ Content.

Twenty-four hours before the end of the fumigation four groups, each of four adult apterous aphids, were placed in 1cm diameter cages, on the underside of the second oldest leaf of 8 plants. One plant was used from each chamber. The cages were spaced along the length of the leaf, separated by approximately 1cm. At the end of the

fumigation the plants were transferred to the C.T. room, and the adults removed leaving the nymphs. Insufficient nymphs were produced on two of the plants which reduced the sample size to 6. The leaf was suspended approximately 2cm above a piece of aluminium foil onto which any honeydew produced by the nymphs would fall. The number of nymphs contributing to the honeydew production was counted daily. After 72 hours the foil was replaced with a second piece, and honeydew collection continued for a further 72 hours. The patches of honeydew were cut from the foil and placed in plastic petri-dishes with ventilation holes so air could circulate freely. The dishes and foils were placed in the freeze drier for 3 days after which the foils were accurately weighed. The foils were then carefully washed with 3ml of 50% aqueous methanol, to remove all the honeydew, the tin foil replaced in the petri dishes, freeze dried and reweighed to give the weight of honeydew. 10ml of Cocktail T was immediately added to a 2ml aliquot of each honeydew solution, left for 24 hours in the dark, and the radioactivity counted as before. The remaining 1ml of each sample was stored at -20°C, and subsequently injected directly into the amino acid analyser. The three data sets were analysed as two way ANOVAs in SX using air quality and collection period as factors.

### 6.2.2 Results

## 1) Amount of Bound $^{35}S$ Within the Plant Material.

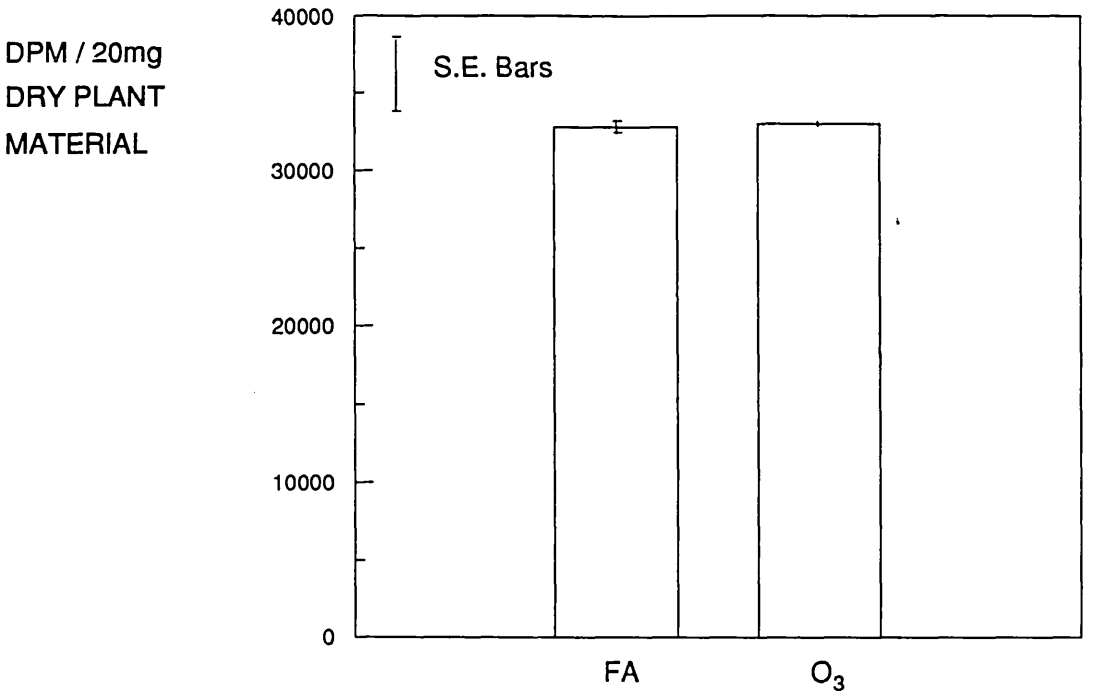


**Figure 6.1** **Amount of $^{35}S$ Bound Sulphur Within Plants Exposed to either Filtered Air or Ozone (at 100 ppb) for 72 hours.**

This clearly shows that there was no difference, due to ozone, in the quantity of radioactive sulphur bound in the plant material. There was no significant difference in radioactivity levels between the chambers.

The levels of soluble radiosulphur were not recorded because the quantity of $^{35}S$ adhering to the leaves following application could not be isolated and would be far greater than any absorbed and incorporated within the plant. Experiment 2 was therefore conducted in which the $^{35}SO_4$ was added via the roots. This permitted a direct measurement of the quantity of $^{35}S$ in the soluble phase of the above ground parts of the plant to be carried out.

## 2 (a) MRGR of the Aphids

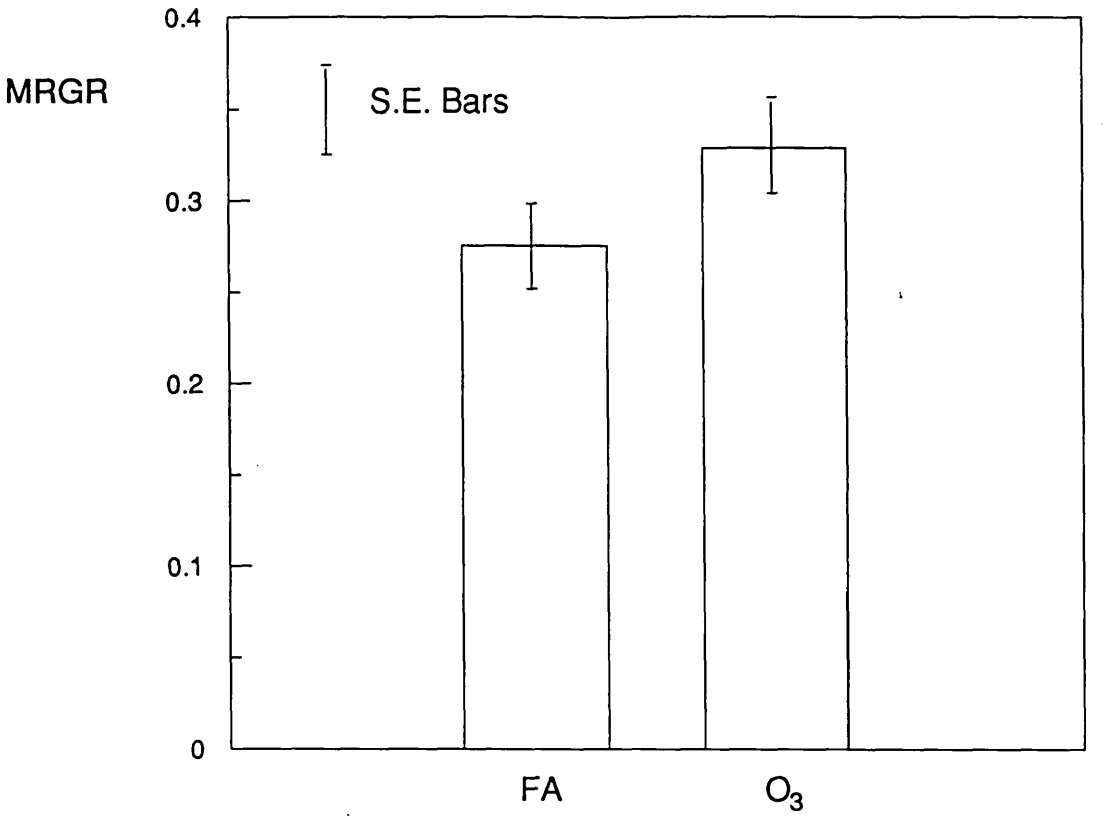


**Figure 6.2** **MRGR of Aphids on $^{35}SO_4$ Treated Plants Exposed to either Filtered Air or Ozone (at 100 ppb) for 72 hours.**

A 15.8% increase in MRGR was seen for the aphids on ozone fumigated plants, although this was not significant ($p < 0.14$). There was no significant difference in growth rate between the chambers. The same fumigation regime in experiments described in chapter 2 produced a significant 28.1% increase in growth rate. The lack of a significant result here may be due to the increased levels of metabolisable sulphur compounds in both treatment and control plants as a result of the $^{35}SO_4$ treatment. This may obscure any biochemical changes due to ozone fumigation, which may be responsible for an increase in growth rate. Alternatively this may be a genuine increase in growth rate, which is masked by the large errors involved possibly induced by the $^{35}SO_4$ treatment.

## 2 (b) Radioactivity of the Aphids.

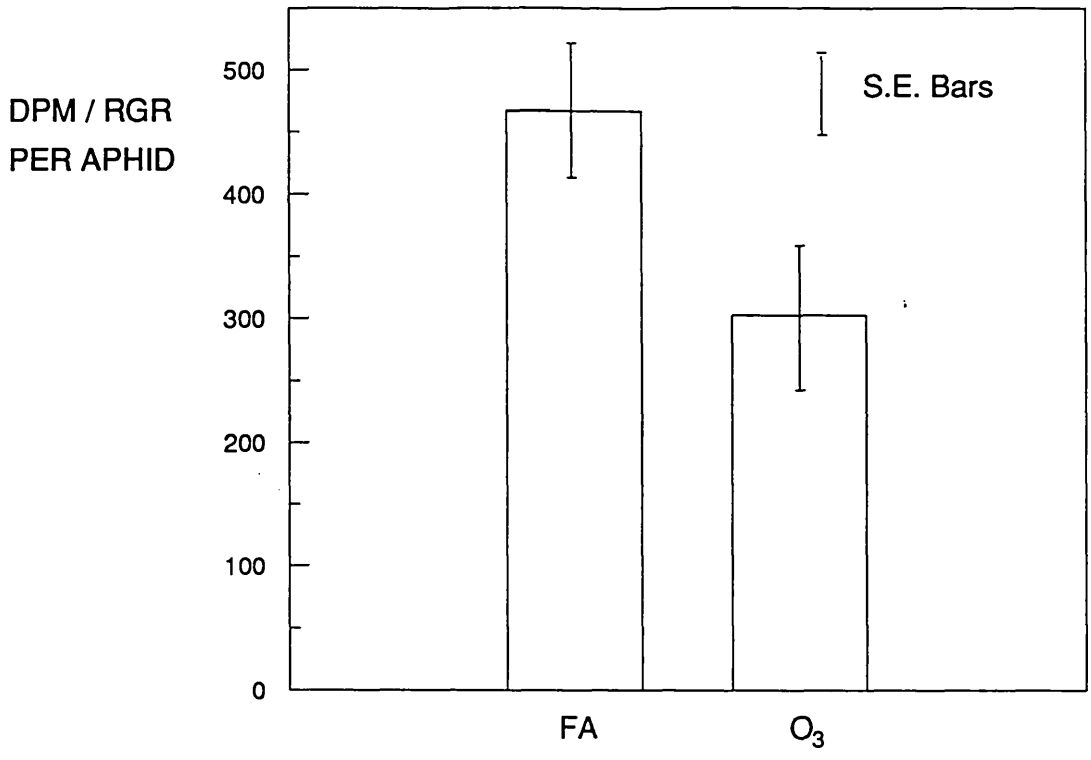


**Figure 6.3** **Radioactivity per Aphid per unit Relative Growth Rate for Aphids on Plants either Exposed to Filtered Air or Ozone (at 100 ppb) for 72 hours.**

There was a decrease in the absolute radioactive count irrespective of the growth rate for aphids on ozone fumigated plants, although this was not significant ($p < 0.29$, 23.3%). The activity per mg of aphid at its final weight was also reduced ($p < 0.22$, 22.1%) on the ozone fumigated plants. However, as figure 6.3 shows, there was a significant decrease ($p < 0.043$) in the radioactive count recorded per aphid per unit relative growth rate, for aphids on ozone fumigated plants. Radioactivity per unit relative growth rate was used since faster growing aphids were shown to contain more radioactivity ($p < 0.004$). There was no significant difference in radioactivity levels between the chambers. The quantity of soluble $^{35}S$ compounds extracted from the plant, per unit weight gain for each aphid is therefore less for the aphids on the ozone fumigated plants. Since these aphids also grow faster it may be that the rate of flow of phloem through the aphid body is reduced, i.e. there is a reduced uptake of $^{35}S$ and therefore phloem sap by the aphids on the ozone fumigated plants.

## 3 (a) Honeydew Quantity

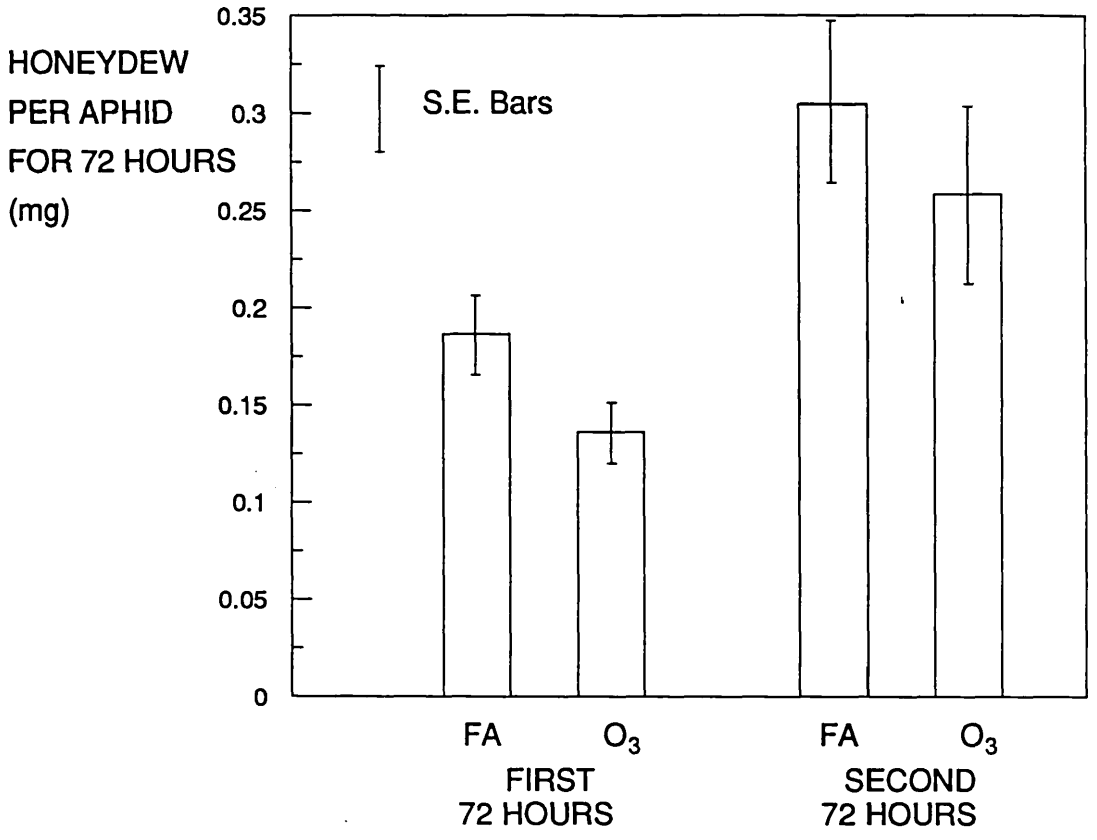


**Figure 6.4** **Quantity of Honeydew Produced per Aphid for Two Consecutive 72 Hour Periods on Plants Previously Exposed to Filtered Air or Ozone (at 100 ppb) for 72 hours.**

This clearly shows a large decrease in the quantity of honeydew produced by aphids on ozone fumigated plants. The differences are, however, not significant probably because of the low level of replication and the considerable variability. A 27% decrease during the first collection period, and a 25% decrease during the second was found. The overall increase in honeydew for the second collection period was because the aphids were larger and therefore extracted more phloem sap.

## 3 (b) Amino Acid Composition of Honeydew

**Table 6.1** **The Concentration of Amino Acids per mg of Honeydew from Aphids on Plants Previously Exposed to Filtered Air or Ozone (at 100 ppb) for 72 hours.**

| Amino Acid | Weight (nM/mg) | | % + or - in $O_3$ | Sig. Level |
|---|---|---|---|---|
| | FA | $O_3$ | | |
| Aspartic Acid | 3.513 | 6.140 | +42.8 | N.S. |
| Threonine | 0.721 | 2.259 | +68.0 | N.S. |
| Amides | 4.832 | 18.64 | +74.1 | ** |
| Glutamine | 7.037 | 11.52 | +38.9 | N.S. |
| Proline | 1.041 | 1.680 | +38.0 | N.S. |
| Glycine | 0.643 | 1.656 | +61.2 | N.S. |
| Alanine | 0.723 | 1.857 | +61.1 | * |
| Cysteine | 0.560 | 2.743 | +79.6 | N.S. |
| Valine | 0.697 | 1.791 | +61.1 | N.S. |
| Methionine | 0.316 | 0.737 | +57.1 | N.S. |
| Isoleucine | 0.676 | 0.764 | +11.5 | N.S. |
| Leucine | 0.702 | 1.001 | +29.9 | N.S. |
| Tyrosine | 0.677 | 0.912 | +25.8 | N.S. |
| Phenylalanine | 1.098 | 0.839 | -30.9 | N.S. |
| GABA | 0.450 | 0.825 | +45.4 | N.S. |
| Histidine | 0.364 | 0.484 | +24.8 | N.S. |
| Lysine | 2.420 | 2.090 | -15.8 | N.S. |
| Arginine | 1.436 | 1.215 | -18.2 | N.S. |
| Total nM amino acid per sample (n = 12) | 27.90 | 57.15 | +51.2 | N.S. |

Key: NS Non significant
* Significant at $p < 0.05$
** Significant at $p < 0.01$

Table 6.1 shows an increase in concentration for almost all of the amino acids in the honeydew. There was considerable variability between the samples and many of the increases are not significant because of this. The total quantity of amino acids excreted

by aphids on ozone fumigated plants was also greater (data not shown), and since the total dry weight of honeydew from these aphids decreased there must have been a considerable reduction in the other components of the honeydew. This data appears to broadly corroborate the amino acid data given in chapter 4 which demonstrated that the concentration of amino acids was increased in the ozone fumigated foliage. The data for the first and the second 72 hour collection periods has been combined in the table for ease of presentation. There was no significant difference in the quality of honeydew produced between the two collection periods.

## 3 (c) Radioactivity of Honeydew

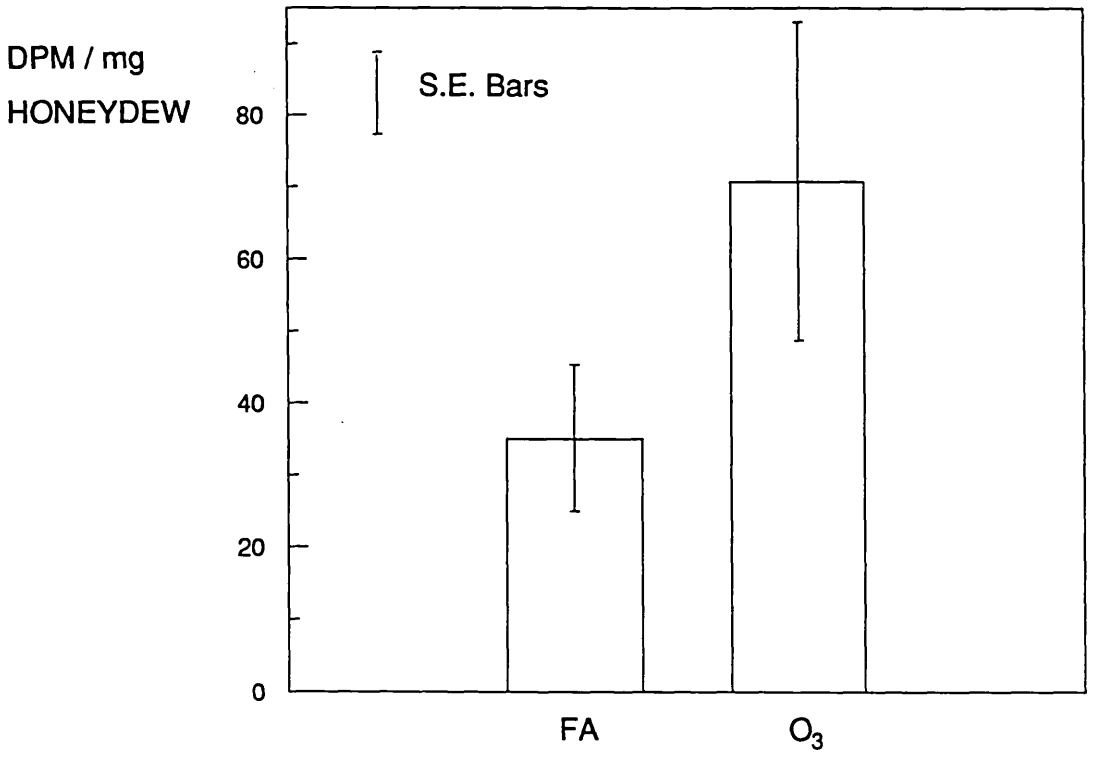


**Figure 6.5** **Radioactivity per mg Honeydew from Aphids on Plants Previously Exposed to Filtered Air or Ozone (at 100 ppb) for 72 hours.**

Figure 6.5 demonstrates that the quantity of soluble radioactive sulphur per unit honeydew was substantially increased in honeydew from aphids on ozone fumigated plants ($p < 0.18$). The total activity was also increased (by 46.5%, $p < 0.17$), but these statistics are not significant probably because of the low level of replication. However, this data corroborates the honeydew amino acid data which showed large increases in

amino acid concentration and in sulphur containing compounds (methionine and cysteine) from aphids on ozone fumigated plants. It is also in accordance with a reduced uptake of a more concentrated phloem sap.

## 6.3 Experiment 2

### 6.3.1 Materials and Methods

Twelve wheat plants were germinated and grown hydroponically, exactly as described in chapter 5. 111KBq of $^{35}SO_4$ (equivalent to 10.5μg of sulphur) was added to the nutrient solution initially. Three weeks after germination the nutrient solution was discarded, the pots carefully washed, and the plants quickly replaced into new nutrient solution, to which 111KBq of $^{35}SO_4$ was added. After 5 weeks, six plants were transferred to each of two chambers in the ozone fumigation greenhouse. It was not possible to use more than two chambers because of technical problems with the air supply to each pot in each chamber. One chamber received ozone at 100 ppb and the other filtered air, for 72 hours. Following fumigation the plants were transferred to the C.T. room and a MRGR study conducted as before. The plants were then cut off at the level of the pot, freeze dried for 3 days, then ground in a Glen Creston Ball Mill for 10 minutes. The powder was transferred to a polytop tube and shaken for 12 hours with 5ml of distilled water. This was centrifuged as in the previous experiment but the supernatant was retained. The pellet was resuspended and shaken with 5ml distilled water for a further 12 hours. This was again centrifuged and the supernatant added to the previous one. In this way the $^{35}S$ was partitioned into a water soluble fraction containing small organic molecules and sulphate, and an insoluble fraction where it was bound to larger and insoluble molecules and tissues.

**Treatment of bound phase:**

The pellet of plant material was dried overnight in an oven at 70°C and 20mg transferred to a glass scintillation vial. This was moistened with a few drops of distilled water, and 1ml of 0.3N NCS added to solubilise the powder. After 24 hours the material was not completely homogeneous and so was further macerated in a hand held homogeniser. The plant material plus NCS was washed out of the vial with 0.5ml of 50% aqueous methanol, and macerated until it appeared homogeneous (approximately 5 minutes). It was then transferred back to the vial with a further 0.5ml of aqueous methanol. The sample was dark green and was decolourised to reduce quenching according to the

method of Burrell and Brunt (1981). 70mg of benzoyl peroxide in 0.5ml of toluene was added and the sample left overnight in the dark. 10ml of Cocktail T was added and the radioactivity assessed as above.

**Treatment of soluble phase:**

1ml from the 10ml of supernatant, was transferred to a glass scintillation vial. The liquid was slightly coloured and was therefore decolourised as above. 10ml of Cocktail T added and the emissions counted as above.

The results of all three data sets were analysed as one way ANOVAs in SX, using air quality as the factor.

### 6.3.2 Results

## 1) Radioactivity of the Bound Phase

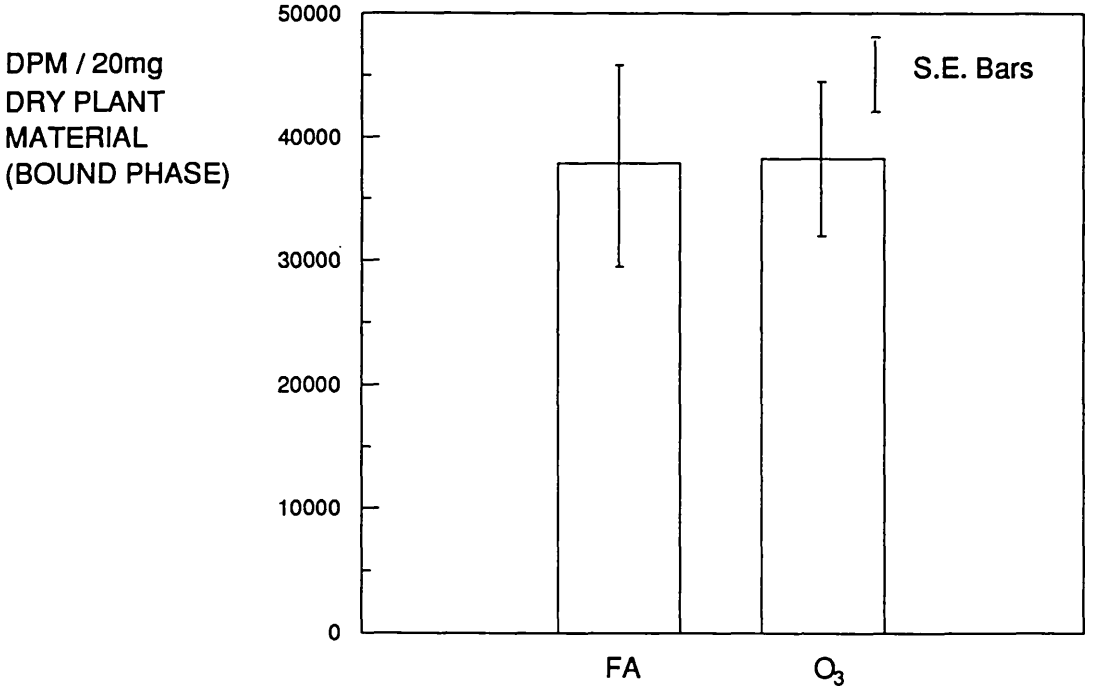


**Figure 6.6** **Radioactivity of the Bound Phase for Plants Exposed to Filtered Air or Ozone (at 100 ppb) for 72 Hours. The Plants were Grown Hydroponically with $^{35}SO_4$ Added to the Nutrient Solution.**

No significant difference was found in the amount of bound $^{35}S$ in the ozone fumigated and the control plants. The levels were comparable with those found in the previous experiment.

## 2) Radioactivity of the Soluble Phase

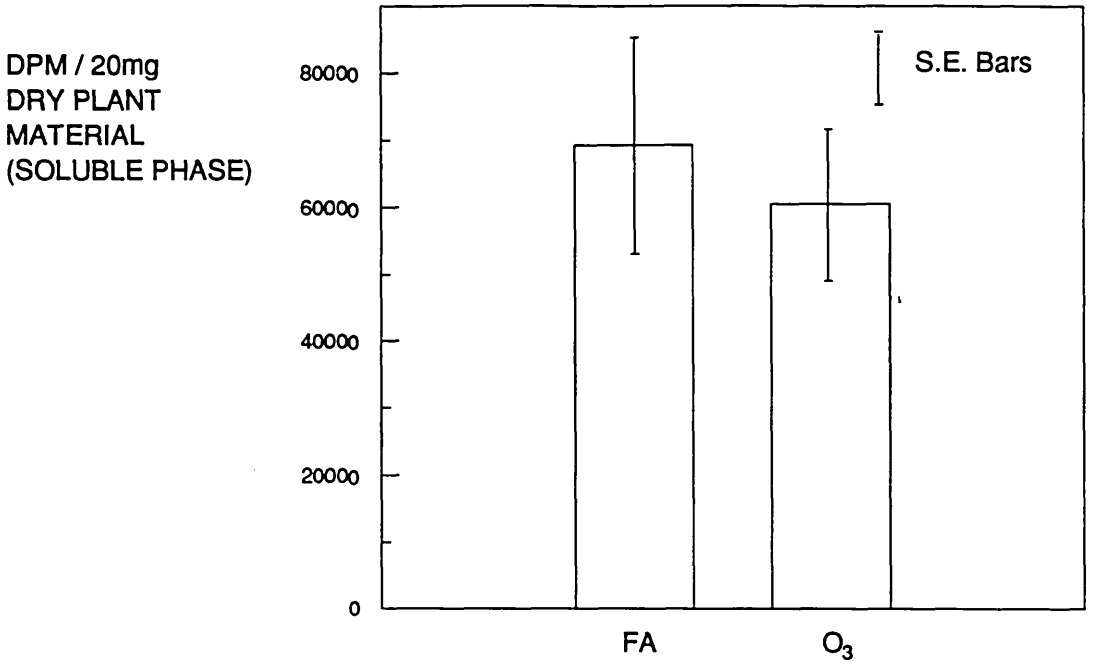


**Figure 6.7** **Radioactivity of the Soluble Phase for Plants Exposed to Filtered Air or Ozone (at 100 ppb) for 72 Hours. The Plants were Grown Hydroponically with $^{35}SO_4$ Added to the Nutrient Solution.**

No significant difference was found between the amount of water soluble $^{35}S$ in the ozone fumigated and the control plants. These results for the plant material appear to firmly reject the hypothesis that the effect of ozone fumigation is to increase the quantity of soluble sulphur containing compounds. The increase in these compounds seen in chapter 4 following this fumigation regime is either not replicable or not possible to detect using this method.

## 3) Aphid MRGR

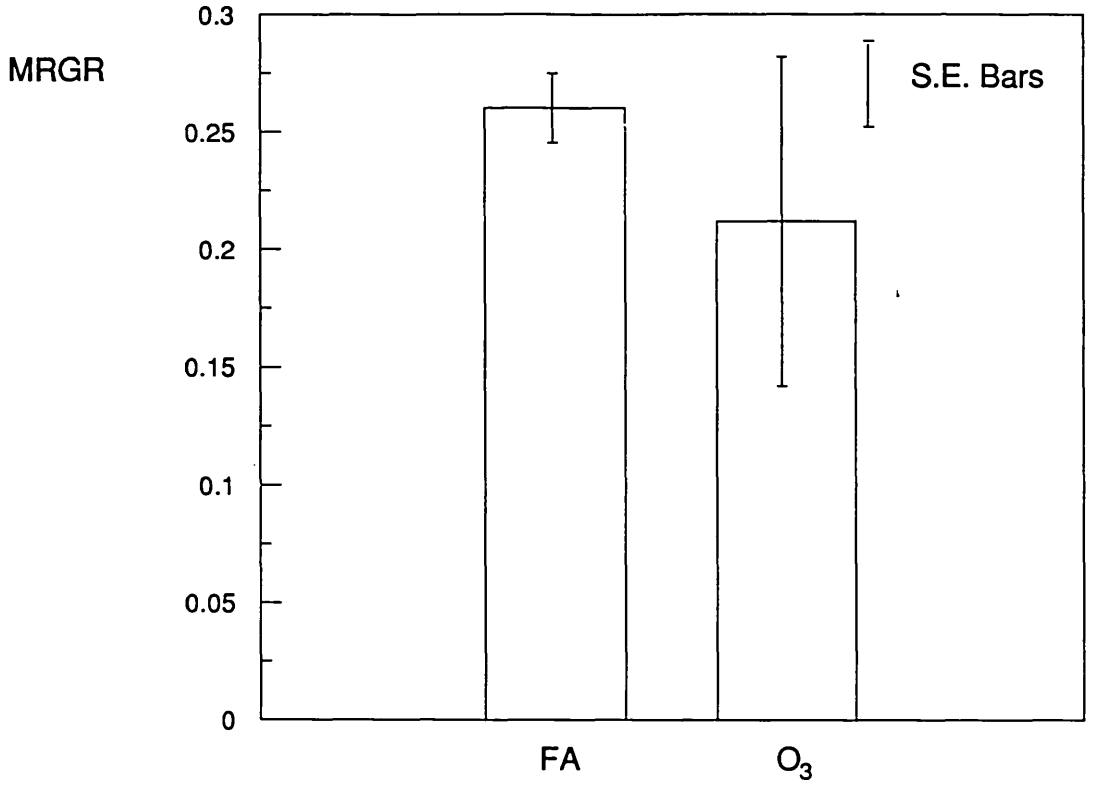


**Figure 6.8** **MRGR of Aphids on Plants Exposed to Filtered Air or Ozone (at 100 ppb) for 72 Hours. The Plants were Grown Hydroponically with $^{35}SO_4$ Added to the Nutrient Solution.**

There was a non-significant decrease ($p < 0.60$) in growth rate of aphids on the ozone fumigated plants, which is contrary to the increase found previously following this regime. The overall growth rate was lower than normally seen on soil grown plants which indicates that the aphids were not feeding properly. The large standard error of the growth rate of the aphids on ozone fumigated plants further indicates that they were not feeding properly. There was a higher than normal aphid mortality (58%), although this was similar for both control and treatment plants. The reason for this is probably because the plants were grown hydroponically and/or because the level of $^{35}SO_4$ was toxic, although the increase above levels present in the nutrient solution is negligible (see method). The plants appeared healthy if a little smaller than plants grown in soil.

## 6.4 Experiment 3

### 6.4.1 Materials and Methods

### 1) Honeydew Quantity

Following the honeydew quantity results discovered in experiment 1, a further experiment to investigate the production of honeydew from aphids on plants exposed to polluted or filtered air was undertaken. Honeydew production was monitored over the course of approximately one week following the end of fumigation. The experiment was repeated twice to increase the replication and was undertaken with both nitrogen dioxide and ozone. The nitrogen dioxide regime was that which was found in chapter 2 to produce a significant increase in MRGR i.e. 48 hours at 100 ppb. The ozone regime was for 72 hours at 100 ppb - as before.

Four plants were grown in the greenhouse for five weeks - as in chapter 2. Two were then fumigated with the pollutant gas and two with filtered air. 24 hours before the end of the fumigation four adult apterous aphids were placed in each of four cages, and placed along the underside of the second oldest leaf of each plant, approximately 1cm apart. Following fumigation the plants were transferred to a controlled environment cabinet and the cages and adults removed to leave nymphs. The temperature was 20°C during the day and 15°C at night. The humidity was maintained at approximately 75% and metal halide lamps were on between the hours of 0600 and 2200, giving a photon flux density of 400μE $m^{-2}s^{-1}$. The leaf was suspended approximately 2cm above aluminium foil positioned on a turntable which was set to turn at the night and day junctions, to produce day and night time honeydew collections. The number of nymphs on the underside of the leaf contributing to the weight of the honeydew were counted at 0900 daily and this was taken to refer to the number present during the previous night, and the following day. The previous 24 hours of honeydew collection was removed at this time, and new foil positioned ready for the following night and day collection. The patches of foil bearing honeydew from the previous night and day collection were cut out and placed in petri-dishes with aeration holes. These were freeze dried for 48 hours, weighed and the honeydew washed off with 3ml of 50% aqueous methanol. The foils were freeze dried again for approximately 48 hours, reweighed, and the weight of honeydew found by subtraction. Collection was continued until the first nymphs of next generation were produced, which occurred after

approximately one week. The honeydew solution from each collection period was analysed for amino acids but the quantities present were not significantly different from the background noise of the analyser.

The data were analysed in SX. Two way ANOVAs of honeydew production per aphid per hour, using experiment repetition and air quality as factors, were carried out for each time period following the fumigations.

## 2) Honeydew Amino Acid Composition

Following the inability to detect amino acids in the small individual quantities of honeydew collected in the previous experiment, a further experiment was conducted to investigate the concentration of amino acids in honeydew from aphids on either filtered air or ozone fumigated plants. The quantity of amino acids in honeydew from aphids on nitrogen oxide fumigated plants was not further investigated. It was important to investigate the possibility that the honeydew amino acid results obtained in Experiment 1 were solely due to the increased sulphur concentration in the plants, rather than due to the ozone fumigation. The 72 hour collection period in the previous successful analysis of honeydew amino acids was repeated in this experiment, to ensure a large enough quantity of honeydew for amino acid detection. Eight plants were grown in the greenhouse until five weeks old - as described in chapter 2. One ozone chamber and one filtered air chamber were used, each containing 4 plants. 24 hours before the end of the fumigation four adult apterous aphids were placed in each of four 1cm diameter cages and positioned along the under side of the second oldest leaf of each plant. Following fumigation the plants, plus aphids were transferred to the C.T. room and the cages plus adult aphids removed to leave nymphs. An insufficient number of nymphs were produced on one ozone fumigated plant which reduced the sample size to 7. Honeydew was collected onto aluminium foil for 72 hours, weighed and washed off, as before, using 3ml of 50% aqueous methanol. The foil was freeze dried, reweighed and the weight of honeydew found by subtraction. Following the first collection period, fresh foil was placed beneath the aphids and collection was continued for a further 72 hours. These samples were treated in the same way as the previous samples. The honeydew solution was analysed for amino acids by injecting it directly into the amino acid analyser. The total weight of honeydew produced per aphid in 3 days, and the total concentration of each amino acid per mg of honeydew, for each collection period were analysed as two way ANOVAs in SX using collection period and air quality as factors.

## 6.4.2 Results

## 1) Honeydew Quantity

### a) Ozone

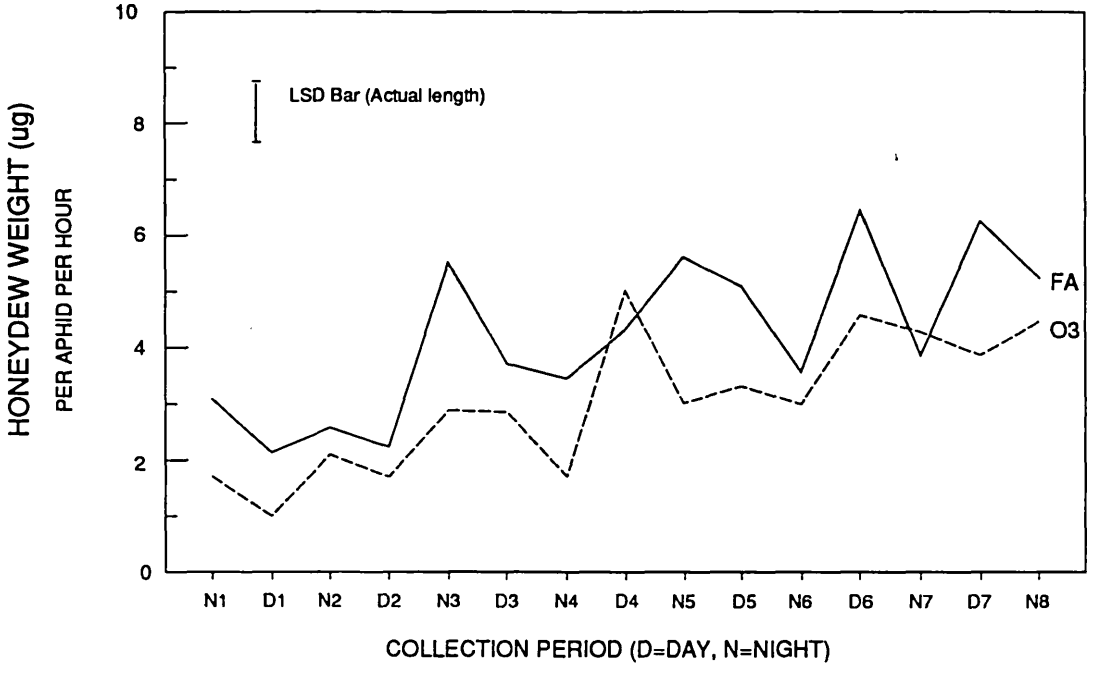


**Figure 6.9** **Weight of Honeydew Produced per Aphid per Hour on Plants Previously Exposed to Filtered Air or Ozone at 100 ppb for 72 hours.**

This clearly shows that honeydew production was greatly reduced from aphids on ozone fumigated plants. The effect occurred immediately following fumigation but was only significant ($p < 0.05$) for days 5, 6 and 7 because of the low level of replication. There was no significant difference in the quantity of honeydew produced per aphid per hour, between the repeat experiments for any of the collection periods. The reduction in honeydew production on ozone fumigated plants could indicate that the quality of the food is improved in terms of aphid nutrition such that they need consume less phloem sap. The graph also shows that the effect persisted for at least 7 days following the fumigation and therefore that the plants did not recover from the fumigation, or that if they did the aphids did not compensate by producing larger quantities of honeydew.

The graph also indicates that there was no reduction in honeydew production during the night. This was unexpected since no photosynthesis occurs at night. This may perhaps be the result of a compensatory mechanism in either the plants or, more

probably, the aphids, which allows no interruption of honeydew production. It can be seen that throughout the experiment an increased quantity of honeydew was produced as the aphids grew.

**b) Nitrogen Dioxide**

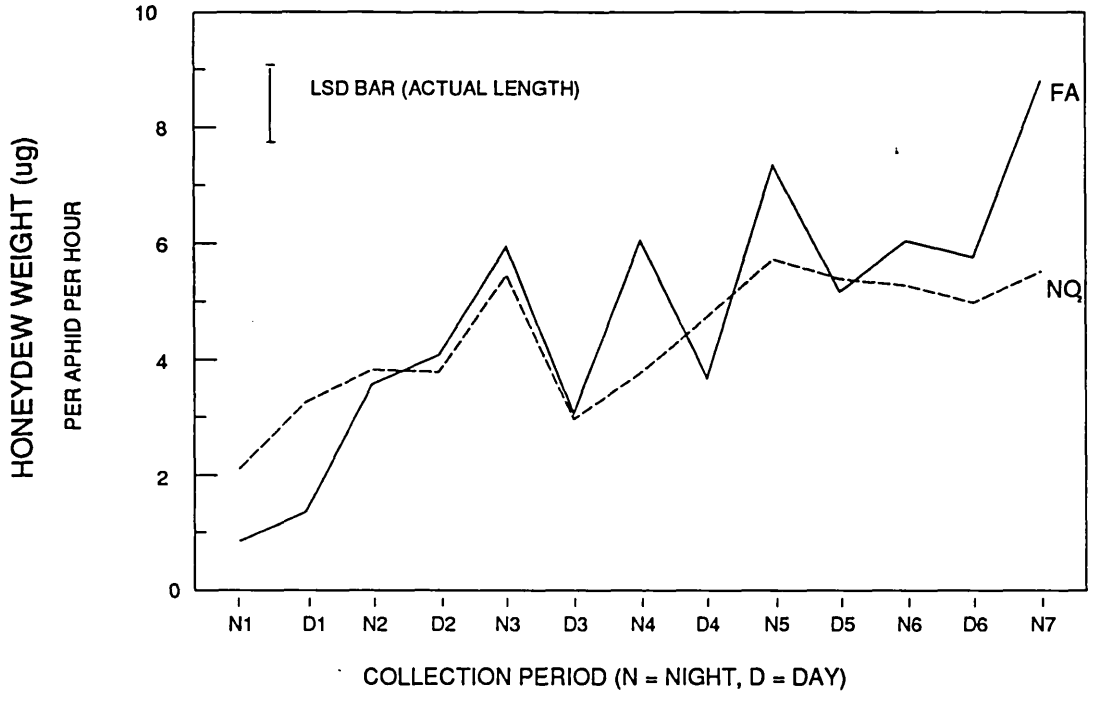


**Figure 6.10** **Weight of Honeydew Produced per Aphid per Hour on Plants Previously Exposed to Filtered Air or Nitrogen Dioxide at 100 ppb for 48 hours.**

There was no significant difference in the quantity of honeydew produced from aphids on plants which received filtered air or nitrogen dioxide. None of the individual ANOVAs for each time period was significantly different for either the air quality or the experiment repetition. Using the arguments above this implies that the quality of the phloem of the fumigated plants was not improved in terms of aphid nutrition, and therefore a different explanation for the increased growth rate observed in chapter 2 at this regime must be sought. Indeed no major biochemical differences were demonstrated between the fumigated and control plants as described in chapter 4. The explanation for the significant increase in growth rate of the aphids may be a statistical artifact, or it may be due to an unknown difference between the fumigated and control plants. Again no difference in honeydew production during the day or night was

observed. A clear increase in the weight of honeydew produced per aphid was observed during the course of the experiment, indicating that as the aphids grew they produced more honeydew.

## 2) Honeydew Amino Acid Composition

**Table 6.2** **The Concentration of Amino Acids per mg of Honeydew from Aphids on Plants Previously Exposed to Filtered Air or Ozone (at 100 ppb) for 72 hours. (No sulphate application).**

| Amino Acid | Weight (nM/mg) | | % + or - in $O_3$ | Sig. Level |
|---|---|---|---|---|
| | FA | $O_3$ | | |
| Aspartic Acid | 1.029 | 3.995 | +74.2 | ** |
| Threonine | 2.189 | 4.653 | +52.9 | ** |
| Amides | 1.711 | 3.427 | +50.1 | * |
| Glutamine | 0.957 | 4.137 | +76.9 | ** |
| Proline | 0 | 0 | 0 | N.S. |
| Glycine | 0.320 | 0.898 | +64.4 | N.S. |
| Alanine | 0.506 | 1.188 | +57.4 | N.S. |
| Cysteine | 0.024 | 0.242 | +90.1 | N.S. |
| Valine | 0.806 | 2.405 | +66.5 | * |
| Methionine | 0.104 | 0.793 | +86.9 | N.S |
| Isoleucine | 0.981 | 2.145 | +54.3 | * |
| Leucine | 0.764 | 1.565 | +51.2 | N.S. |
| Tyrosine | 0.887 | 1.232 | +28.0 | N.S. |
| Phenylalanine | 2.040 | 2.873 | +29.0 | N.S. |
| GABA | 1.526 | 1.590 | +4.02 | N.S. |
| Histidine | 2.734 | 2.412 | -13.3 | N.S. |
| Lysine | 1.391 | 2.817 | +50.6 | N.S. |
| Arginine | 0.903 | 1.270 | +28.9 | N.S. |
| Total nM amino acid per sample (n = 14) | 17.04 | 37.642 | +54.7 | ** |

**Key:** NS Non significant

* Significant at $p < 0.05$

** Significant at $p < 0.01$

For ease of presentation the data in Table 6.2 are for the first and the second three day collection periods combined. There was no significant difference in the quality of honeydew produced between the two collection periods. It can be seen that again there was an increase in the concentration of amino acids from the aphids on ozone fumigated plants. This probably reflects an increase in amino acid concentration within the phloem and also indicates that this quantity is excess to the needs of the aphid.

The table again compares the quantity of amino acid per mg of honeydew. The absolute quantities of honeydew produced are presented below.

## Honeydew Quantity

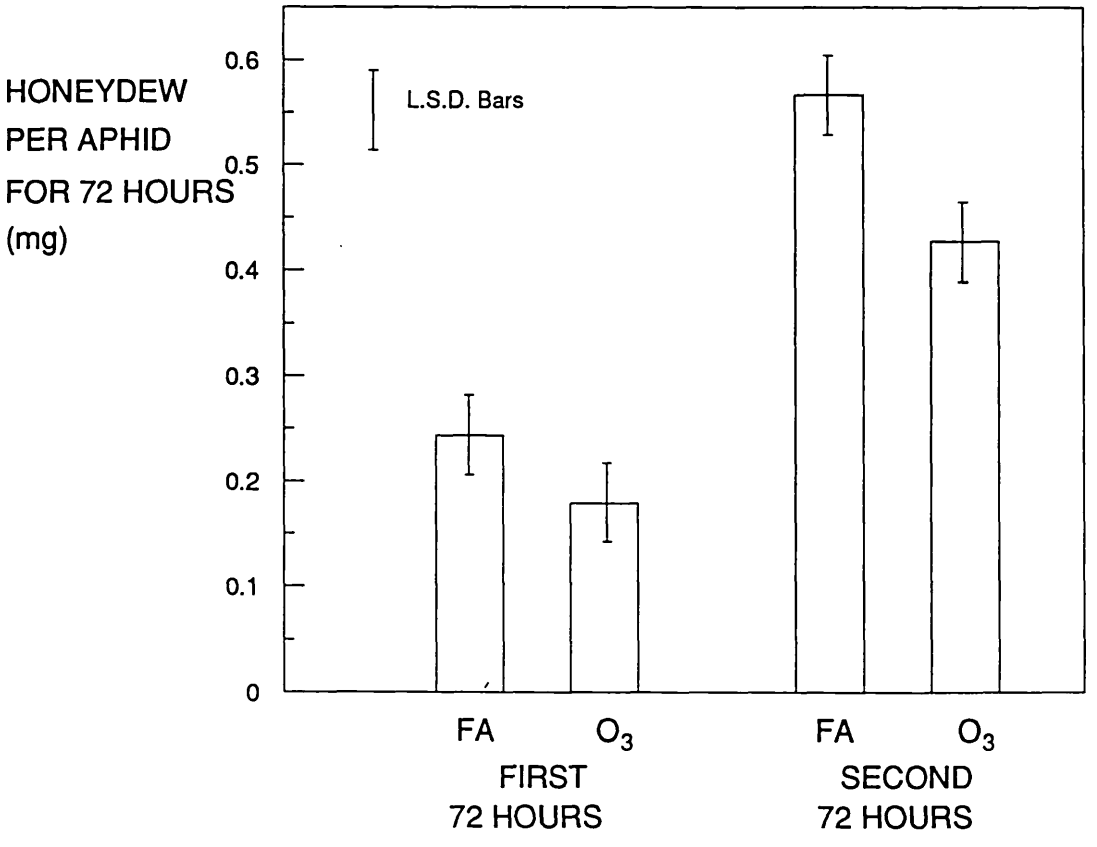


**Figure 6.11** **Quantity of Honeydew Produced per Aphid for 72 hours on Plants Exposed to Filtered Air or Ozone (at 100 ppb) for 72 hours. (No sulphate Application)**

Figure 6.11 clearly shows a significant decrease ($p < 0.02$) in the quantity of honeydew produced by aphids on ozone fumigated plants, compared with those on filtered air plants. This is apparent for the collection period immediately following fumigation, and for the period beginning 72 hours after the end of the fumigation.

## 6.1 Conclusions

The aim of experiments 1 and 2 was to investigate whether, following fumigation with ozone, there was an increase in the soluble sulphur in the plants, and therefore an increase in that available to the aphids, leading to an increase in growth rate. In experiment 1 the sulphate applied to the leaves, would have appeared in any extract of soluble sulphur and which was not incorporated into the plant metabolism. This prevented any calculation of the quantity of soluble $^{35}S$ within the plant. The ratio of the bound to the soluble sulphate could therefore not be calculated, and only a reduction in the quantity of bound $^{35}S$ would have provided any support for the hypothesis. However, no difference in the quantity of bound organic $^{35}S$ was seen. In experiment 2 the sulphate was applied via the roots and the $^{35}S$ found in the shoots was therefore in the vascular tissues of the plant, or incorporated in the plant metabolism. There was a non-significant slight increase in the quantity of bound $^{35}S$ and a non-significant decrease in the quantity of soluble $^{35}S$ in the ozone fumigated plants. This provides no support for the hypothesis and demonstrates that it may not be a greater availability of soluble sulphur containing compounds which leads to an increase in aphid MRGR following ozone fumigation.

In addition to providing information concerning the quantity of bound $^{35}S$ compounds in the plant material, Experiment 1 provided interesting information concerning the uptake by aphids of sulphur containing compounds. If there is no selective uptake of the $^{35}S$ its concentration within the aphid and the honeydew also provides information concerning the bulk uptake and fate of phloem sap. As shown in chapter 4, the amino acid concentration of ozone fumigated plants and probably phloem sap is higher than that of control plants. This might be the stimulus for a reduced uptake and slow passage of phloem sap through the aphid. This would explain the reduced quantity of honeydew from aphids on ozone fumigated plants. Within the aphid it is broadly the case that required nutrients are extracted from the phloem sap, and those remaining are excreted in the honeydew. The increased concentration of amino acids, and $^{35}S$ in the honeydew from aphids on ozone fumigated plants indicates that these may be in excess of the aphid requirements. It is evident from the lack of an increase in $^{35}S$ in the bodies of

aphids on fumigated plants that the sulphur is not selectively retained and assimilated within the aphid body and therefore is not the factor which promotes the increase in growth rate. It may be speculated that it is the general increase in the levels of amino acids in the phloem sap which produces the increases in MRGR.

In experiment 1 radiolabelled sulphate was applied directly to the leaves with the intention that it should distribute itself to all the foliage. Biddulph *et al* (1956) found that $^{35}SO_4$ applied to the leaves of *Phaseolus vulgaris*, either by injection or by spraying directly onto the foliage, could be detected in all parts of the plant within 30 minutes. Bukovac and Wittwer (1957) separated the process into uptake and translocation, and stated that the initial penetration most likely involved passage through the cuticle and epidermal cells, with the stomata playing only a minor role. They continued by saying that transport of foliar applied nutrients undoubtedly occurs via the phloem. They applied a drop of various radioactive isotopes onto the upper surface of a primary leaf blade of *P. vulgaris* and studied its absorption and transport throughout the plant. They found that carrier free $^{35}SO_4$ was absorbed through the leaf at an intermediate rate, but was highly mobile within the plant. Rennenberg *et al* (1979) presented evidence that translocation of reduced sulphur towards the roots, applied via the foliage occurs, in the phloem. Chromatography revealed that the main transport form of reduced sulphur in *Nicotiana tabacum* was as glutathione with 67-70%, methionine as 27-30% and cysteine as 2-8%. Bonas *et al* (1982) continued this work with *Ricinus communis* and concluded that reduction of sulphate occurred predominantly in the leaves in the light. 20-40% of the $^{35}S$ moved in the form of organic sulphur compounds, but the bulk was transported as inorganic sulphate. They again found that the most important translocated organic sulphur compound was glutathione.

Garsed and Mochrie (1980) found that radiolabelled sulphur applied to the leaves of *Vicia faba* was incorporated into *Megoura viciae*. The sulphur was applied as $Na_2{}^{35}SO_3$ and was subsequently present in the leaves as sulphite, sulphate, and organic material. The aphids contained sulphate and soluble organic compounds but no sulphite, and the honeydew contained only sulphite and sulphate. In the work presented here no attempt was made to distinguish between sulphur containing compounds in the aphid, or in the honeydew, but on the basis of this paper it seems likely that since the $^{35}S$ was applied as sulphate this would be found both within the aphid and the honeydew, but that organic sulphate would be found within the aphid only with none found within the honeydew. It is important to note that the $^{35}S$ present within the plant, aphid and honeydew is not

the only sulphur present in the system (Canny and Askham, 1967), since it is also present in trace amounts in both the seed and the nutrient solution. This however, cannot be included in the interpretation of the results.

Despite the literature reported above, in the experiments presented here the use of a hand held radioactive monitor suggested that the distribution of labelled sulphur within the plant was not extensive following direct application to the leaves. Hot spots of activity on the surface of the leaves at the points of application were observed, which suggested poor absorption of sulphate and poor mobility within the phloem. The soluble sulphur phase, if retained, would also have given no information concerning the sulphur within the leaf, and therefore that which was accessible to the aphid, since a large proportion of the sulphate was that from the leaf surface.

Experiment 2 was conducted in an attempt to overcome these problems. The isotope was added to the nutrient solution of hydroponically growing plants and any radioactivity isolated from the dried aerial tissues could therefore be attributed solely to sulphur within, as opposed to on, the leaf. Some of the soluble phase is that accessible to the aphid. Biddulph *et al* (1956) grew red *Phaseolus vulgaris* in a Hoagland type nutrient solution supplemented with $^{35}SO_4$ and found extensive movement of the isotope within the plant. They state that part of the sulphate being moved upwards in the xylem stream was trapped in organic synthesis. This is the fraction in the two experiments reported here which is designated 'bound' sulphur, and it is assumed to be unavailable to the aphid. Kylin and Hylmo (1957) grew wheat hydroponically and found good distribution of $^{35}S$ to the aerial parts of the plant. They found that uptake and transport was due to two different processes, one passive, depend nt upon transpiration, and the other active depending on bleeding mechanisms. The metabolic process dominated at low external concentrations, whereas the passive process predominated under conditions of high sulphate in the medium. Kylin (1953) found that the methionine and cysteine, and possibly the glutathione of hydroponically growing deseeded wheat plants were labelled with $^{35}SO_4$, which indicates that these important amino acids in terms of aphid nutrition would also be labelled in the experiments presented here. These would therefore form part of the radioactively labelled soluble phase. Banks and Nixon (1959) conducted aphid feeding and excretion studies using a similar method to that employed here. They grew *Vicia faba* hydroponically, incorporated $^{32}P$ into the nutrient solution, and subsequently measured the radioactivity found in *Aphis fabae* and its honeydew.

Kylin (1960) classifies the incorporation of radio-sulphate into three fractions; sulphate, soluble organic sulphur and protein sulphur fractions. The fractions separated in the investigations presented here are the protein sulphur fractions - termed 'bound sulphur', and the sulphate and the soluble organic sulphur - termed the 'soluble sulphur'. Both the sulphate and the soluble organic sulphur can be utilized by the aphid, as discussed in chapter 5.

Busgen (1891 - in Gray and Fraenkel, 1954) first proposed a theory for the production of honeydew. He suggested it was necessary since 'the insects are attempting to obtain a needed supply of protein which apparently is present in small quantities in plant sap.' The theory continues that 'in the process of obtaining the protein supply, excess carbohydrates and water are imbibed, and these are eliminated as honeydew.' This proposition is still believed broadly to be the case. The results presented in this chapter conclusively demonstrate a reduction in the quantity of honeydew produced by aphids on ozone fumigated plants. This decrease is concomitant with an increase in aphid growth rate which strongly suggests an improvement in the quality of the phloem sap, in terms of aphid nutrition. The decrease was seen both for the plants to which sulphate was applied, and for the untreated plants.

The reduction in honeydew quantity on a superior host plant indicates that aphids are able to tap phloem sap as required (see Jones and Jones, 1984). The controlling mechanism is in the insects head and thorax. When muscles of the anterior alimentary canal wall contract, sap under pressure is admitted from the stylet food canal into the oesophagus and stomach and, when the muscles relax, the anterior wall returns by elasticity to its normal position, and shuts off the flow of sap. Mittler (1958) suggested that lower nitrogen levels in plant sap are compensated for by a higher uptake rate which in turn leads to a greater production of honeydew. If the phloem sap is of better quality aphids need consume less, and if they consume less it passes through the gut at a reduced rate. This facilitates improved efficiency of extraction of nutrients which therefore permits reduced phloem sap uptake, and reduced honeydew production. This is similar to a positive feedback mechanism. (see Scriber and Slansky, 1981). An individual aphid on a polluted plant therefore may exert a less detrimental effect on the plant, than an aphid on a plant not exposed to ozone, since it withdraws a reduced

quantity of phloem sap. However, since that aphid is then able to grow more quickly and be more fecund the effect will be an increase in the size of the resulting population and probably greater damage in the longer term.

Srivastava and Auclair (1974) demonstrated, using artificial diets, that the amino acid concentration reached an optimum for aphid performance, above which performance was impaired. The optimum for the pea aphid (*Acyrthosiphon pisum*) was 3.5%. The literature concerning ant tended aphids provides further information concerning the ability of aphids to alter the rate of production and composition of honeydew. For example Banks and Nixon (1958) state that ant attended aphids (*Aphis fabae*) directly control their rates of exretion and feeding, and that these are not determined solely by forces within the plant. Maxwell and Painter (1959) found that the uptake rates of the greenbug (*Toxoptera graminum*) and the spotted alfalpha aphid (*Therioaphis maculata*) were influenced by changes in temperature, different parts of the plant as feeding sites, different varieties of plants, and the amount of light reaching the host plant and feeding site.

Excess quantities of honeydew can be unfavourable, especially on a crop plant. For example honeydew produced by the vine mealy bug (*Planococcus vitis*) causes infection of the fruit with a black sooty fungus, decreasing its market value, and honeydew encourages the growth of saprophytic moulds (Warrington *et al*, 1987). It may also be a hindrance in the harvesting of certain crops where cutting or threshing is necessary, and may interfere with photosynthesis and pollination of flowers (Maxwell and Painter, 1959). Crops growing in an environment experiencing ozone episodes may therefore be prone to fewer problems of excess honeydew.

No decrease in honeydew production is observed on the plants polluted with nitrogen dioxide, and this together with no difference in the intrinsic rate of natural increase data (see chapter 4), suggests that the increase in mean relative growth rate observed following this pollution regime, although significant at the 4% level, is the result of a type 1 statistical error and is in fact a spurious result. Further unmeasured parameters may be the explanation for the increase in MRGR, but additional MRGR studies and possibly plant physiological and biochemical would be needed to confirm this.

Both the studies presented here of the composition of honeydew, indicate that the patterns of amino acids found do not reflect the amino acid composition of the plant - as elucidated in chapter 4. This may be for a number of reasons. Firstly, the composition

of amino acids of the plant material is a sample of the whole plant material and is not necessarily the same as the phloem contents. Secondly the quality of the honeydew may be the result of digestive processes within the aphid. Alternatively the amino acid composition of the plant following sulphate application may be different from that observed without sulphate application, and the honeydew may in fact be an accurate reflection of the plant composition. This could have been tested by amino acid analysis of the plant material, however this was not possible due to technical problems. Honeydew was therefore collected and analysed from aphids on plants which were not treated with sulphate. This produced different results, and demonstrated that the honeydew was not a reflection of the amino acid constituents of the plant as found in chapter 4.

The first examination of the amino acid constituents of aphid honeydew found 22 free amino acids and amides (Maltais and Auclair, 1952). Initial studies comparing the amino acid quality of the host plant and the honeydew produced by aphids feeding on the plant, proposed that the honeydew was a direct reflection of the phloem constituents. A short review paper by Mittler (1953) supported this view and stated that; "The free amino acids detected in the honeydew seem to come directly from the ingested sap, and are not to be regarded as products of protein breakdown or of the fixation of atmospheric nitrogen supposedly through the agency of the aphid's symbionts." Mittler (1958) reiterated this using willow (*Salix acutifolia*) and *Tuberolachnus salignus*. He found that the composition of phloem sap was identical to that of the honeydew, but within the honeydew each amino acid and amide was present at a lower concentration. Saleh and Salama (1971) also presented supporting evidence for this using their work with the vine mealy bug (*Planococcus vitis*) and its host plant - potato sprouts (*Solanum tuberosum*). If this is indeed the case the explanation for the differences in the honeydew composition and the plant material composition, found in the work reported here, must be that the total plant material composition does not accurately reflect the composition of the ingested phloem sap. This is however, rejected by van Emden's statement (1966) that the amino acid composition of the whole plant material does accurately reflect the composition of the phloem, since most of the free amino acids within the whole plant material are situated within the phloem.

Work contesting the view that honeydew composition is simply a reflection of the phloem sap was initially suggested by Gray (1952). His work with the pineapple mealy bug (*Pseudococcus brevipes*) demonstrated the presence of amino acids in honeydew which were not present in the food source. He suggested that this could be the result

of the activity of symbionts within the insect. Gray and Fraenkel (1954) produced evidence from work with carbohydrates of honeydew of the citrus mealy bug (*Pseudococcus citri*) which indicated that honeydew was a digestive product. Bragdon and Mittler (1963) reported differential absorption of amino acids from artificial diets, and concluded that the amino acid composition of honeydew excreted by aphids feeding on a plant may not always be a reliable guide to the quantitative or the qualitative amino acid composition of the phloem. Salama and Rizk (1969) found five amino acids in the honeydew of the sugar cane mealy bug (*Saccharicoccus sacchari*) which were not present in the plant sap, which they proposed may be due to the presence of peptidases in the insect gut which digested imbibed protein molecules.

The experiments reported here support the proposition that the phloem sap constituents are changed on passage through the aphid, but the question remains unresolved. The only similarity between the two honeydew data sets is the significant increase in the 'amide' fraction in honeydew from aphids on ozone fumigated plants, which probably indicates that this is in excess within the plant. The overall increase in amino acids is a further similarity and it does appear that this is due to an increased concentration within the phloem - especially when the plant amino acid data is considered.

# CHAPTER 7

# A Long Term, Low Level Ozone Fumigation Study.

## 7.1 Introduction

The experiments of the previous chapters considered the effects of acute fumigations with levels of pollutants designed to elicit a response. This study considers the effect of a longer term (5 weeks), low level (30 ppb) exposure to ozone on various wheat and aphid responses. An acute ozone dose was delivered at the end of the fumigation, to half of the plants, to investigate the importance of the previous history of the plant in its response to such a stress. Ozone delivered for 72 hours at 100 ppb was chosen for this final fumigation since previous experiments demonstrated a significant increase in aphid growth rate following this regime. The plant and aphid responses considered were:

1) The subsequent mean relative growth rate (MRGR) and intrinsic rate of natural increase ($r_m$) of the aphid
2) The photosynthetic rate and leaf conductance of the plant
3) The visible damage to the plant
4) The dry weight of the plant
5) Biochemical analysis of the plant, including:-
   Soluble nitrogen
   Total nitrogen
   Reducing sugars and
   Glutathione.

The ethylene inhibitor AVG (aminoethoxyvinylglycine) was applied to half of the plants, 24 hours before the final fumigation, to attempt to protect the plants from the adverse effects of the fumigation. An additional separate experiment was subsequently carried out to further investigate the effects of AVG. Ethylene is normally produced by all plants, in trace amounts and it is thought to interact with a wide variety of developmental processes. However, when plants experience environmental stress they produce large amounts of ethylene - called stress ethylene. AVG is used to inhibit the formation of stress ethylene. It inhibits 1-aminocyclopropane-1-carboxylic acid (ACC) synthase during ethylene biosynthesis, and if ethylene evolution is responsible for ozone injury, AVG should provide some protection. Taylor *et al* (1988), for example, demonstrated the successful suppression of ethylene production using AVG, and found

that following ozone stress, the response of stomatal conductance to water vapour, and carbon dioxide assimilation, was substantially diminished. Melhorn and Wellburn (1987) suggested that damage due to ozone was caused by its reaction with small unsaturated hydrocarbons, such as ethylene. This produces water soluble, highly reactive free radicals, which may initiate peroxidative events inside plant cells, which could eventually cause leaf necrosis.

## 7.2 Materials and Methods

Ninety six pots were filled with California D3 mix (as in previous chapters) and four wheat (cv. Ralle) seeds planted in each. The pots were placed on 8 well watered trays for 4 days until coleoptiles appeared, at which point the plants were randomised within and between the trays, and one tray was placed in each of the 8 chambers of the ozone greenhouse. The greenhouse specifications were as described in chapter 2. 10 days after planting the seedlings were thinned to one per pot, with the strongest seedling retained in each case. Thirty two of the 'thinnings' were replanted and 4 plants placed in each chamber. These were used for an assessment of the effects of the long term fumigation, prior to the final acute fumigation. During the long term fumigation half of the chambers received ozone at 30 ppb from 0900 to 1700, and filtered air at night, whilst the other half constantly received filtered air. The experiment was conducted in January and February and the temperature in the chambers therefore remained at 20°C during the day (0600 - 2200) and 15°C at night.

24 hours before the start of the acute fumigation a 1mM solution of AVG in 0.05% Tween 20 (used to improve leaf wetting ability) was 'painted' onto all the above ground parts of half of the plants. The other half of the plants were painted with Tween 20 only.

Immediately before the start of the final acute fumigation the 32 plants used for the long term low level study only were removed from the chambers and leaf conductance and photosynthetic rate measurements made (see below). The section of leaf used to make these measurements was used for the ethylene evolution study (see below). The remainder of the plant was cut off at soil level and immediately freeze dried. The dry weight was recorded five days later.

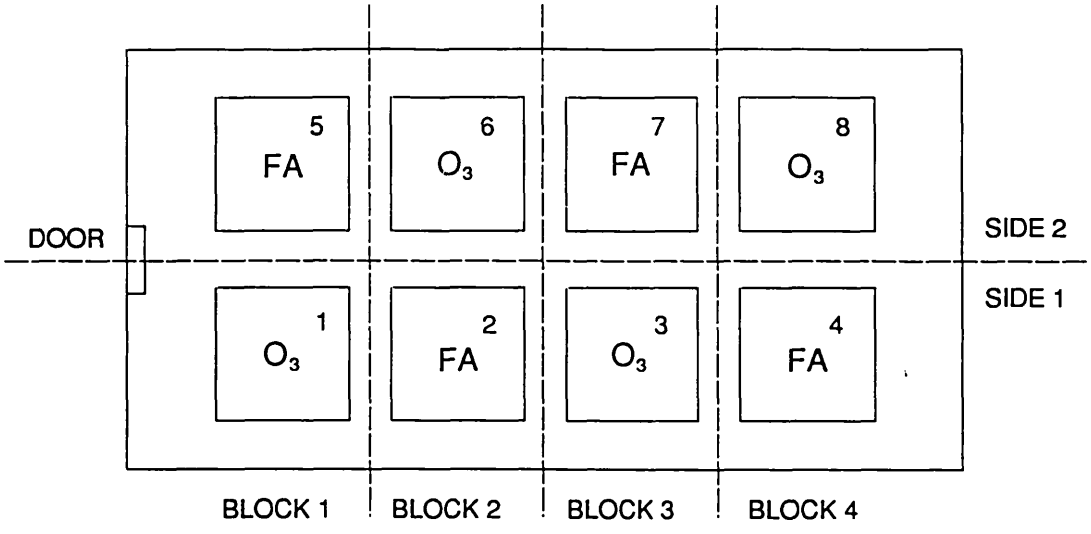


**Figure 7.1 Position of Fumigation Chambers**

Immediately before the acute fumigation half of the plants in each chamber were exchanged with plants in the neighbouring chamber. (i.e. 6 plants were transferred from chamber 1 to chamber 2, and 6 transferred from chamber 2 to chamber 1. Plants were similarly exchanged between chambers 3 and 4, 5 and 6, and 7 and 8.) This meant that half of the plants experiencing the acute fumigation had previously experienced filtered air, and half had experienced 30 ppb ozone. Plants were exchanged between chambers on the same side of the greenhouse. Three of the plants exchanged in each case had received AVG with Tween 20 and three Tween 20 only.

There were therefore 4 treatments in each chamber, each with three replicates. These were;

1 Filtered Air Chambers containing plants which had received:-

(i) Filtered air for 5 weeks, followed by filtered air for 3 days - no AVG
(ii) Filtered air for 5 weeks, followed by filtered air for 3 days - plus AVG
(iii) Ozone at 30 ppb for 5 weeks, followed by filtered air for 3 days - no AVG
(iv) Ozone at 30 ppb for 5 weeks, followed by filtered air for 3 days - plus AVG

2 Ozone Chambers containing plants which had received:-

(i) Filtered air for 5 weeks, followed by ozone at 100 ppb 3 days - no AVG
(ii) Filtered air for 5 weeks, followed by ozone at 100 ppb for 3 days - plus AVG
(iii) Ozone at 30 ppb for 5 weeks, followed by ozone at 100 ppb for 3 days - no

AVG

(iv) Ozone at 30 ppb for 5 weeks, followed by ozone at 100 ppb for 3 days - plus AVG

Following the acute fumigation various measurements were made:-

### 7.2.1 MRGR and $r_m$

Four plants from each chamber (one from each treatment class) were removed to the C.T. room and a MRGR assessment undertaken (as in chapter 2). Following this the 5 day old aphids were retained and returned to the plants for an intrinsic rate of natural increase study (as in chapter 2).

There were therefore 8 remaining plants in each chamber, 2 of which had received exactly the same treatment. Various measurements were made on these plants:-

### 7.2.2 Photosynthetic Rate and Leaf Conductance

A portable infra red gas analyser (IRGA) system - the LCA-2 Leaf Chamber Apparatus (Analytical Development Company Ltd., Hoddesdon, Herts.) was used to measure these parameters. The system consists of an IRGA, a leaf cuvette, an apparatus to supply air to the cuvette (the Air Supply Unit), a data processor and logger.

The LCA-2 is an open system; the leaf is enclosed in a cuvette into which there is a measured supply flow of air of known water vapour and carbon dioxide concentration. The gas exchange rates are determined from the flow rate and concentration differences between the inlet and exhaust air. The cuvette is designed for broad leaved species where the lamina of the leaf should fill the whole pod. Since the elongate cereal leaf did not fill the pod, the leaf area within the pod, contributing to the measurements, was found (to the nearest 0.01 of a mm), using a computer leaf area meter.

The cuvette contains an air temperature sensor, a PAR sensor, and a humidity sensor, positioned in the exhaust air from the cuvette, and a fan. The fan ensures there is little boundary layer resistance and therefore that there is a rapid response. Air emerging from the cuvette (the analysis stream), and a sample of air emerging directly from the air supply unit (the reference gas stream) are passed to the IGRA. The inlet tube to the air supply unit was suspended in the greenhouse roof. The cuvette was normally enclosed around the leaf for a minute, with a stable reading generally obtained after 45 seconds. The IRGA measures the difference in carbon dioxide concentration of these two air streams, and the product of this and the flow rate per unit leaf area gives the

photosynthetic rate. The leaf conductance is given by the saturated vapour pressure (at leaf temperature), divided by the water vapour pressure in the air emerging from the cuvette, all divided by the difference between the mass flow of air through the cuvette (per unit leaf area) minus the boundary layer resistance of the leaf. Further details of the calculations can be found in the company manuals (Analytical Development Corporation), and in Parkinson *et al* (1988).

The cuvette was placed to enclose the leaf at the position at which the aphids are put in MRGR studies. This was to ensure that the parameters found are those which the aphid would experience.

### 7.2.3 Visible Damage Assessment

The assessment of visible damage was made without knowledge of the treatment each plant had received. It was made for each leaf individually. Leaves were looked at in terms of tillers. The oldest tiller was designated tiller 1, and the oldest leaf within that tiller, leaf 1. The second oldest leaf within the tiller was designated leaf 2, and so on. The second oldest tiller was designated tiller 2, and the oldest leaf designated leaf 1 as before. Up to 4 tillers per plant were recorded and some tillers comprised 5 leaves. However, this varied greatly between plants. The leaves were scored as the percentage area showing any visible damage. This ranged from slight flecking to severe necrosis, but no account was taken of this in the scoring. Assessment was made to the nearest 10%. The data were analysed using binomial errors.

### 7.2.4 Dry Weight

Following the visible damage assessment the plant was cut off at soil level and immediately freeze dried. The total above ground dry weight was recorded five days later.

### 7.2.5 Biochemical Analysis

The dry plant was ground using a ball mill, and retained for biochemical analysis. Analyses of soluble and total nitrogen, reducing and total soluble sugars and glutathione concentration were carried out (as in chapter 4). The total soluble sugar results were unreliable and are therefore not presented.

### 7.2.6 Ethylene Evolution

The particular portion of leaf used for the photosynthesis and leaf conductance measurements was retained for the ethylene study. The protocol used was exactly as that of Melhorn and Wellburn (1987), but unfortunately the ethylene peaks recorded were not significantly different from the background flame noise level, and no firm conclusions could be drawn. This was probably because the detection limit of the gas liquid chromatograph was too high. The results are therefore not presented.

### Data Analysis

During the experiment it became apparent that side 1 of the greenhouse received more light than side 2 (see Figure 7.1 - greenhouse diagram). The data was therefore blocked, as shown in the diagram, and this used as a factor in the analysis. The results from the plants used prior to the final acute fumigation were analysed as two-way ANOVAs in SX, using block and air quality as factors. The results from the plants used in the final acute fumigation were analysed as 4-way ANOVAs in Glim, using chronic air quality, acute air quality, AVG application and block as factors.

## 7.3 Additional Experiment

### 7.3.1 Introduction

Following the analysis of the results it became apparent the application of AVG did not have the predicted effect. This was therefore further investigated.

### 7.3.2 Materials and Methods

Forty wheat plants were grown in the greenhouse until five weeks old, as described in chapter 2. 24 hours prior to fumigation a 2mM solution of AVG in 0.2% Tween 20 was painted onto all the above ground parts of half of the plants. 0.2% Tween 20 only was painted onto the other plants. Immediately prior to fumigation the plants were transferred to the ozone fumigation chambers so that half the plants in each chamber were painted with AVG and half with Tween 20 only. Two filtered air and two ozone chambers were used. Fumigation was conducted at 100 ppb for 72 hours as before. The temperatures in the chambers did not exceed 20°C.

Following fumigation a visible damage assessment was made of each leaf of each plant without prior knowledge of the exposure regime of each plant. Damage was scored in the same way as above. A MRGR assessment using two day old *M. dirhodum* nymphs

was then conducted in the C.T. room. (as in chapter 2). The data was analysed in Glim as three way ANOVAs using air quality received, AVG application or not, and block (where each block contained a treatment and a control chamber) as factors.

## 7.4 Results of Long Term Low Level Ozone Study.

### 7.4.1 Results of Plants Assessed Prior to the Final Acute Fumigation.

#### 7.4.1.1 Photosynthetic Rate and Leaf Conductance.

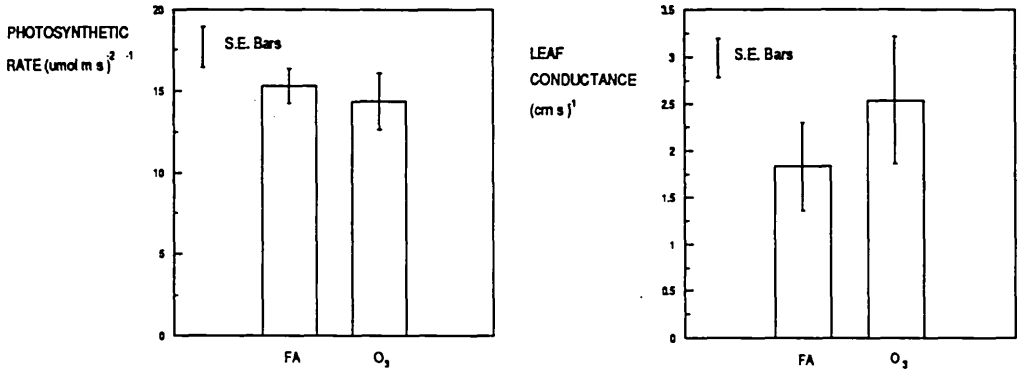


**Figure 7.1** **Photosynthetic Rate and Leaf Conductance Following 5 Weeks of Fumigation With Filtered Air or Ozone (at 30 ppb).**

There was no significant difference in either parameter. The level of each parameter is high as a result of the very strong sunlight whilst the measurements were being made. There were no significant differences between the blocks, or the interaction between blocks and air quality.

### 7.4.1.2 Dry Weight Measurements

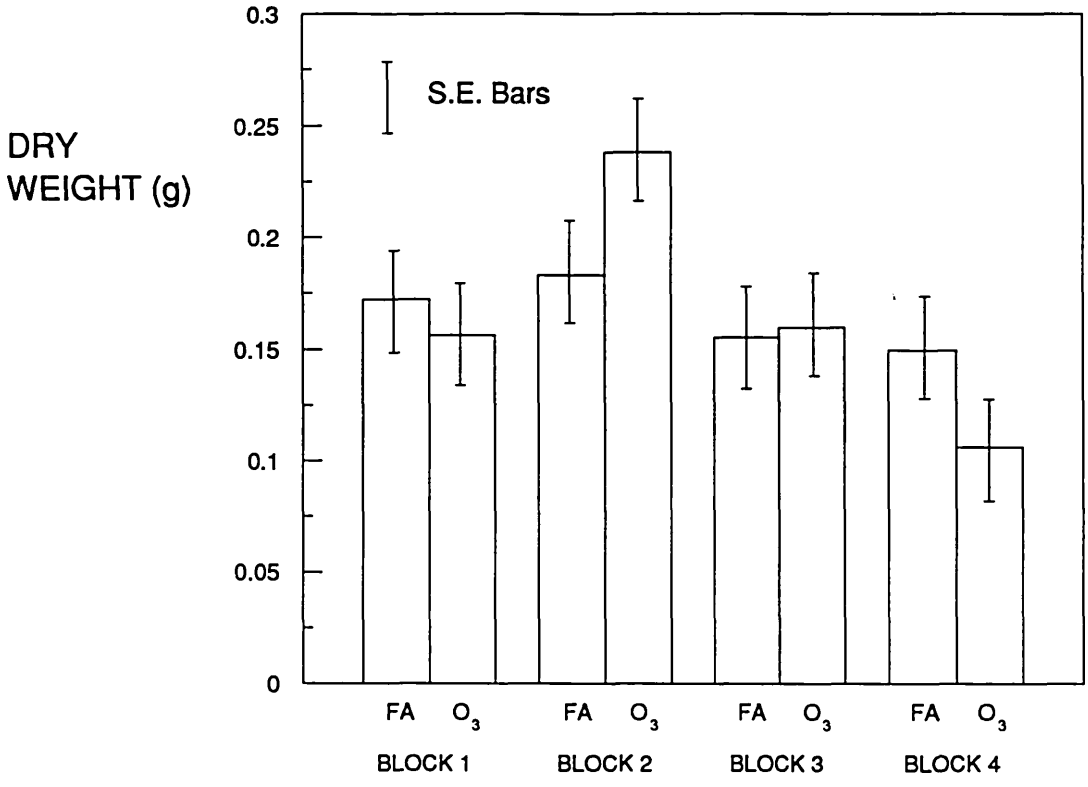


**Figure 7.2** **Above Ground Plant Dry Weight Following 5 Weeks of Fumigation With Filtered Air or Ozone (at 30 ppb).**

This shows that there was no overall difference in the dry weight of the plants in either the filtered air or the ozone treated plants. However, as Table 7.1 shows there was a significant difference in the dry weights between the blocks ($p < 0.012$). There was no significant interaction between the air quality received (chronic) and the block.

**Table 7.1** **Analysis of Variance Table for Pre-Fumigation Above Ground Plant Dry Weight.**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Chronic | 1 | 0 | 0 | - | - |
| Block | 3 | 0.0282 | 0.0094 | 4.54 | 0.012 |
| Chronic.Block | 3 | 0.0103 | 0.0034 | 1.66 | 0.202 |
| Error | 24 | 0.0498 | 0.0021 | | |
| Total | 31 | 0.0884 | | | |

### 7.4.2 Results of Plants Assessed Following the Final Acute Fumigation.

#### 7.4.2.1 MRGR and $r_m$

**1 Mean Relative Growth Rate (MRGR).**

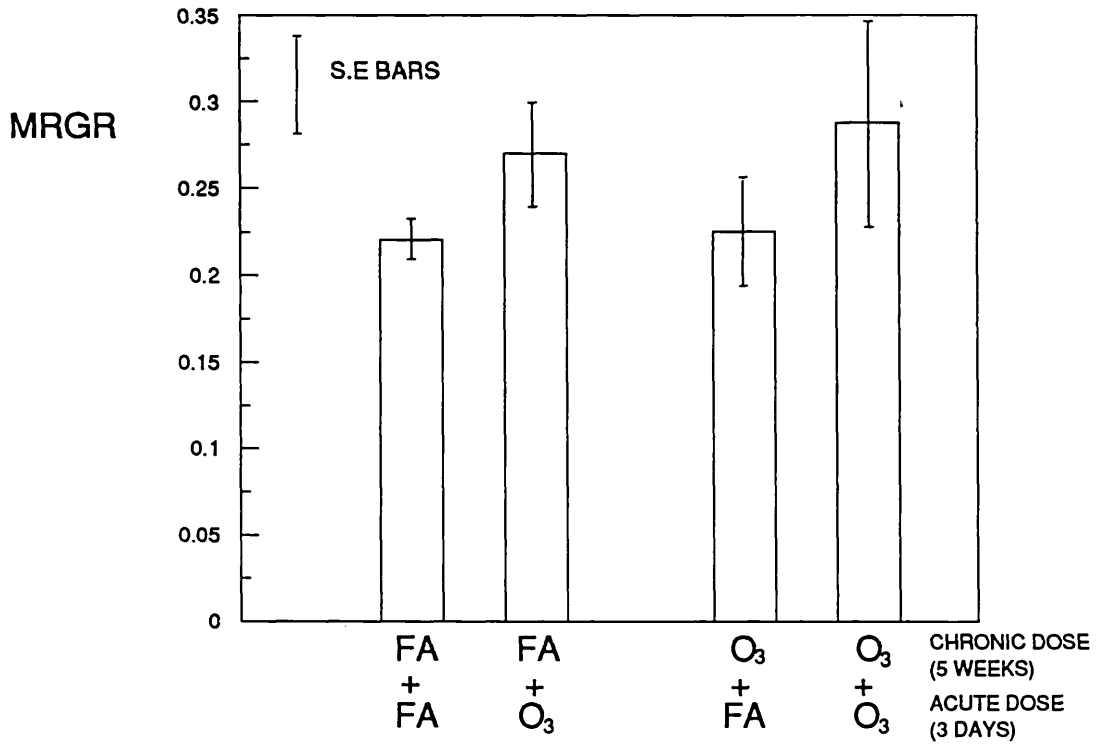


**Figure 7.3** **MRGR of Aphids Following Fumigation of Plants With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days.**

The only main effect which differed significantly ($p < 0.05$) from the control was the effect of the final acute fumigation. This caused a significant increase in the growth rate of the aphids. The five week chronic fumigation had no effect on the growth rate and neither did the interaction between this and the acute fumigation. This indicates that the previous exposure of the plants to either ozone or filtered air did not alter the response of the plants, as measured by aphid performance, to the final acute dose.

The AVG also had no effect on the growth rate of the aphids. There were no significant interactions between AVG application and the other factors, or between the blocks and the block interactions. The analysis of variance was therefore repeated excluding these factors and the results of the two-way analysis are presented below.

**Table 7.2 Analysis of Variance Table for MRGR of the Aphids.**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 0.03056 | 0.03056 | 4.29 | 0.05 |
| Chronic | 1 | 0.00126 | 0.00126 | 0.18 | 0.67 |
| Acute.Chronic | 1 | 0.00043 | 0.00043 | 0.06 | 0.81 |
| Error | 23 | 0.16367 | 0.00712 | | |
| Total | 26 | 0.19601 | | | |

## 2 Intrinsic Rate of Natural Increase ($r_m$)

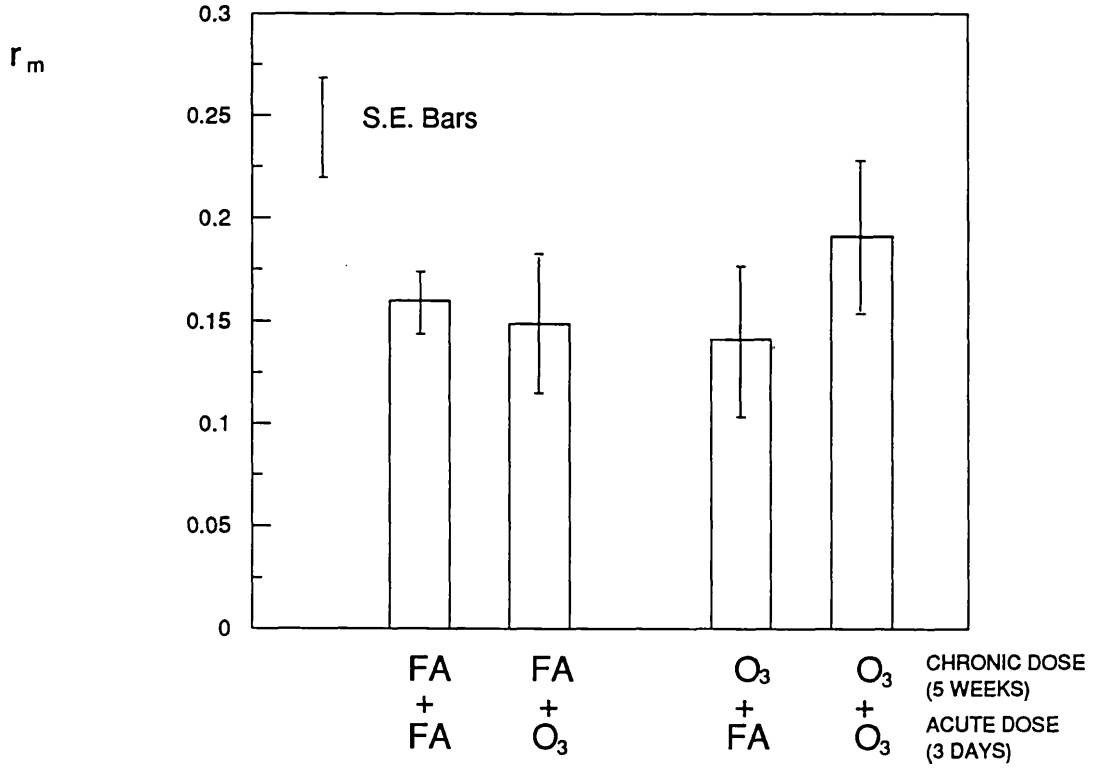


**Figure 7.4** **$r_m$ of Aphids Following Fumigation of Plants With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days**

None of the main effects or the interaction terms were significantly different from the controls. The MRGR study indicated that an increase in $r_m$ following the final acute fumigation irrespective of the chronic fumigation would have led to an increase in $r_m$.

However, a slight decrease following the acute fumigation of plants previously grown in filtered air was seen. This may be due to the death of many aphids and therefore decreased accuracy of the statistic (n = 19 out of 128 initially). The visible damage experienced by the ozone fumigated plants (see below), and the long time needed to record this parameter combined to reduce the aphid survival. The two-way ANOVA table is presented below.

**Table 7.3 Analysis of Variance Table for $r_m$**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 0.000620 | 0.000620 | 0.15 | 0.70 |
| Chronic | 1 | 0.000654 | 0.000654 | 0.16 | 0.69 |
| Acute.Chronic | 1 | 0.005452 | 0.005452 | 1.33 | 0.27 |
| Error | 15 | 0.061615 | 0.004108 | | |
| Total | 18 | 0.068340 | | | |

## 7.4.2.2 Photosynthetic Rate and Leaf Conductance

### 1 Photosynthetic Rate

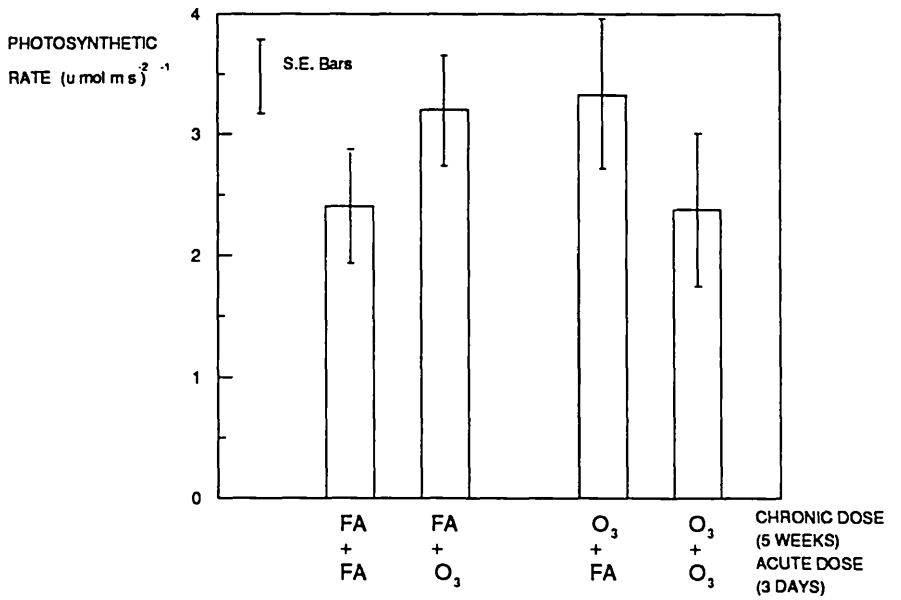


**Figure 7.5** **Photosynthetic Rate Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days.**

None of the main effects or the interaction terms were significantly different from the controls. There is a suggestion that the response to the final ozone fumigation may have depended on the chronic fumigation, but the interaction term is not significant. The four-way analysis of variance table is presented in the appendix.

## 2 Leaf Conductance

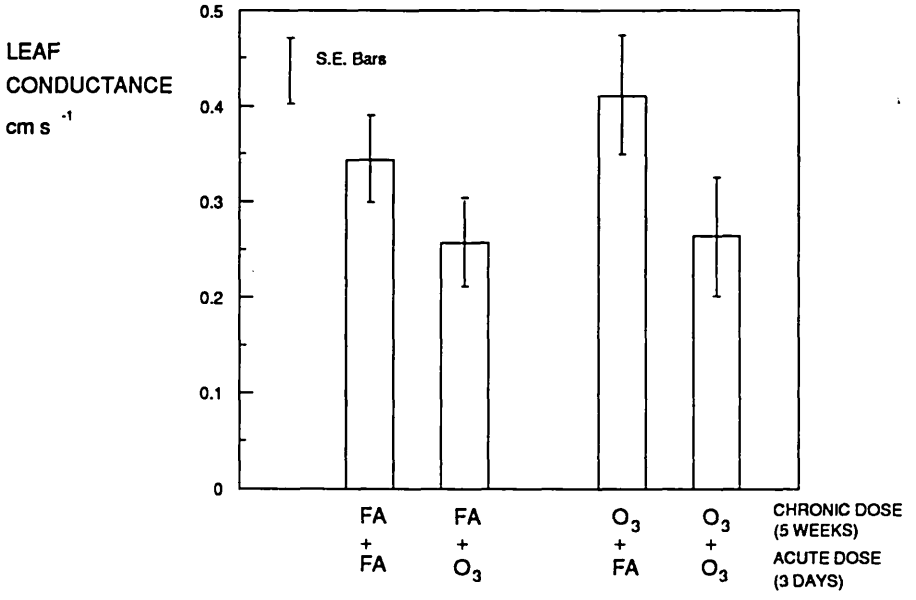


**Figure 7.6** **Leaf Conductance Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days.**

None of the main effects or the interaction terms were significantly different from the controls, but an almost significant ($p < 0.073$) decrease due to the acute fumigation was seen. There was no interaction with this and the previous chronic fumigation, indicating that the previous exposure to either ozone or filtered air was unimportant in the response to the acute fumigation. The four-way analysis of variance table is presented in the appendix.

### 7.4.2.3 Visible Damage

#### 1 For Tiller 1, Leaf 2

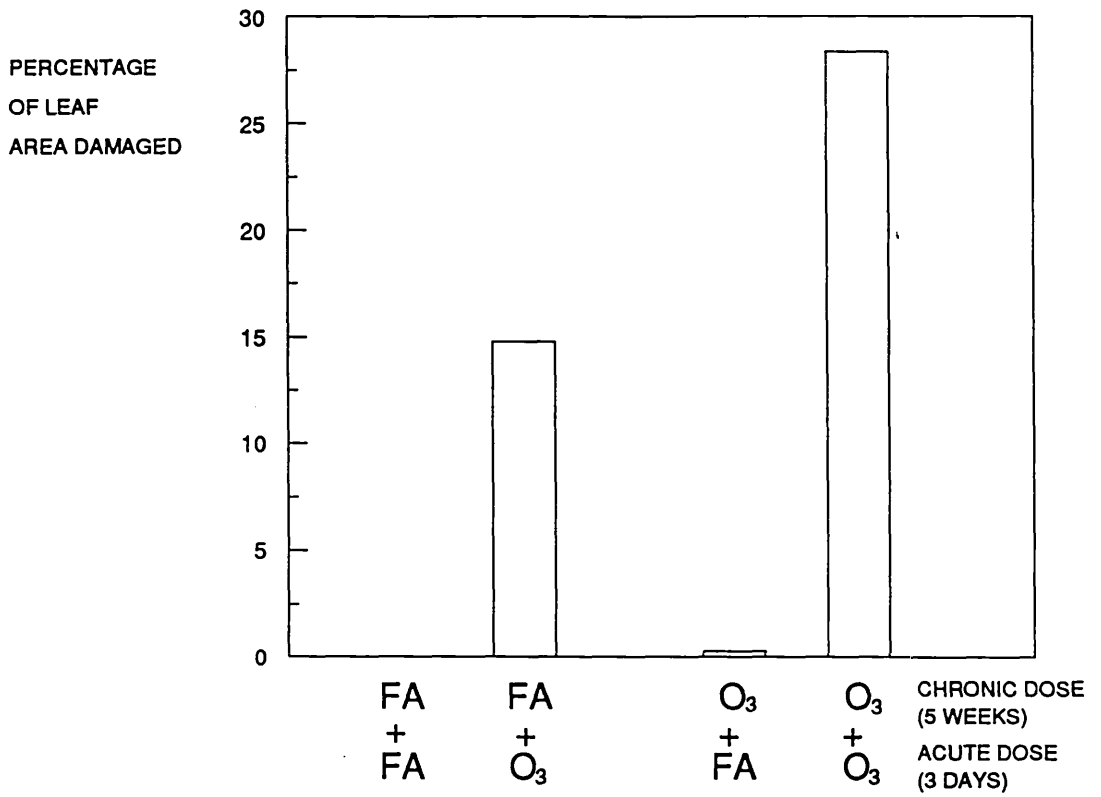


**Figure 7.7** **Percentage of Leaf Area Damaged (For Tiller 1, Leaf 2), Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days**

Figure 7.7 is for the damage experienced by the leaf which the aphid is placed on in the MRGR studies. The chronic ozone fumigation produced a significant increase ($p < 0.032$) in damage, as did the acute fumigation ($p < 0.0001$). Data for the other leaves demonstrate similar results. There was no significant interaction between the damage caused by the final fumigation and the previous air quality. The application of AVG did not alter the observed levels of damage, and therefore did not appear to provide protection as expected. There was also no significant difference between the damage experienced in each block. The data was therefore re-analysed as a two-way ANOVA, excluding the AVG and block factors, and the results are presented below.

**Table 7.4 Analysis of Variance Table for Visible Damage to Tiller 1 Leaf 2.**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 79.9 | 79.9 | 4.82 | 0.032 |
| Chronic | 1 | 973.0 | 973.0 | 58.8 | <0.001 |
| Acute.Chronic | 1 | 3.64 | 3.64 | 0.22 | 0.641 |
| Error | 57 | 943.1 | 16.54 | | |
| Total | 60 | 1999.8 | | | |

## 2 For the Whole Plant

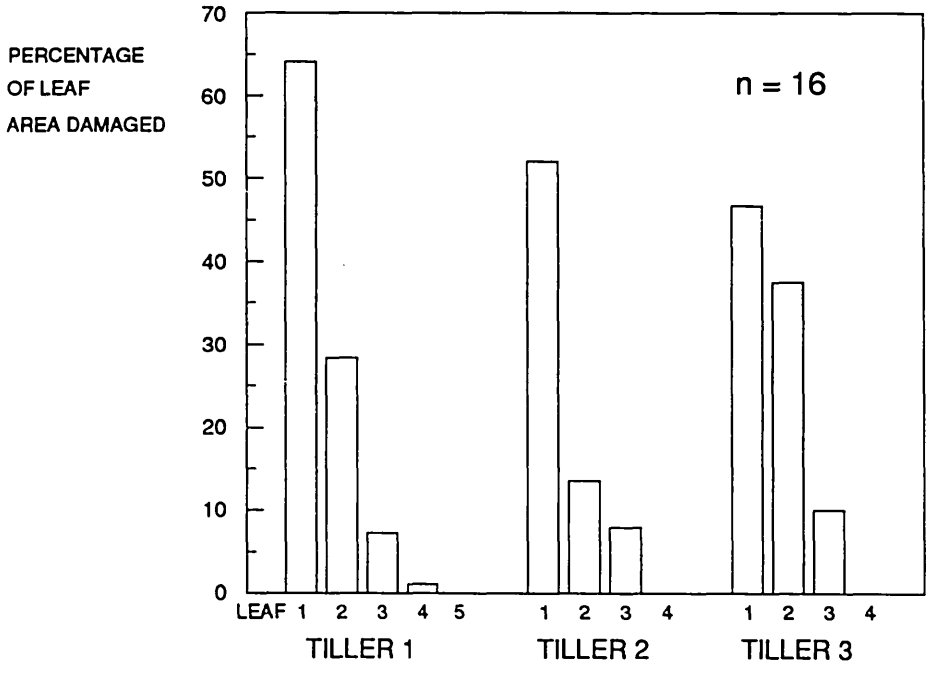


**Figure 7.8** **Percentage of Leaf Area Damaged for Each Leaf Following Fumigation With Ozone (at 30 ppb) for 5 Weeks, and Ozone (at 100 ppb) for 3 Days.**

This clearly shows a marked increase in visible damage to the older leaves. A greater degree of damage to leaf 1 of the older tillers was also seen, with leaf 1, tiller 1 showing greater damage than leaf 1 tiller 2, and with leaf 1 tiller 2 showing greater damage than leaf 1 tiller 3. There was also more visible damage the oldest part of the leaves - the tips, although this was not scored.

### 7.4.2.4 Dry Weight

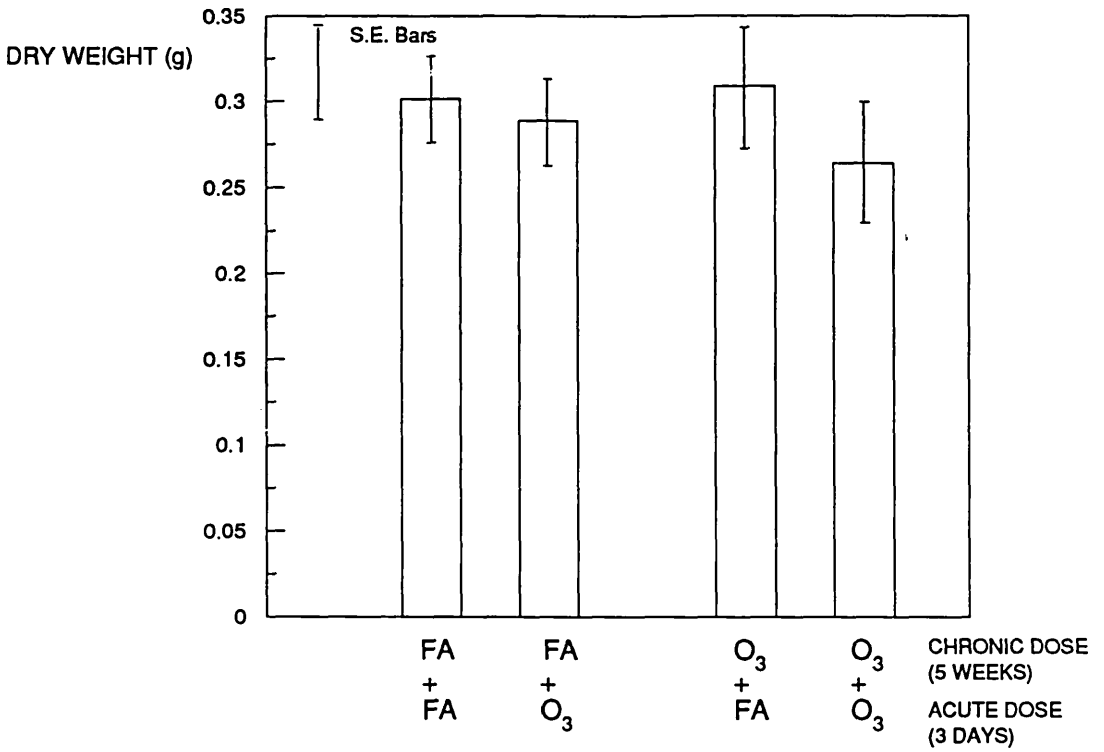


**Figure 7.9** **Dry Weight Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days.**

None of the main effects (chronic fumigation, acute fumigation, AVG application or block) were significantly different from control levels. All the interactions were also non-significant. This result endorses that found with the plants assessed prior to the final fumigation - when the dry weight also did not differ between control and treatment plants. The two way ANOVA table is presented below.

Table 7.5 Analysis of Variance Table for Post-Fumigation Above Ground Plant Dry Weight.

| SOURCE | d.f. | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 0.00034 | 0.00034 | 0.03 | 0.83 |
| Chronic | 1 | 0.00241 | 0.00241 | 0.23 | 0.63 |
| Acute.Chronic | 1 | 0.00542 | 0.00542 | 0.53 | 0.48 |
| Error | 60 | 0.61616 | 0.01027 | | |
| Total | 63 | 0.62434 | | | |

### 7.4.2.5 Biochemical Analysis

## 1 Soluble Nitrogen

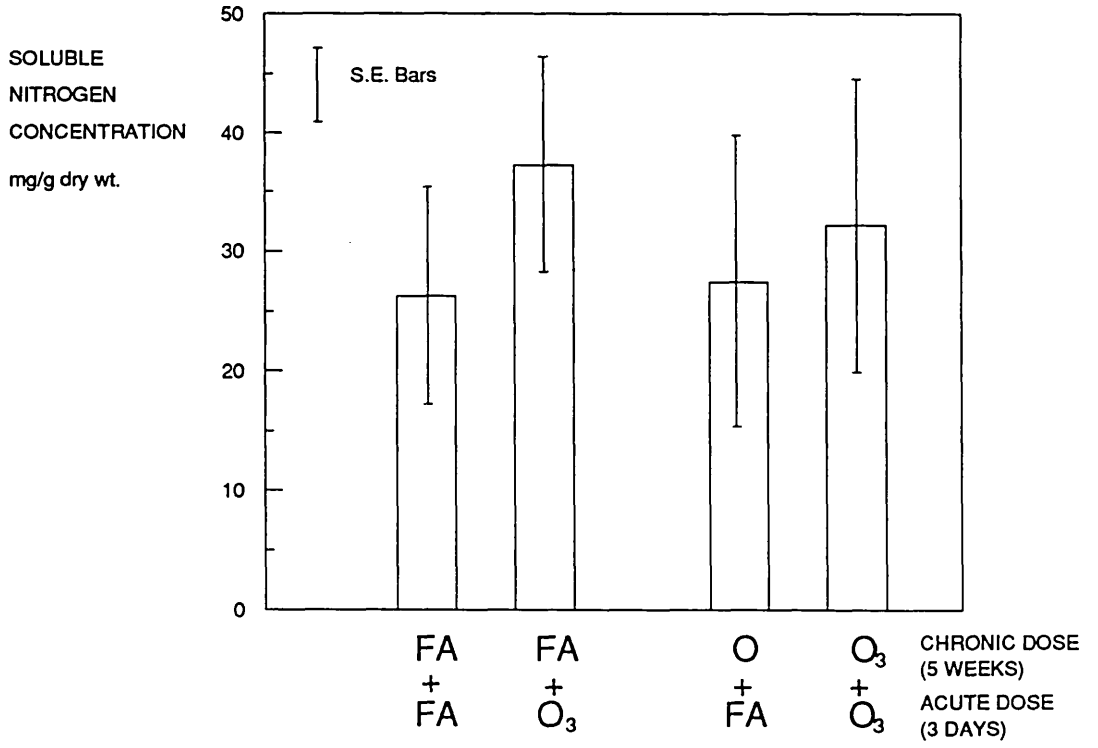


Figure 7.10 Soluble Nitrogen Levels Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days.

The soluble nitrogen levels are increased following the final acute fumigation, irrespective of the chronic fumigation exposure. However this is not significant because of the large errors involved. None of the main effects or the interaction terms are significantly different. The simplified two-way analysis is presented below.

**Table 7.6 Analysis of Variance Table for Soluble Nitrogen Levels.**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 942.4 | 942.4 | 0.924 | 0.34 |
| Chronic | 1 | 25.07 | 24.07 | 0.024 | 0.88 |
| Acute.Chronic | 1 | 75.71 | 75.71 | 0.070 | 0.79 |
| Error | 45 | 45912 | 1020 | | |
| Total | 48 | 46955 | | | |

## 2 Total Nitrogen

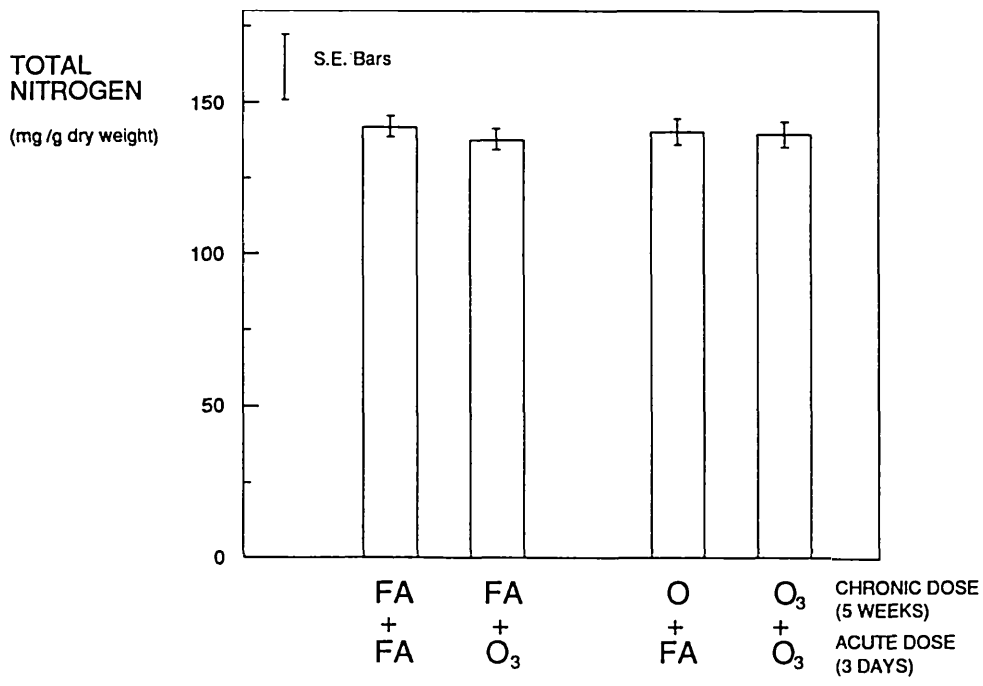


**Figure 7.11** **Total Nitrogen Levels Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days**

The total nitrogen levels show no significant difference due to any of the main effects or any of the interactions. The simplified two-way analysis is presented below.

**Table 7.7 Analysis of Variance Table for Total Nitrogen Levels.**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 130.0 | 130.0 | 0.700 | 0.41 |
| Chronic | 1 | 19.58 | 19.58 | 0.105 | 0.74 |
| Acute.Chronic | 1 | 2.723 | 2.723 | 0.015 | 0.87 |
| Error | 60 | 11131 | 185.5 | | |
| Total | 63 | 11284 | | | |

## 3 Reducing Sugars

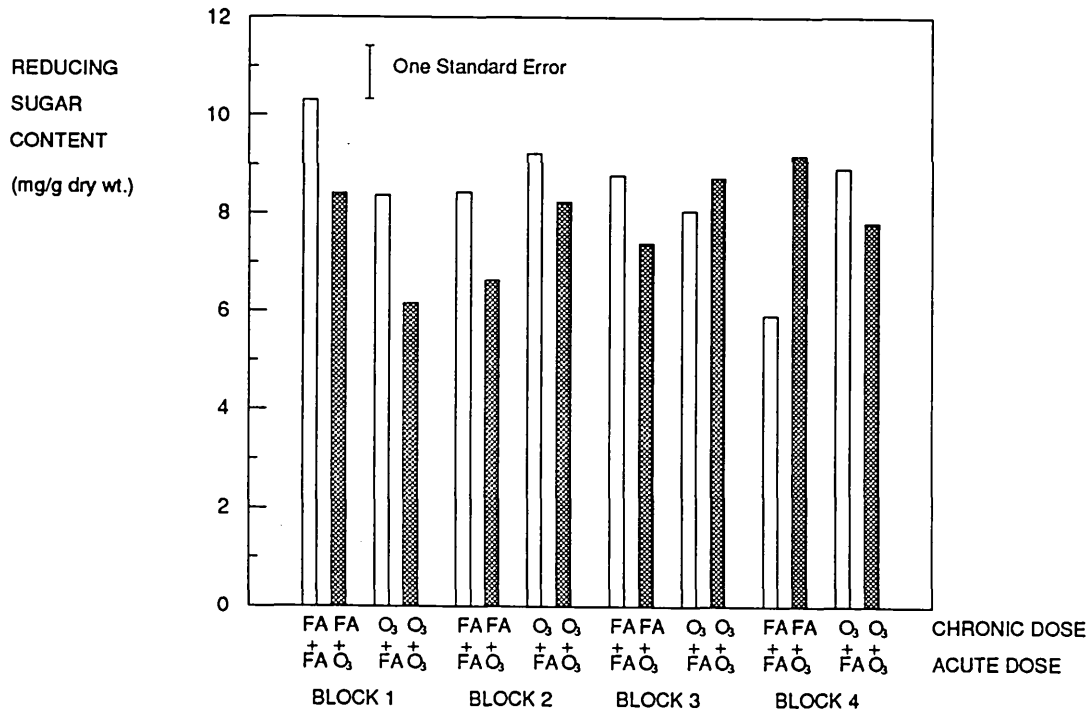


**Figure 7.12** **Reducing Sugar Levels in Each of the Four Blocks Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days**

The four way ANOVA demonstrated that there were significant interaction terms, but that none of them involved the effect of the AVG application. The data was therefore re-analysed as a three way ANOVA using chronic air quality, acute air quality and block as factors. This showed that there was a significant ($p < 0.048$) decrease in the levels of reducing sugars due to the final acute ozone fumigation. There was no significant difference in the concentration due to either the block or the chronic fumigation. However, there were significant interaction terms as shown in Table 7.8. The four-way analysis is presented in the appendix.

**Table 7.8 Analysis of Variance Table for Reducing Sugar Levels.**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 7.635 | 7.635 | 4.12 | 0.048 |
| Chronic | 1 | 0.032 | 0.032 | 0.02 | 0.888 |
| Block | 3 | 1.132 | 0.377 | 0.20 | 0.657 |
| Acute.Chronic | 1 | 0.918 | 0.918 | 0.49 | 0.487 |
| Acute.Block | 3 | 22.90 | 7.63 | 4.12 | 0.011 |
| Chronic.Block | 3 | 25.51 | 8.50 | 4.59 | 0.007 |
| Acute.Chronic.Block | 3 | 23.29 | 7.76 | 4.19 | 0.010 |
| Error | 47 | 87.05 | 1.85 | | |
| Total | 62 | 168.47 | | | |

# 4 Glutathione Levels

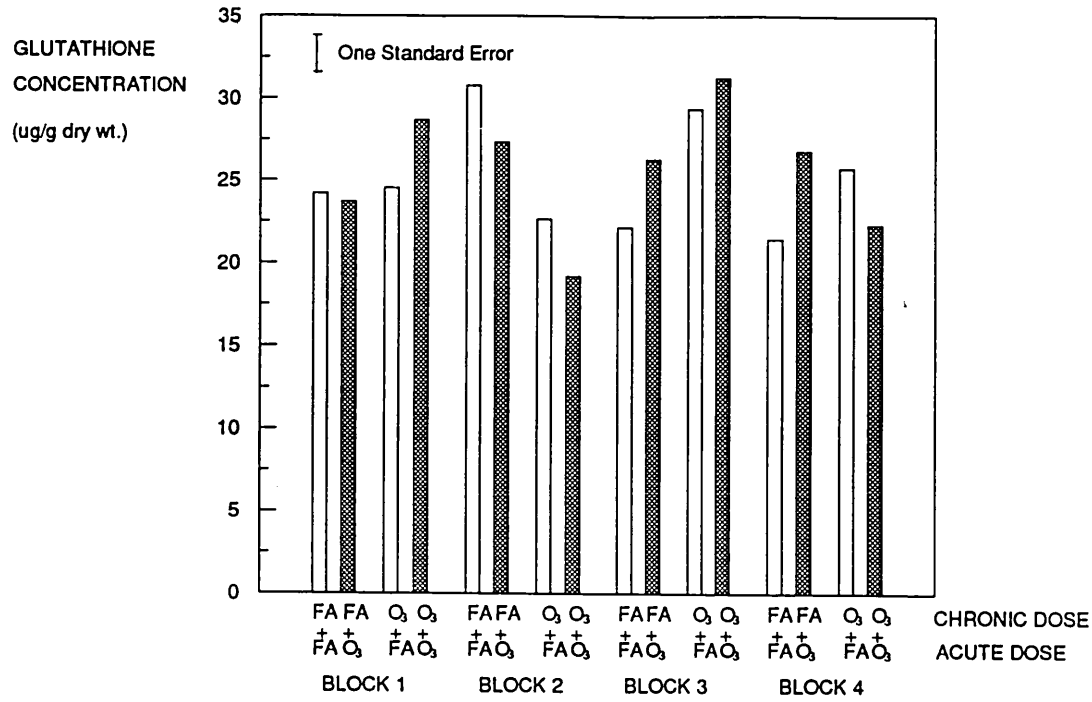


**Figure 7.13** **Glutathione Levels in Each of the Four Blocks Following Fumigation With Filtered Air or Ozone (at 30 ppb) for 5 Weeks, and Filtered Air or Ozone (at 100 ppb) for 3 Days**

The analysis was initially carried out as a four way analysis of variance. Since the AVG was shown to have no significant effect the data was re-analysed as a three way ANOVA. This showed that there was a significant interaction between the air quality received during the chronic fumigation and the block ($p < 0.001$). The air quality received during the chronic and the acute fumigations produced slight increases in the levels of glutathione (1.8% and 2.3% respectively) although these were not significant. The three-way ANOVA table is presented in Table 7.9, and the four-way analysis is presented in the appendix.

**Table 7.9 Analysis of Variance Table Glutathione Levels.**

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 5.410 | 5.410 | 0.27 | 0.61 |
| Chronic | 1 | 0.036 | 0.036 | 0.001 | 0.97 |
| Block | 3 | 86.64 | 28.88 | 1.42 | 0.25 |
| Acute.Chronic | 1 | 10.16 | 10.16 | 0.50 | 0.48 |
| Acute.Block | 3 | 95.13 | 31.71 | 1.56 | 0.21 |
| Chronic.Block | 3 | 440.1 | 146.7 | 7.22 | <0.001 |
| Acute.Chronic.Block | 3 | 93.69 | 31.23 | 1.54 | 0.22 |
| Error | 48 | 975.63 | 20.32 | | |
| Total | 63 | 1707 | | | |

## 7.5 Results of Additional Experiment

### 7.5.1 Visible Damage Assessment

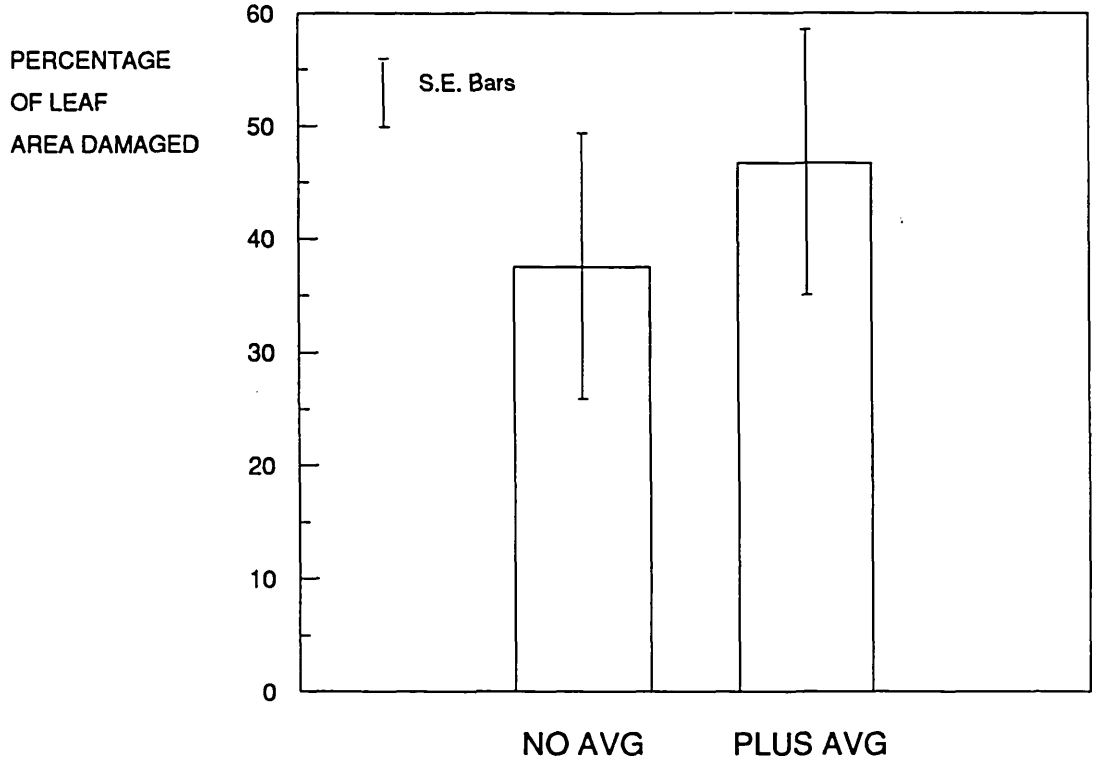


**Figure 7.14** **Visible Damage to Tiller 1, Leaf 2 of Plants Treated with Tween 20 or with Tween 20 plus AVG, and Fumigated with Ozone (at 100 ppb) for 72 Hours.**

No visible damage was apparent on any of the plants which had received filtered air. The graph therefore is for the damage only to the ozone fumigated plants. It can be seen that there is no significant difference in the damage with or without AVG application. The total visible damage per plant was also evaluated and was found to not differ significantly between control and treatment plants. The damage was not significantly different for any of the leaves, and in half the cases greater damage to leaves receiving Tween 20 only was seen, and in the other half greater damage occurred to leaves receiving AVG. No significant difference in damage was attributable to the blocks.

### 7.5.2 MRGR Assessment

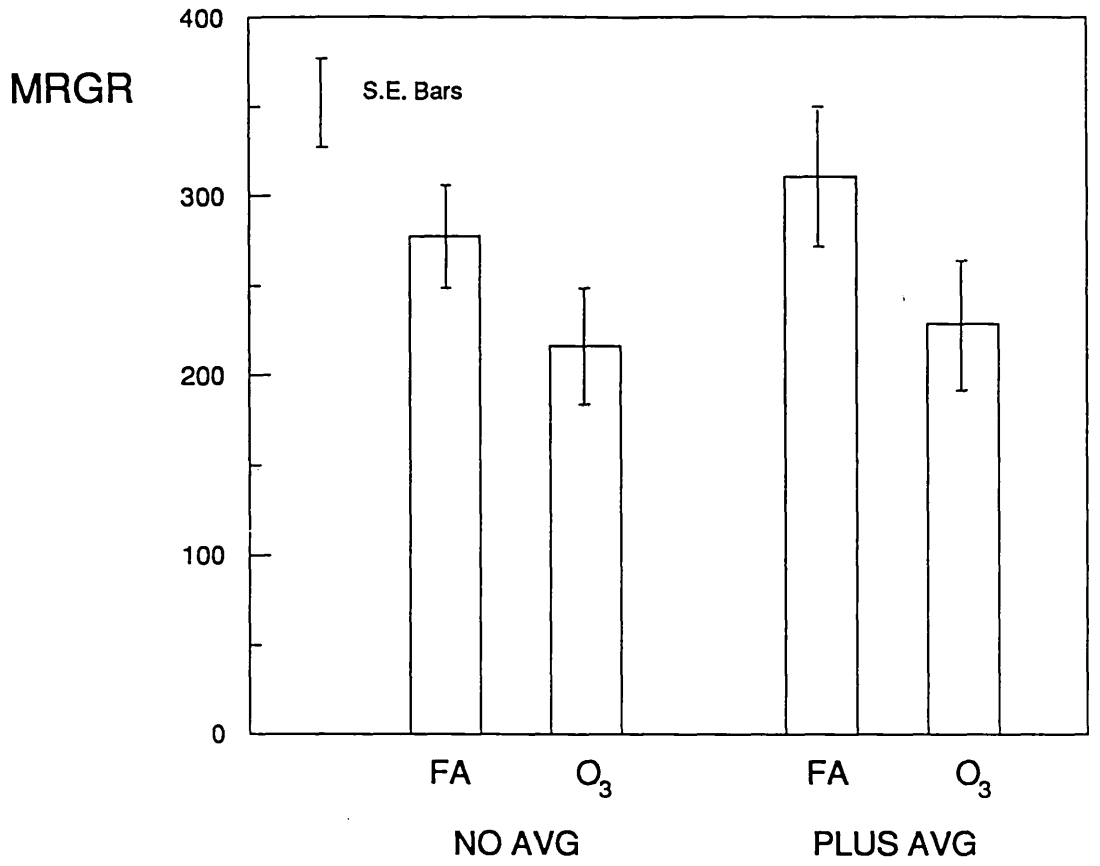


**Figure 7.15** **MRGR of Aphids on Plants Treated with Tween 20 or with Tween 20 plus AVG, and Fumigated with Filtered Air or Filtered Air plus Ozone (at 100 ppb) for 72 Hours.**

The results demonstrated a non-significant reduction in MRGR following ozone fumigation irrespective of AVG application. This is contary to all previous results at this fumigation duration which show a significant increase in performance following ozone fumigation. The AVG did not produce any difference in the growth rate, and there was no significant interaction between the air quality received and the AVG application. A possible explanation for the decrease in growth rate observed following ozone fumigation is the effect of the interaction with the Tween 20 (which was applied at a greater concentration in this experiment than in the last), but the inclusion of control plants minus Tween 20 would be needed to investigate this possibility. No significant difference in growth rate of the aphids was attributable to the blocks.

## 7.6 Conclusions

The results presented here in general show that the ozone pretreatment had no effect on the subsequent response of the plants to the acute ozone dose. This is contary to much of the published literature which indicates that ozone pretreatment is important, although whether it provides protection from or increases susceptibility to an acute dose depends on the experimental conditions. For example McDowall (1965) found that exposure of tobacco (*Nicotiana tabacum*) to low or high (unspecified) ozone doses increased or decreased, respectively, the amount of injury to subsequent exposure. Heagle and Heck (1974) showed increased susceptibility of tobacco (*N. tabacum*) resulting from pretreatments with ambient oxidant concentrations above 30 ppb. Runeckles and Rosen (1974) found that mint plants (*Mentha arvensis* L.) pretreated daily with ozone at 20 ppb were more susceptible to damage following an acute ozone exposure for three days at 300 ppb, than plants previously exposed to ozone free air. The opposite was found with beans (*Phaseolus vulgaris*) where the pre-treatment later reduced their susceptibility to an acute dose. When, however, the pretreatment concentration was 40 ppb the susceptibility of beans to an acute dose was increased.

In the light of the above findings it is therefore perhaps surprising that the results presented here indicate so little effect of the pretreatment on the final response of the plants to the acute ozone dose. The final acute fumigation produced an increase in MRGR irrespective of the chronic exposure of the plants. The suppression of leaf conductance following the final fumigation was also irrespective of the chronic fumigation. The levels of visible damage observed following the final fumigation were also irrespective of the chronic fumigation. This is also the case for the levels of soluble nitrogen, reducing sugars and glutathione. Exceptions to this are seen in the intrinsic rate of natural increase data and in the photosynthesis data. Levels of photosynthesis are stimulated following acute ozone fumigation when the plants have been exposed to filtered air, but depressed when they have been exposed to ozone. Neither of these results, however, are significantly different. The explanation for the lack of a difference in response to the acute fumigation due to the pretreatment must lie with the conditions of this treatment. Any suggestions are purely speculative but may involve the duration of the exposure, the level of exposure, timimg of exposure, temperature during the exposure, soil moisture level and so on. Only further experimentation could resolve the question.

The MRGR data endorses that of chapter 2, however, the $r_m$ study does not. This may be due to the poor level of replication since many of the aphids died during the calculation of this statistic. This is an inherent problem of the $r_m$ technique and highlights the superiority of the MRGR technique for assessing general aphid performance.

The photosynthesis data for the plants assessed after the final fumigation show that there is no significant difference in rates between the treatment and control plants. This is contary to the results of Black *et al* (1982) who found that ozone fumigation of *Vicia faba* at concentrations greater than 50 ppb for 4 hours, produced significant reductions in net rates of photosynthesis, with greater reductions seen at higher concentrations. Of thirteen studies cited in Tingey and Taylor (1982) twelve demonstrated a significant reduction in photosynthesis with ozone fumigation. Black, however, found that concentrations of less than 40 ppb produced no significant difference in rates which suggested that the plants were able to tolerate the absorption of $O_3$ at a certain rate, or to repair the injury arising. This would depend on many factors, such as the rate of $O_3$ uptake by the leaf and environmental parameters which influence either stomatal aperture or biochemical protection mechanisms. No significant difference in photosynthetic rate was observed in this study for the plants fumigated with ozone at 30 ppb, and those experiencing filtered air only. An alteration in the rate of photosynthesis would result in an alteration in the levels of soluble sugars. A decrease in rates of photosynthesis may benefit the aphid since this would lead to a decrease in the levels of soluble sugars, and consequently a more concentrated nitrogen source. However, it would be extremely difficult to discover if a change in photosynthetic rate in isolation from other changes, alters the performance of the aphid.

Leaf conductance is primarily due to stomatal conductance, and the leaf conductance value can therefore be looked upon as an index of stomatal aperture. Five out of the six studies cited in Winner *et al* (1987) show that stomatal closure is seen following exposure to ozone. Unsworth and Black (1981) state that 'with a few exceptions it is now generally agreed that exposure to ozone leads to stomatal closure.' This is corroborated by the results of this study. Changes in conductance can either be due to the direct effect of pollutants on the guard cells or via changes in photosynthesis altering the carbon dioxide exchange within the mesophyll. Winner favours the later explanation but does not rule out the direct effect possibility, and notes that no studies concerning this have been conducted with ozone. If stomata are the major route of entry for ozone it seems likely that stomatal closure would confer ozone resistance upon the plant. Fletcher *et al* (1972) demonstrated this by artificially stimulating stomatal closure using

ABA, and observed increased ozone resistance. The response of the stomata to ozone may be a crucial determinant of the degree of ozone resistance or sensitivity of a plant (Unsworth and Black, 1981).

The visible damage results indicated that the plants were only very slightly damaged following chronic fumigation but seriously damaged following the acute fumigation. 64% of the leaf area of the oldest leaf displayed some form of visible damage. The most severely damaged leaves demonstrated curling and drying edges and tips. Necrosis appeared as yellowing brown patches with less severe chlorosis of the inter-veinal areas also occurring. It was very clear that the older leaves were more severely affected than the younger leaves. This may be related to the stomatal closure response to ozone, since as Olszyk and Tibbits (1981) demonstrated the stomata of expanding leaves of *Pi*sum sativum closed more during pollutant exposure than stomata of already expanded leaves. They also found that there was significantly less injury on expanding compared with expanded leaves. Craker and Starbuck (1973) also reported differential sensitivity of leaves of *Nicotiana tabacum* of different ages. They found that younger leaves were less sensitive to ozone than intermediately aged leaves, and that differences in ozone uptake were sufficient to explain this. Menser *et al* (1963) similarly reported increased ozone sensitivity of mature *N. tabacum* leaves, compared with younger leaves, and also noted increased damage to the tips of the leaves (see also Price, 1990). Increased damage to the tips of the leaves was also noted in the study reported here, although this was not quantified. Very severely damaged areas cannot support aphids, but the mildly chlorotic areas can. Throughout the study aphids were placed on the second oldest leaf close to the axis of the plant, and were therefore rarely positioned on severely damaged tissue.

It is not surprising that the dry weight accumulation is unaffected by the final acute fumigation since this only occurred for three days. However, differing levels may have been apparent due to the chronic fumigation but this was not the case for either the plants assessed prior to the final acute fumigation or for the plants assessed after the acute fumigation. An attempt was made to measure the root dry weight but because of the great difficulty encountered in separating the soil from the small monocotyledonous roots, this was not possible. This is unfortunate since the shoot dry weight results alone can give no indication as to the allocation of dry matter. Much evidence has now been gathered which indicates that during exposure to air pollution resources are directed towards the shoots (e.g. Oshima *et al*, 1978), and roots therefore have proportionately much greater growth reductions.

Broadly similar results were obtained in the biochemical analysis as those obtained in chapter 4 - i.e. an increase in soluble nitrogen, no change in total nitrogen, and an increase in levels of glutathione. A significant decrease in levels of reducing sugars was observed, which contradicts the significant increase observed in chapter 4. This could only be explained by further experimentation. Unfortunately it was not possible to assess the levels of amino acids.

All the plants in the experiment (except those assessed prior to the final acute fumigation) were treated with either Tween 20 or Tween 20 plus AVG. This produced absolutely no difference, in any of the measured parameters, to the final response of the plants or the aphids, to the acute fumigation. The additional experiment used twice the concentration of AVG and four times the concentration of Tween 20 to ensure the solution properly wet the leaf. This did indeed remove the increase in aphid growth rate normally seen following this ozone fumigation regime, however no increase was also seen on the plants which had not received AVG. The reduction in growth rate following fumigation was therefore not as a result of the AVG application itself, but may be due to an interaction effect with Tween 20, or to the handling of the plants prior to fumigation. These treatments are the only differences between this and other experiments in which an increase in growth rate is seen following this ozone fumigation regime. The lack of alteration in levels of visible damage with AVG application also indicates that it is providing no protection effect.

It is unfortunate that no measure of ethylene evolution above background could be recorded, as this might have provided an explanation for the above results. Melhorn and Wellburn (1987) found that application of AVG dramatically reduced the formation of stress ethylene which in turn appeared to prevent the formation of visible damage. They conducted a similar experiment to that described above, but found very different results. They fumigated pea seedlings (*Pisum sativum*) with either 50, 100 or 150 ppb ozone for 7 hours every day for the first three weeks of their growth. Other seedlings were grown in filtered air for three weeks and then exposed to ozone at 150 ppb for 7 hours. The extent of visible damage to the clean air grown seedlings following the final fumigation was far greater than the damage to the plants grown in ozone. The level of stress ethylene production was also much higher. The application of AVG 24 hours before the acute ozone fumigation successfully prevented the formation of visible damage, and reduced the quantity of ethylene evolved. They concluded that the degree of stress ethylene production determined the sensitivity of plants to atmospheric levels of ozone. In their experiment the plants were sprayed twice until run-off with the AVG

solution, whereas in this experiment AVG was applied to the plant using a paint brush. However, Brown (pers. comm.) also applied AVG using a paint brush to 3 week old *Vicia faba* and found that the increase in growth rate of *Aphis fabae* following ozone fumigation (at 100 ppb for 8 hours) was no longer evident, and that the degree of visible damage was greatly reduced. This indicates that it is unlikely to be the application method which is the reason for the contradictory results. Zhen-guo *et al* (1987) successfully demonstrated suppression of ethylene biosynthesis by AVG in wheat leaves exposed to sulphur dioxide, and demonstrated the operation of the same enzyme system as found in dicotyledonous plants. There is no mention of visible damage in their studies, but despite this their results can still not provide any explanation for the lack of an effect with AVG application apparent in this study.

# Chapter 8
# Final Discussion

The equilibrium evolved for insect levels on plants can be altered through increased levels of atmospheric pollutants (Jeffords and Endress, 1984). Plant species originally present only in their wild form are now cultivated in vast areas for use as food, and pest numbers have increased concomitantly. Results of this study and results recorded in the literature have demonstrated that under certain conditions there is an increase in insect growth rate on certain plants exposed to air pollution, which can lead to an increase in population growth rates. In agriculture increased insect populations beyond economic threshold values normally cannot be tolerated, so leading to further environmental pollution in the form of increased pesticide usage. In natural ecosystems new equilibrium levels result with consequences for plant and insect species richness. Forest systems may in rare cases also be sprayed, but probably will simply suffer the consequences of increased insect attack.

The results of this study, however, have suggested that an increase in insect growth rate, on crop plants, is much less of a general phenomenon than has been found by previous workers (e.g. Houlden *et al*, 1990). An initial aim of the thesis was to elucidate trends in the response of the rose grain aphid, *Metopolophium dirhodum*, to acute fumigation of its host plants for short durations. However, no trend in response could be detected for any combination of pollutant and cereal, with increases and decreases observed following different fumigation durations. Significant increases were observed in isolated cases and were only observed for one of the host plants - wheat. The lack of any significant increases following fumigation of barley may be due to the variety chosen for the study. Large differences in varietal resistance to air pollution have been demonstrated for *Vicia faba* (Masters, pers. com.), and it may be the case that the variety of barley (Atem) used in this study, and chosen for its resistance to powdery mildew, was also particularly resistant to air pollution. This may be true particularly since significant increases in aphid growth rate following short nitrogen dioxide fumigations of barley cv. Igri were observed by Houlden *et al* (1991).

Work with sulphur dioxide and nitrogen dioxide has frequently demonstrated an increase in aphid performance on polluted plants (e.g. Houlden *et al*, 1990, Aminu-Kano, 1987 and Dohmen *et al*, 1984). In general, work with ozone has proved more ambiguous (Whittaker and Warrington, 1990), with increases and decreases in insect performance observed depending on the species used and the conditions of the

fumigation. For example Brown *et al* (1991) found that the exposure pattern and timing of fumigation of *Vicia faba* with ozone could crucially affect the response of *Aphis fabae*. Brown and Bell (1990) also found that the temperature of the fumigation could have a significant effect on the response of the insect following fumigation with ozone. This could be particularly significant for the work described here, but unfortunately the experimental design was such that no firm conclusions could be drawn. The timing of air pollution incidence throughout the growth of the crop is also likely to have a crucial influence on the establishment of the aphid and its subsequent effects on the yield. Watt and Wratten (1984) demonstrated that an outbreak of *Metopolophium dirhodum* on wheat only caused significant reductions in yield when it occurred at an early growth stage of the plant. Therefore a pollution incident, potentially leading to an increase in insect numbers, occurring towards the end of the growth of the crop would be of less importance than one occurring when the crop was at an early growth stage.

Improvement in insect performance can be attributed to either a decrease in the controlling influence of natural enemies or an improvement in host plant food quality (assuming no changes in weather conditions) (Lawton and McNeill, 1979). This study considered only the effects of food quality, since natural enemies were irrelevant under the controlled conditions used. The plant biochemical studies indicated that following particular conditions alteration in biochemistry did occur in directions known to favour insect herbivores. Whether this was due to the overall increase in available nitrogen levels or to specific changes in amino acid levels can not be determined. The improved performance following the nitrogen oxide fumigations could only be tenuously linked with the recorded changes in plant biochemistry, but must be attributable to changes at the plant level. The importance of changes in levels of soluble sulphur compounds in isolation from other biochemical changes has been discounted, but its relevance in association with the changes in levels of amino acids has not been investigated and may be of significance. The regulating effect of certain natural enemies of cereal aphids, following air pollution, was studied by Aminu-Kano *et al* (1991), and was found to be of little consequence to aphid numbers. There is, however, a possibility that subtle interactions occur, especially with parasitoids. The number of parasitised aphids in an open air field fumigation experiment of wheat and barley was found to remain constant despite an increase in the overall number of aphids, but it is not clear if this was due to the direct effect of the gas on the parasitoids, or to the overall increase in aphid numbers in fumigated plots. Preliminary work of Frizzell (1991, pers. com.) demonstrated that

ozone reduced the efficiency of the parasitoid *Asobara tabida* to parasitise its natural host *Drosophila subobscura*. A review of this subject by Riemer and Whittaker (1989) indicates that parasites and predators are of potential significance and justify further study.

The direct effects of atmospheric pollutants at realistic levels on insects have in most studies cited in the literature been shown to be negligible. Studies frequently utilise artificial diets as a means of separating the effects solely due to the direct effect on the insect, from those due to effects mediated via the plant. Chapter 3 demonstrates the need to be cautious in the interpretation of the results of artificial diet studies. It would appear solely from the results on artificial diets that ozone at 100 ppb reduces the growth rate and survival of the aphids. Fumigation of the aphids on artificial diet exposes the insect when under physiological stress, and the pollution stress and exposures in warm, probably dry chambers compounds this. This study demonstrates the need to fumigate the plant and insect together before drawing firm conclusions concerning the direct effect on the insect.

Ozone as a rural pollutant is potentially important to the growth of cereals. This study demonstrated that longer term, background levels of ozone, such as those persisting in rural areas, have no effect on aphid performance and also do not modify the aphid response to an acute pollution episode. This is contrary to evidence in the literature which demonstrates that the previous history of the plant can predispose the plant to air pollution injury (see chapter 7). This would also be expected to be evident in the aphid performance. Of the parameters measured the results also indicate that the chronic levels of ozone have no effect on the plant. This does not rule out the possibility that background ozone levels are important to either the plant or the aphid, only that under these particular conditions it does not appear to be the case. In some agricultural areas more frequent exposures to higher background levels of ozone are experienced which may modify subsequent aphid performance. From the perspective of plant performance, it is the synergistic effects of the pollution acting independently of the insect interaction, and the additional effects of the insect on the plant which must be considered. Further studies of the multi-pronged attack on the plant due to air pollution, i.e. directly due to the pollution itself, and indirectly due to secondary interacting factors, such as insects, drought, mineral deficiency, frost and cold stress etc. are required to quantify the change in plant performance due to air pollution. Combinations of all these factors are crucial to understanding the final response of the plant, but study of all such effects is difficult

and it is only when the basic responses have been elucidated that combinations of factors can be considered. It is probably also at this point that work in the field should be initiated.

## Future Work

The results of chapter 2 clearly demonstrate the need for growth rate studies to firstly be carried out under more controlled conditions, and secondly to investigate results at different temperatures. The $r_m$ studies indicate that direct assumptions concerning increase in population growth rates suggested by MRGR studies are not necessarily valid. Studies involving a count of population numbers following the introduction of a certain number of adult aphids on treatment and control plants are probably necessary to measure increases in population numbers. The progression of this work in to a full field exposure system, involving all the stresses placed on a growing crop, is the only truly accurate method to quantify increases in aphid numbers.

A field exposure system is not necessary for mechanistic studies underlying changes in insect performance on polluted plants. The most practical system is one in which as much of the environmental variability as possible is removed. The small chamber system is therefore ideal for this work. Initial suggestions of mechanisms can be tested in this way, and later extrapolated to and modified in a field situation. Further investigation into ethylene as the causative agent mitigating ozone damage should be investigated in this way. The inhibitor AVG should be sprayed onto the plants as in the study by Melhorn and Welburn (1987), instead of being applied with a paint brush. Measurement of levels of ethylene production following fumigation could also be further considered, especially since the saliva of many plant sucking insects contain enzymes known to stimulate the production of ethylene (Martin *et al*, 1988).

It appears from this study that the mechanism underlying the increase in aphid performance following 72 hours of ozone fumigation is the increased concentration of amino acids in the plant tissues. The exact location of the changes, for example in the phloem sap, needs to be elucidated. The biochemical data suggests that the metabolite pools are in flux for at least four days following the fumigation. The possible persistence of an increase in aphid performance in subsequent days is important in the field, especially following ozone episodes. This could initially be investigated by conducting growth rate studies following a delay after the end of the fumigation. Small controlled environment chambers are again ideal for this kind of study.

A final important feature of the increase in aphid performance on polluted plants is the effect this can have on plant performance. This must be evaluated over the longer time periods necessary for the plant development. Potentially one ozone episode could reduce the performance of the plant substantially by facilitating the growth of an aphid population. The timing of the ozone episode during the plant development could be crucial and could be investigated using small chambers. The work of Watt and Wratten (1984) supports the hypothesis that the time of infestation is crucial since an infestation on young wheat produced a reduction in grain weight, whereas infestation of older plants did not.

## Conclusions

1) This project has demonstrated the potential for ozone and nitrogen oxides to produce increases in aphid performance. This, however, is dependant on fumigation duration and probably fumigation conditions.

2) Direct fumigation of the aphid with nitrogen oxides, whilst feeding on artificial diets, demonstrated no difference in performance compared with that in filtered air. There was a suggestion of a decrease in performance following ozone exposure. This direct deleterious effect was not seen when the aphid was fumigated on a plant, which was probably indicative of a stronger physiological status of an aphid on a plant, compared with on an artificial diet sachet.

3) The improvement in aphid performance following ozone fumigation was concomitant with a change in biochemical parameters known to be beneficial to the aphid. An increase in soluble nitrogenous compounds, particularly an increase in free amino acids was observed. Increases in sulphur containing amino acids and the tripeptide glutathione also occurred. Certain biochemical changes were shown to persist for at least four days following the end of the fumigation. However, no such changes accompanying an increase in MRGR following nitrogen oxide fumigation were seen. The increases must therefore be attributable to unmeasured factors, or alternatively may be due to a type I statistical error in the measurement of the MRGR.

4) The increase in sulphur containing compounds, in isolation from other biochemical changes, was found not to be the causal mechanism behind the increase in aphid performance.

5) Further investigation into changes in the partitioning of sulphur containing compounds following ozone fumigation demonstrated no change in the proportion of aphid imbibable sulphur, compared with sulphur bound in water insoluble compounds.

6) The use of a radioactive tracer demonstrated that an aphid imbibes less phloem sap, on an ozone fumigated plant and that the honeydew produced is more concentrated. A reduction in honeydew production and an increase in the concentration of amino acids within it, further indicated a more concentrated phloem sap.

7) Long term low level ozone fumigations appear to have little effect on plant and aphid performance, and also on the subsequent response to an acute ozone episode.

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# APPENDIX

## Samples of the Analysis of Covariance Tables from Chapter 2

Analysis for Ozone and Barley, for 3 covariates;

1) Maximum temperature in the chambers

| Source | d.f. | SS | MS | F | p |
|---|---|---|---|---|---|
| Air | 1 | 0.01246 | 0.01246 | 0.53 | 0.47 |
| Maximum Temp | 1 | 0.07780 | 0.07780 | 3.31 | 0.08 |
| Air.Max T | 1 | 0.00139 | 0.00139 | 0.06 | 0.81 |
| Error | 20 | 0.47016 | 0.02351 | | |
| Total | 23 | 0.56181 | | | |

2) Mean temperature in the chambers

| Source | d.f. | SS | MS | F | p |
|---|---|---|---|---|---|
| Air | 1 | 0.01246 | 0.01246 | 0.51 | 0.48 |
| Mean Temp | 1 | 0.04523 | 0.04523 | 1.84 | 0.19 |
| Air.Mean T | 1 | 0.01330 | 0.01330 | 0.54 | 0.47 |
| Error | 20 | 0.49082 | 0.02454 | | |
| Total | 23 | 0.56181 | | | |

3) Time in the chambers (Duration)

| Source | d.f. | SS | MS | F | p |
|---|---|---|---|---|---|
| Air | 1 | 0.01246 | 0.01246 | 0.49 | 0.49 |
| Duration | 1 | 0.03067 | 0.03067 | 1.21 | 0.28 |
| Air.Dur | 1 | 0.01286 | 0.01286 | 0.51 | 0.48 |
| Error | 20 | 0.50582 | 0.02529 | | |
| Total | 23 | 0.56181 | | | |

## Analysis of Variance Tables from Chapter 7

### Photosynthetic rate

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 3.189 | 3.189 | 0.85 | 0.36 |
| Chronic | 1 | 1.728 | 1.728 | 0.46 | 0.50 |
| AVG | 1 | 0.595 | 0.595 | 0.16 | 0.69 |
| Block | 3 | 10.63 | 3.543 | 0.95 | 0.43 |
| Acute.Chronic | 1 | 3.582 | 3.582 | 0.96 | 0.43 |
| Acute.AVG | 1 | 0.564 | 0.564 | 0.15 | 0.69 |
| Acute.Block | 3 | 1.283 | 0.428 | 0.11 | 0.95 |
| Chronic.AVG | 1 | 3.106 | 3.106 | 0.83 | 0.49 |
| Chronic.Block | 3 | 15.33 | 5.110 | 1.37 | 0.25 |
| Block.AVG | 3 | 10.99 | 3.663 | 0.98 | 0.33 |
| Acute.Chronic.AVG | 1 | 0.181 | 0.181 | 0.05 | 0.98 |
| Acute.Chronic.Block | 3 | 22.51 | 7.503 | 2.01 | 0.13 |
| Chronic.AVG.Block | 3 | 9.150 | 3.050 | 0.82 | 0.49 |
| AVG.Block.Acute | 3 | 8.587 | 2.862 | 0.77 | 0.52 |
| Acute.Chronic. AVG.Block | 3 | 5.165 | 1.722 | 0.46 | 0.71 |
| Error | 32 | 119.39 | 3.731 | | |
| Total | 63 | 215.98 | | | |

### Leaf Conductance

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 0.111 | 0.111 | 3.44 | 0.07 |
| Chronic | 1 | 0.079 | 0.079 | 2.45 | 0.13 |
| AVG | 1 | 0.000 | 0.000 | < 0.00 | > 0.99 |
| Block | 3 | 0.128 | 0.043 | 1.33 | 0.28 |
| Acute.Chronic | 1 | 0.000 | 0.000 | < 0.00 | > 0.99 |
| Acute.AVG | 1 | 0.011 | 0.011 | 0.34 | 0.56 |
| Acute.Block | 3 | 0.126 | 0.042 | 1.30 | 0.29 |
| Chronic.AVG | 1 | 0.004 | 0.004 | 0.12 | 0.73 |
| Chronic.Block | 3 | 0.033 | 0.011 | 0.34 | 0.80 |
| Block.AVG | 3 | 0.082 | 0.027 | 0.84 | 0.48 |
| Acute.Chronic.AVG | 1 | 0.040 | 0.040 | 1.24 | 0.27 |
| Acute.Chronic.Block | 3 | 0.054 | 0.018 | 0.56 | 0.64 |
| Chronic.AVG.Block | 3 | 0.181 | 0.060 | 1.86 | 0.16 |
| AVG.Block.Acute | 3 | 0.110 | 0.037 | 1.15 | 0.34 |
| Acute.Chronic. AVG.Block | 3 | 0.174 | 0.058 | 1.80 | 0.17 |
| Error | 32 | 1.031 | 0.032 | | |
| Total | 63 | 2.166 | | | |

## Reducing Sugars

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 7.698 | 7.698 | 3.636 | 0.07 |
| Chronic | 1 | 0.028 | 0.028 | 0.013 | 0.91 |
| AVG | 1 | 0.413 | 0.413 | 0.195 | 0.66 |
| Block | 3 | 1.132 | 0.377 | 0.178 | 0.91 |
| Acute.Chronic | 1 | 0.982 | 0.982 | 0.464 | 0.50 |
| Acute.AVG | 1 | 0.223 | 0.223 | 0.105 | 0.75 |
| Acute.Block | 3 | 22.98 | 7.660 | 3.61 | 0.02 |
| Chronic.AVG | 1 | 6.457 | 6.457 | 3.05 | 0.09 |
| Chronic.Block | 3 | 25.22 | 8.407 | 3.97 | 0.02 |
| Block.AVG | 3 | 1.565 | 0.522 | 0.246 | 0.86 |
| Acute.Chronic.AVG | 1 | 2.787 | 2.787 | 1.32 | 0.26 |
| Acute.Chronic.Block | 3 | 23.74 | 7.913 | 3.73 | 0.02 |
| Chronic.AVG.Block | 3 | 0.368 | 0.123 | 0.058 | 0.98 |
| AVG.Block.Acute | 3 | 4.776 | 1.592 | 0.752 | 0.53 |
| Acute.Chronic. AVG.Block | 3 | 4.472 | 1.491 | 0.704 | 0.56 |
| Error | 31 | 65.63 | 2.117 | | |
| Total | 62 | 168.47 | | | |

## Glutathione

| Source | df | SS | MS | F | p |
|---|---|---|---|---|---|
| Acute | 1 | 5.410 | 5.410 | 0.29 | 0.59 |
| Chronic | 1 | 0.363 | 0.363 | 0.02 | 0.89 |
| AVG | 1 | 11.29 | 11.29 | 0.60 | 0.44 |
| Block | 3 | 86.64 | 28.88 | 1.53 | 0.23 |
| Acute.Chronic | 1 | 10.16 | 10.16 | 0.54 | 0.40 |
| Acute.AVG | 1 | 0.067 | 0.067 | 0.01 | 0.921 |
| Acute.Block | 3 | 95.13 | 31.71 | 1.68 | 0.19 |
| Chronic.AVG | 1 | 1.255 | 1.255 | 0.07 | 0.79 |
| Chronic.Block | 3 | 440.1 | 146.7 | 7.77 | 0.009 |
| Block.AVG | 3 | 38.43 | 12.81 | 0.68 | 0.416 |
| Acute.Chronic.AVG | 1 | 0.007 | 0.007 | 0.00 | 0.99 |
| Acute.Chronic.Block | 3 | 93.69 | 31.23 | 1.65 | 0.20 |
| Chronic.AVG.Block | 3 | 16.18 | 5.393 | 0.28 | 0.84 |
| AVG.Block.Acute | 3 | 130.3 | 43.43 | 2.30 | 0.096 |
| Acute.Chronic. AVG.Block | 3 | 173.3 | 57.77 | 3.06 | 0.042 |
| Error | 32 | 604 | 18.87 | | |
| Total | 63 | 1717 | | | |